A Heritable Structural Alteration of the Yeast Mitochondrion

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ABSTRACT

Prions have revived interest in hereditary change that is due to change in cellular structure. How pervasive is structural inheritance and what are its mechanisms? Described here is the initial characterization of \( [\text{Leu}^+ \] \), a heritable structural change of the mitochondrion of \( \text{Saccharomyces cerevisiae} \) that often but not always accompanies the loss of all or part of the mitochondrial genome. Three phenotypes are reported in \( [\text{Leu}^+] \) vs. \( [\text{Leu}^-] \) strains: twofold slower growth, threefold slower growth in the absence of leucine, and a marked delocalization of nuclear-encoded protein destined for the mitochondrion. Introduction of mitochondria from a \( [\text{Leu}^-] \) strain by cytoduction can convert a \( [\text{Leu}^+] \) strain to \( [\text{Leu}^-] \) and vice versa. Evidence against the Mendelian inheritance of the trait is presented. The incomplete dominance of \( [\text{Leu}^+] \) and \( [\text{Leu}^-] \) and the failure of \( \text{HSP104} \) deletion to have any effect suggest that the trait is not specified by a prion but instead represents a new class of heritable structural change.

Heritable change that results from modification of nonnuclear cellular structure has been documented in a number of biological systems (for reviews, see Nanney 1968; Frankel 1989; Grimes and Aufderheide 1991; Wickner and Chernoff 1999). This was emphatically demonstrated in Paramecium by the 180° inversion of several rows of cilia. Beisson and Sonneborn (1965) showed this change in structure of the cellular cortex to be a heritable change that was completely independent of any type of nuclear change. Other less widely noted studies have established cortical inheritance to be a general property of the ciliated protozoa [see, for example, the work of Tartar (1961) on Stentor and Ng and Frankel (1977) on Tetrahymena].

Heritable structural change has received renewed interest with the discovery of prions in mammals (Prusiner 1982) and in fungi (Wickner 1994; Coustou et al. 1997). \( [\text{PSI}^+] \), one of two extensively characterized yeast prions [discovered by Cox (1965) and reviewed by Liebman and Derratch (1999)], is specified by one of two conformational states of \( \text{Sup35p} \). In its nonprion conformation, \( [\text{psi}^-] \), \( \text{Sup35p} \) plays an essential role in translation termination (Zhou, Avila et al. 1995). \( [\text{PSI}^+] \) is heritable and dominant to \( [\text{psi}^-] \) because the \( [\text{PSI}^+] \) conformers of \( \text{Sup35p} \) appear to recruit nonprion \( \text{Sup35p} \) subunits to the prion conformation, producing large multimeric aggregates (Patino et al. 1996; Paushkin et al. 1996). In its aggregated form \( \text{Sup35p} \) is presumed to be inactive in translation termination. Thus, as first proposed by Wickner (1994), one heritable state, \( [\text{PSI}^+] \), exists when \( \text{Sup35p} \) adopts the prion conformation and a second heritable state, \( [\text{psi}^-] \), exists when \( \text{Sup35p} \) adopts its other conformation. Two structural states of \( \text{Sup35p} \) are thus manifested as two alternate translational phenotypes, which, in turn, specify alternate sets of diverse colony morphology and growth phenotypes (True and Lindquist 2000).

Prions, the first examples of structural inheritance understood in molecular terms, have stimulated the search for additional types of heritable change that are not specified by genetic or nuclear change. The mitochondrion is a likely place to look. That this organelle has never been seen to form de novo suggests that information intrinsic to its very structure may be required to specify its reproduction. While the mitochondrion itself is essential for viability (Yaffe 1999), the ~80-kb mitochondrial genome of \( \text{Saccharomyces cerevisiae} \), termed \( \rho \), can be completely (Nagley and Linnane 1970) or partially (Mounolou et al. 1966) lost to yield strains termed \( \rho^- \) and \( \rho^+ \), respectively. Because some of the proteins required for oxidative phosphorylation are encoded by the mitochondrial genome, the resulting “petite” strains are unable to grow on nonfermentable carbon sources (such as glycerol) yet have not been shown to be defective in any other metabolic process. I report here a non-Mendelian trait that often accompanies the loss of mitochondrial DNA (mtDNA). This trait, termed \( [\text{Leu}^+] \), is characterized by slow growth and an additional requirement for leucine. Data presented here demonstrate that \( [\text{Leu}^+] \) is the result of a structural alteration of the mitochondrion.

MATERIALS AND METHODS

Materials, media, and strains: YPD, YPG, and HC growth media have been described (Adams et al. 1997). Strains 2247-21-2a (from Lee Hartwell), 132 (from Bob Sclafani), and 10507...
(from Peter Pryciak), the three \( p^+ \) parent strains from which all the \( p^- \) and \( p^- \) strains described in this work were derived, are all congenic with A364A. YL608 was from Mark Johnston. The 11 \( m+ \) strains used to genetically define the \( p^- \) strains described here were from Alexander Tzagoloff. YDL121 was gene on yeast mtDNA, respectively. DL106 and BLU/0 were obtained by growing cultures and allowed to mate overnight on a YPD plate. leucine to complete synthetic medium did not enhance Cells grown in HC liquid medium onto HC plates lacking leucine dependently of the manner in which mtDNA was lost. The growth properties of two such strains was done by \( Ura^+ \) \( \times \) 16kar/0. DL124 was obtained by growth of YDL121 in 3, 0.3, and 1 \( \mu\)g EB/ml YPD, respectively. DL106 and BLU/0 were obtained by growing BLU in 0.3 and 10 \( \mu\)g/ml EB, respectively. 3Z and 27Z were obtained after growth of YDL121 in 0 and 3 \( \mu\)g/ml EB, respectively. DL125 and DL123 were isolated as \( p^- \) and \( p^- \) coryx- tants, respectively, from the cross BLU \( \times \) 16kar/0. DL124 was obtained by growth of DL125 in 3 \( \mu\)g/ml EB. Homozygous diploid derivatives of strains 3Z and 27Z were obtained by first transforming each to Ura\(^+\) with the HO endonuclease-expressing plasmid pCY204 (from Dan Gottschling). Candidate spontaneously diploidized transformants were grown briefly on 5-fluoroorotic acid plates; nonmating, Ura\(^-\) strains whose cells were twofold larger than those of the parental strains were presumed to be homozigous diploids. A fusion between Cox4p and green fluorescent protein (GFP), which is localized to the mitochondrial inner membrane, was expressed from the plasmid pCox4-GFP. A fusion between Cig1 and GFP, which is localized to the mitochondrial matrix, was expressed from the plasmid pRS416/CSI-GFP (from Ron Butow; Zelenaya-Troitskaya et al. 1998). Abf2p was overexpressed from the URA3 plasmid pGAL68-AFB (from Ron Butow; Zelenaya-Troitskaya et al. 1998).

**Methods:** Yeast was stained with 4′,6-diamidino-2-phenylindole (DAPI) dihydrochloride by first fixing in methanol and then resuspending in 1 \( \mu\)g DAPI/ml water. GFP fusion proteins were visualized in living yeast cells, grown from single cells to microcolonies in situ as described by Kohlweiss (2000), using a Zeiss fluorescent microscope equipped with DeltaVision stage movement motors. Images of the strains were collected identically: 20 exposures of each field were taken at 0.4-\( \mu m \) focal intervals. The exposures were first deconvoluted to eliminate signal that is out of focus and then superimposed.

Cytoductive mating was done by combining freshly grown strains on YPD for 3 hr, after which an aliquot of mating mixture, resuspended in water, was spotted onto YPD. Only trefoil-shaped zygotes (bearing medial buds) were chosen for isolation by micromanipulation. Buds forming over the next several hours were isolated until each zygote yielded at least six buds. Normal-growing derivatives of strain 3Z and 16kar/0 were obtained by plating a freshly grown stationary culture grown in HC liquid medium onto HC plates lacking leucine to give 9 \( \pm \) \( 3 \times 10^5 \) colonies/100-mm plate. Determination of mtDNA sequences retained in the \( p^- \) strains was done by genetic complementation as follows: a \( p^- \) strain was mixed with one of each of five or six relevant \( m+ \) strains (whose names begin with M and aM in Table 1) as droplets of freshly grown cultures and allowed to mate overnight on a YPD plate. Cells from each mating were resuspended in water and spotted onto YPG plates, where growth of the diploid demonstrated complementation while failure to grow demonstrated non-complementation.

**Loss of mtDNA yields strains with either of two patterns of growth:** While studying the effect of a nuclear gene on yeast mtDNA (Lockshon et al. 1995), a single \( p^- \) parent strain was found to give rise to two types of \( p^- \) strains. For example, the \( p^- \) strain DL156 and its \( p^- \) progenitor (strain 10507) grew equally well on complete synthetic media whereas DL035, another independent \( p^- \) derivative that also arose spontaneously from the same \( p^- \) parent, grew somewhat more slowly (Figure 1A). All slow-growing \( p^- \) derivatives grew even more slowly in the absence of leucine, yet all normal-growing \( p^- \) derivatives had no leucine requirement for optimal growth (Figure 1B).

*S. cerevisiae* is also capable of losing only part of its mitochondrial genome to yield so-called \( p^- \) respiratory-deficient strains (Dujon 1981). Since the mass of mtDNA is the same in \( p^- \) strains and their \( p^- \) parents (Nagley and Linzane 1972), \( p^- \) and \( p^- \) derivatives can be easily distinguished by DAPI staining. As is the case with \( p^- \) derivatives of 10507, two types of \( p^- \) derivatives were also readily isolated: those such as DL110 that grow slowly and have a partial leucine requirement and those such as 16K31 that show no growth defect and no leucine requirement (Figure 1, A and B). The fraction of spontaneous \( p^- \) derivatives of 10507 that demonstrated this pair of growth phenotypes was high; 2160 colonies grown from an untreated culture gave 26 \( p^- \) and 3 \( p^- \) derivatives of which 12 and 2, respectively, were slow growing and leucine dependent. Overexpression of Abf2p, which is known to cause the loss of mtDNA (Zelenaya-Troitskaya et al. 1998), also yielded both normal-growing and leucine-dependent \( p^- \) derivatives of strain 10507, as did ethidium bromide treatment. Thus, slow-growing, leucine-dependent derivatives of strain 10507 arose independently of the manner in which mtDNA was lost.

Two other \( p^- \) strains were also examined for their ability to yield slow-growing, partially leucine-dependent \( p^- \) and \( p^- \) derivatives. Loss of mtDNA directly from the \( p^- \) strain BLU yielded only normal-growing \( p^- \) derivatives, such as BLU/0. Slow-growing, leucine-dependent \( p^- \) derivatives such as DL106 could also be readily isolated directly from BLU, yet normal-growing \( p^- \) derivatives of BLU were not found. The \( p^- \) strain YDL121 readily yielded \( p^- \) and \( p^- \) derivatives, which exhibited both normal growth and slow growth that was leucine dependent. The growth properties of two such \( p^- \) strains, 3Z and 27Z, were examined in more detail. In liquid synthetic medium 3Z grew 2.1 \( \pm \) 0.2-fold slower than 27Z in the presence of leucine and 3.1 \( \pm \) 0.3-fold slower than 27Z in its absence. Addition of up to 8-fold more leucine to complete synthetic medium did not enhance the growth rate of 3Z, suggesting a more global cellular defect. Removal of no single amino acid (or uracil or adenine) other than leucine (aside from His and Trp,
Nuclear mutation must also be considered. Data and an additional diploid was therefore made by mating alternative, LeuP could be due to a heritable structural revealed LeuP or caused it to arise.

Alternatively, LeuP could not be due to alteration of the mitochondrial genome. On the other hand, because removal of mtDNA from the spore colonies to attempt to reveal the LeuP phenotype required the tester strain to be.

In summary, three ρ+ strains yielded ρ+ and ρ− derivatives that either grew as well as their ρ+ parents did or grew slowly and showed an additional partial requirement for leucine. This pair of growth defects is hereafter referred to as the Leu· phenotype (partial). What is the nature of the heritable change that led to Leu·? Since a single ρ− parent yielded derivatives completely lacking mtDNA that were either normal growing or leucine dependent (cf. DL156 and DL035), the heritable change could not be due to alteration of the mitochondrial genome. On the other hand, because removal of mtDNA might be expected to influence organelar structure and because part of leucine biosynthesis occurs in the yeast mitochondrion (Ryan et al. 1973), Leu· could be caused by an alteration in structure of the mitochondrion itself. Alternatively, Leu· could be due to a heritable structural change in some nonmitochondrial cellular component. Nuclear mutation must also be considered. Data addressing these hypotheses are presented below.

Analysis of meiotic products of a cross between a ρ+ strain and a ρ0 Leu+ strain: Two ρ+ strains (10507 and YDL121) gave a far greater fraction of ρ0 derivatives that were Leu+ than would be expected by nuclear mutation. Moreover, it is difficult to understand how the biosynthetic (or utilization) pathway of only leucine could be affected by such mutations. Nevertheless, this possibility was directly addressed by examining the segregation of Leu+ in meiotic products of a diploid made by crossing a Leu+ ρ0 strain (3Z) with a Leu+ ρ0 tester strain to be.

<table>
<thead>
<tr>
<th>Yeast strains</th>
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<tbody>
<tr>
<td>10507</td>
<td>MATa aro2 his7 ura3 kar1 [ρ+]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16kar/0</td>
<td>MATa aro2 his7 ura3 kar1 [ρ+ Leu+]</td>
<td></td>
<td></td>
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<tr>
<td>DL035</td>
<td>MATa aro2 his7 ura3 kar1 [ρ+ Leu+]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL124</td>
<td>MATa aro2 his7 ura3 kar1 [ρ+ Leu+]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL156</td>
<td>MATa aro2 his7 ura3 kar1 [ρ+ Leu+]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL110</td>
<td>MATa aro2 his7 ura3 kar1 [ρ- cox1 atp6 COB COX3 Leu·]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16K31</td>
<td>MATa aro2 his7 ura3 kar1 [ρ- atp6 COB COX3 Leu·]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>132</td>
<td>MATa ade1,2 ura3 leu2 tyr1 lys2 his7 [ρ+]</td>
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<td></td>
</tr>
<tr>
<td>BLU</td>
<td>MATa ade1,2 ura3 leu2::URA3LEU2 tyr1 lys2 his7 [ρ+]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BLU/0</td>
<td>MATa ade1,2 ura3 leu2::URA3LEU2 tyr1 lys2 his7 [ρ+ Leu+]</td>
<td></td>
<td></td>
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<tr>
<td>DL125</td>
<td>MATa ade1,2 ura3 leu2::URA3LEU2 tyr1 lys2 his7 [ρ+ Leu+]</td>
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<td>DL106</td>
<td>MATa ade1,2 ura3 leu2::URA3LEU2 tyr1 lys2 his7 [ρ+ Leu+]</td>
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<tr>
<td>2247-21-2a</td>
<td>MATa his7 trp5 kar1 can1 sap3 [ρ+]</td>
<td></td>
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</tr>
<tr>
<td>YDL121</td>
<td>MATa ura3Δ his7 trp5 kar1 can1 sap3 (pCox4-GFP) [ρ+]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3Z</td>
<td>MATa ura3Δ his7 trp5 kar1 can1 sap3 (pCox4-GFP) [ρ+ Leu+]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27Z</td>
<td>MATa ura3Δ his7 trp5 kar1 can1 sap3 (pCox4-GFP) [ρ+ Leu+]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>33-4C</td>
<td>MATa ura3Δ his7 trp5 kar1 can1 sap3 (pCox4-GFP) [ρ- Leu+]</td>
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</tr>
<tr>
<td>YM608</td>
<td>MATa ura3-52 his3-200 ade2-101 lys2-801 tyr1-501 gal80-538 [ρ+]</td>
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<tr>
<td>aM10-150/4D</td>
<td>MATa ade1 [cox1-I]</td>
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<tr>
<td>aM9-94/A7</td>
<td>MATa ade1 [cox2-I]</td>
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<td>aM9-3/6C</td>
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<tr>
<td>aM6-200/2C</td>
<td>MATa ade1 [cob-3]</td>
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<tr>
<td>aM28-82/11A</td>
<td>MATa ade1 [atp6-I]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aM17-102/4D</td>
<td>MATa ade1 [cob-1]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M10-150/A3</td>
<td>MATa [cox1-I]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M9-3/AS</td>
<td>MATa Met+ [cox3-I]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M6-200/A1</td>
<td>MATa ade5 [cob-3]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M28-82</td>
<td>MATa met6 [atp6-I]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M17-162</td>
<td>MATa met6 [cob-1]</td>
<td></td>
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two auxotrophies) affected the growth rate of 3Z. However, growth in minimal medium caused some reduction in growth rate of 3Z relative to that seen in complete medium, whether this comparison was done in the presence or absence of leucine. The ρ+ parent (YDL121) and the normal-growing ρ0 strain (27Z) grew at the same rate in minimal vs. complete medium (data not shown).

In summary, three ρ+ strains yielded ρ+ and ρ− derivatives that either grew as well as their ρ+ parents did or grew slowly and showed an additional partial requirement for leucine. This pair of growth defects is hereafter referred to as the Leu· phenotype (partial). What is the nature of the heritable change that led to Leu·? Since a single ρ− parent yielded derivatives completely lacking mtDNA that were either normal growing or leucine dependent (cf. DL156 and DL035), the heritable change could not be due to alteration of the mitochondrial genome. On the other hand, because removal of mtDNA might be expected to influence organelar structure and because part of leucine biosynthesis occurs in the yeast mitochondrion (Ryan et al. 1973), Leu· could be caused by an alteration in structure of the mitochondrion itself. Alternatively, Leu· could be due to a heritable structural change in some nonmitochondrial cellular component. Nuclear mutation must also be considered. Data addressing these hypotheses are presented below.

Two ρ+ strains (10507 and YDL121) gave a far greater fraction of ρ0 derivatives that were Leu· than would be expected by nuclear mutation. Moreover, it is difficult to understand how the biosynthetic (or utilization) pathway of only leucine could be affected by such mutations. Nevertheless, this possibility was directly addressed by examining the segregation of Leu· in meiotic products of a diploid made by crossing a Leu+ ρ0 strain (3Z) with a ρ+ “tester” strain (the dependence of sporulation on respiration required the tester strain to be ρ+). Twenty-six of 31 tetrads from this diploid yielded four respiratory-competent spores. None of the colonies from any of the spores were Leu·. Since ρ+ strains that are Leu· have not been found, the failure of any of the ρ− meiotic products to exhibit Leu· could be due to the masking of its expression by the ρ+ genome. It was therefore necessary to remove the mtDNA from spore colonies to attempt to reveal the Leu· phenotype that would have been due to a hypothesized nuclear mutation in 3Z. However, the inability to distinguish whether removal of mtDNA from the spore colonies revealed Leu· or caused it to arise de novo required the use of a different experimental strategy.

An additional diploid was therefore made by mating the normal-growing ρ0 strain 27Z (derived from the
same $\rho^+$ parent as was the Leu$^+$ $\rho^+$ strain 3Z) to the $\rho^+$ tester strain. If the trait specifying Leu$^+$ persisted through meiosis, as would be expected of a nuclear mutation, a higher incidence of Leu$^+$ should have been seen in $\rho^+$ derivatives of spores derived from the 3Z-containing diploid than in $\rho^+$ derivatives of spores derived from the 27Z-containing diploid. The two types of diploids were sporulated and the 16 $\rho^+$ strains representing 2 tetrads from each diploid were treated with EB to produce $\rho^+$ derivatives. Of five randomly chosen $\rho^+$ derivatives of each of the 16 strains, the frequency of Leu$^+$ is similar for the two types of diploids (Table 2). In other words, the presence of Leu$^+$ in a mating partner had little if any influence on the frequency of Leu$^+$ in $\rho^+$ derivatives of meiotic products. In summary, no evidence could be found for the persistence of Leu$^+$ through meiosis, as would be expected of a trait specified by a nuclear mutation.

**Cytoductive transfer of Leu$^+$:** If the heritable change that causes Leu$^+$ is not a nuclear change, then Leu$^+$ must instead be specified by nonnuclear cellular material. Specification of the trait by the mitochondrion, moreover, predicts that transfer of the trait should accompany mitochondrial transfer. Nonnuclear components of yeast can be readily transferred (“cytoded”) between mating partners if one of them is kar1-1 since haploid nuclei seldom fuse within zygotes thus formed. Buds arising from such a zygote are therefore mainly of one haplotype or the other (Conde and Fink 1976).

![leucine image]  
**Figure 1.—Growth properties of a $\rho^+$ strain and its $\rho^+$ and $\rho^-$ derivatives.** The $\rho^+$ strain 10507 and its $\rho^-$ (DL156 and DL035) and $\rho^-$ (16K31 and DL110) derivatives were streaked onto two plates from a single batch of HC minus leucine plates to which a standard amount of leucine was (A) or was not (B) added. Photos were taken after 3 days of growth.

<table>
<thead>
<tr>
<th>Diploid</th>
<th>Tetrad, spore No.</th>
<th>$\rho^+$ derivatives that were Leu$^+$</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>3Z $\times$ YM608</td>
<td>1, a, b, c, d</td>
<td>3, 5, 0, 1</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>2, a, b, c, d</td>
<td>0, 0, 4, 2</td>
<td></td>
</tr>
<tr>
<td>27Z $\times$ YM608</td>
<td>3, a, b, c, d</td>
<td>2, 3, 1, 0</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>4, a, b, c, d</td>
<td>2, 0, 5, 1</td>
<td></td>
</tr>
</tbody>
</table>

Colonies derived from the spores of two tetrads from each of the 3Z $\times$ YM608 and 27Z $\times$ YM608 diploids were grown in 10 μg EB/ml YPD and plated for single colonies. Ten randomly chosen colonies from the plating of each of the 16 EB-treated strains were patched onto YPD. mtDNA content was then assayed by DAPI staining. Five randomly chosen $\rho^+$ derivatives of each of the spore colonies were streaked onto HC media in the presence and absence of leucine to determine which were Leu$^+$. The number of the $\rho^+$ derivatives (of five tested) of each spore whose growth was Leu$^+$ is listed. The percentage of the 40 $\rho^+$ derivatives of both types of diploids that expressed Leu$^+$ is also listed.

A Leu$^+$ $\rho^+$ strain (DL125) was crossed to a normal-growing $\rho^-$ kar1 strain (16K31). Zygotes and their buds were isolated and colonies grown from each bud were examined for nuclear markers. All 12 bud-derived strains that were found to have the same nucleus as the Leu$^+$ $\rho^+$ mating partner (DL125) were streaked onto plates lacking leucine (Figure 2). While 4 of these 12 cytoductants retained the Leu$^+$ phenotype of the Leu$^+$ $\rho^+$ mating partner (DL125), the other 8 switched to normal growth (compare to BLU/0, an isogenic normal-growing $\rho^+$ strain). The act of mating itself was not the cause of the conversion in growth properties: in control 3Z $\times$ YM608 and 27Z $\times$ YM608 diploids were grown in 10 μg EB/ml YPD and plated for single colonies. Ten randomly chosen colonies from the plating of each of the 16 EB-treated strains were patched onto YPD. mtDNA content was then assayed by DAPI staining. Five randomly chosen $\rho^+$ derivatives of each of the spore colonies were streaked onto HC media in the presence and absence of leucine to determine which were Leu$^+$ The number of the $\rho^+$ derivatives (of five tested) of each spore whose growth was Leu$^+$ is listed. The percentage of the 40 $\rho^+$ derivatives of both types of diploids that expressed Leu$^+$ is also listed.

Which nonnuclear component, when cytoded, caused the conversion of DL125 from Leu$^+$ to normal growth? For each of the 12 strains, the percentage of cells that contained mtDNA, as determined by DAPI staining, is also presented (Figure 2). Cytoductants that contain mtDNA and must therefore have received mitochondria from the normal-growing mating partner became normal-growing themselves. Those cytoductants that received no mtDNA—and hence possibly no mitochondrial material whatsoever—from the mating partner, retained Leu$^+$. This experiment demonstrates not only that a Leu$^+$ strain can be converted by cytoduction...
Mitochondrial Structural Inheritance

Figure 2.—Growth of strains derived from a cytoductive cross. After mating a normal-growing, \( \rho^- \ kar1 \) strain (16K31) with a Leu\(^r\) \( \rho^- \) strain (DL125), 27 zygotes were isolated. From these zygotes, 87 viable buds were isolated, 75 of which were of 16K31 and 12 of which were of DL125 nucleotype. All 75 strains of 16K31 nucleotype contained mtDNA and grew identically to 16K31 (data not shown). Cells from the colonies that grew from the 12 buds of the DL125 nucleotype were picked directly off the dissection plate and streaked onto a plate lacking leucine. These were then photographed after 3 days of growth. The number indicates the zygote from which the bud was dissected and the following letter indicates the order in which the bud was formed. Liquid cultures, each grown directly from cells of a dissection plate colony, were DAPI stained and the percentage of cells containing mtDNA is shown.

to one that is normal growing, but also that this conversion strictly correlates with the transfer of mtDNA from the normal-growing mating partner.

In the converse experiment, I sought to determine whether a normal-growing strain could be converted by cytoduction to one that is Leu\(^r\). A normal-growing \( \rho^- \ kar1 \) strain (27Z) was mated to a Leu\(^r\) \( \rho^- \) strain (DL106), zygotes and buds were isolated, and bud colonies were nucleotyped and DAPI stained as described above. In two independent experiments, 99 zygotes yielded a total of 275 viable buds of which 225 had the nucleotype of 27Z, the normal-growing parental strain. Two of the colonies that grew directly from the 27Z-nucleotype buds grew far more slowly than did the other 223 colonies of this nucleotype. None of the normal-growing colonies had mtDNA by DAPI staining. However, one of the slow-growing colonies, derived from bud 33-4C, stably maintained mtDNA and stably displayed the Leu\(^r\) phenotype (data not shown).

The other slow-growing colony, derived from bud 21-6B, was grown a minimal amount in YPD liquid media for DAPI staining and replating. About half of the cells of this culture contained mtDNA (data not shown). Plating of this liquid culture of 21-6B onto YPD gave mostly small colonies (Figure 3A). Three of the small colonies (\( i, ii, \) and \( iii \)) and one large one (\( iv \)) from Figure 3A were then restreaked onto plates lacking or containing leucine. The growth of a large fraction of colonies that grew from the cells of colony \( i \) were Leu\(^r\) yet all colonies that grew from cells of \( iv \) had no leucine requirement (Figure 3B). Colonies \( ii \) and \( iii \) behaved the same as did colony \( i \) (data not shown). Cells grown from colonies \( i-iv \) of Figure 3A were assayed for mtDNA as well: between 25 and 50% of the cells grown from colonies \( i, ii, \) and \( iii \) had mtDNA; cells grown from colony \( iv \) were devoid of mtDNA. In summary, it was possible, at a low frequency, to convert a normal-growing \( \rho^- \) strain into one that is Leu\(^r\) \( \rho^- \) by cytoduction. As most clearly demonstrated by bud 21-6B, the retention of the Leu\(^r\) phenotype strongly correlates with retention of mtDNA and, therefore, presumably of mitochondria, from the \( \rho^- \) mating partner. The ability to cytoduce the Leu\(^r\) phenotype into a normal-growing strain (Figure 3) and to cytoduce normal growth into a Leu\(^r\) strain (Figure 2) supports the hypothesis that a mitochondrial structural change is the basis of Leu\(^r\).

**Is Leu\(^r\) specified by a prion?** The only heretofore known heritable structural changes in yeast are specified by prions. What aspects of the heritable trait that specifies Leu\(^r\) are shared with prions? Since prions are fully dominant (Wickner and Chernoff 1999), the interaction of the traits that specify Leu\(^r\) and normal growth
was investigated. The growth of six diploid strains was examined in the absence (Figure 4A) and presence (Figure 4B) of leucine. These strains were made by crossing either normal growing (Figure 4, DL124; c, e, and g) or Leu$^\circ$ (Figure 4, 16kar/0; d, f, and h) $\rho^\circ$ derivatives of one $\rho^+$ strain with a second $\rho^+$ strain (Figure 4, BLU; c and d) or its normal-growing (Figure 4, BLU/0; e and f) or Leu$^\circ$ (Figure 4, DL125; g and h) $\rho^\circ$ derivatives. Note the large difference in growth rates between the normal-growing (Figure 4, DL124) and Leu$^\circ$ (16kar/0) haploid control strains (Figure 4, a vs. b). When each type of $\rho^\circ$ strain was crossed to a $\rho^+$ strain (BLU) to form $\rho^\circ$ diploids, no detectable difference in growth rate was seen (Figure 4, c vs. d).

When the normal-growing (DL124) and Leu$^\circ$ (16kar/0) $\rho^\circ$ strains were mated instead to a normal-growing $\rho^\circ$ strain (BLU/0), a difference in growth between these two $\rho^\circ$ diploids was apparent (Figure 4, e vs. f), yet this difference was smaller than the difference between the haploids (Figure 4, a vs. b). Such incomplete dominance of the Leu$^\circ$ phenotype with respect to the normal growth phenotype was seen in all cases when comparing the growth of diploids formed by mating a normal-growing $\rho^+$ to normal-growing vs. Leu$^\circ$ $\rho^\circ$ strains (BLU/0 × 3Z vs. BLU/0 × 27Z; DL124 × DL125 vs. DL124 × BLU/0; data not shown). Sectors g vs. h (Figure 4) further demonstrate the incomplete dominance of Leu$^\circ$: crossing a Leu$^\circ$ $\rho^\circ$ strain (DL125) to a normal-growing (DL124) vs. a Leu$^\circ$ (16kar/0) $\rho^\circ$ strain produced diploids with a smaller difference in growth compared to that seen between the haploids (Figure 4, compare a vs. b with g vs. h). This trend was borne out in all comparisons between diploids made by mating a Leu$^\circ$ $\rho^\circ$ strain with normal-growing vs. Leu$^\circ$ isogenic $\rho^\circ$ strains (DL125 × 3Z vs. DL125 × 27Z; 16kar/0 × DL125 vs. 16kar/0 × BLU/0; data not shown). As a result, the magnitude of the difference in growth rate between Leu$^\circ$ × Leu$^\circ$ vs. normal-growing homozygous diploids (3Z × 3Z vs. 27Z × 27Z), as judged by colony size on plates lacking leucine, was the same as the difference between their haploid counterparts 3Z vs. 27Z (data not shown). In summary, Leu$^\circ$ exhibited incomplete dominance when a comparison was made of the growth of diploids formed by every combination of the normal-growing and Leu$^\circ$ strains described here. Furthermore, incomplete dominance was stable: after >100 generations of growth in the absence of leucine, the difference in colony size between the diploids shown in Figure 4, sectors e and f, was just as great as those seen in Figure 5 (data not shown).

Spontaneous conversion of yeast from the nonprion to prion state occurs at a low frequency but the opposite spontaneous transition has not been seen. I have not observed spontaneous conversion of $\rho^\circ$ or $\rho^+$ strains from normal growth to Leu$^\circ$, although the slow growth of Leu$^\circ$ strains may prevent this conversion from being observed. On the other hand, conversion of Leu$^\circ$ strains to normal growth was observed to occasionally occur and was therefore examined in detail. Plating of 3 × 10$^7$ cells from a nonselective HC liquid culture of 3Z onto plates lacking leucine gave 12 fast-growing colonies whose growth properties on HC ± leucine and YPD plates were indistinguishable from those of 27Z. Since the assumption that these derivatives arose in the last generation of liquid growth is not necessarily valid, the observed frequency with which they arise (4 × 10$^{-7}$) is an upper limit of the actual frequency of their occurrence. Plating of 9.8 × 10$^6$ cells of a second Leu$^\circ$ strain, 16kar/0, onto HC without leucine gave 25 normal-growing colonies. The upper limit of the actual conversion frequency in this second genetic background is therefore 2.6 × 10$^{-6}$.
Mitochondrial Structural Inheritance

note the tight localization of GFP to the mitochondria, which smoothly arc around the cortex of each cell. In the normal-growing \( \rho^+ \) derivative (Figure 5b), although the pattern of the mitochondria is not as smooth as that seen in the \( \rho^+ \) parent, GFP is still localized to the organelle; the cytoplasm is in most cases invisible. In contrast, the \( \rho^0 \) derivative (Figure 5c) shows a substantial amount of GFP that has failed to be localized to the mitochondrion. In multiple independent experiments no difference in shape between the mitochondria of normal-growing vs. \( \rho^0 \) strains of a given nucleotype was discernible. The difference between the tight localization of GFP to the normal-growing \( \rho^+ \) strain’s mitochondria compared to the delocalized GFP in the \( \rho^0 \) strain, however, was highly reproducible. The overall fluorescent intensity seen in microcolonies of normal-growing vs. \( \rho^0 \) strains was never seen to differ appreciably. Results similar to those seen in Figure 5, a, b, and c, were obtained by expressing Cit1p-GFP, a fusion protein destined instead for the mitochondrial matrix, in these three strains (data not shown).

The ability of strain 3Z to switch to normal growth at a low frequency provided an opportunity to further examine the correlation between the growth and cytological phenotypes. Three of the 12 normal-growing derivatives of 3Z described above were examined by mitochondrial microscopy (Figure 5, d, e, and f). All three converted to the high degree of GFP localization seen in 27Z (see Figure 5b). The \( \rho^0 \) growth phenotype is therefore well correlated with the failure of substantial amounts of two known mitochondrial proteins to fully localize to mitochondria. The identification of this cytological phenotype as an additional property of \( \rho^0 \) strains further supports the hypothesis that \( \rho^0 \) is caused by a structural alteration of the mitochondrion.

DISCUSSION

Yeast strains lacking all or part of the mitochondrial genome have long been known to be incapable of growth on nonfermentable carbon sources. I describe here a second heritable change that often, but not always, accompanies the partial or complete loss of mtDNA. Affected strains grow slowly relative to the \( \rho^+ \) parent and require leucine for optimal growth. These phenotypes appear to be the result of a heritable alteration in mitochondrial structure because:

1. Half of the spontaneous \( \rho^0 \) derivatives from one \( \rho^+ \) strain (strain 10507) exhibited \( \rho^0 \), an incidence far higher than could be explained by nuclear mutation.
2. The frequency of \( \rho^0 \) derivatives, relative to normal-growing \( \rho^+ \) derivatives, was no greater in meiotic products from a diploid made using a \( \rho^0 \) mating partner than from a diploid made using a normal-growing \( \rho^+ \) mating partner. Again, a nonnuclear basis of the trait specifying \( \rho^0 \) is indicated.
3. A Leu\textsuperscript{p} strain was converted to normal growth when the mitochondrion of a normal-growing mating partner was transferred by cytoduction. Conversion from the Leu\textsuperscript{p} to the normal growth phenotype never occurred when there was no evidence for transfer of the mitochondrion from the normal-growing mating partner (Figure 2). Conversely, a normal-growing strain was converted by cytoduction to a Leu\textsuperscript{p} strain, but only when there was positive evidence for transfer of the mitochondrion from the Leu\textsuperscript{p} mating partner (Figure 3).

4. Fluorescence microscopy demonstrated a phenotypic difference between mitochondria of normal-growing and Leu\textsuperscript{p} \(\rho^0\) strains (Figure 5).

I conclude that \(\rho^0\) and \(\rho^-\) strains that grow normally compared to the \(\rho^+\) parent contain mitochondria that are structurally sound, whereas Leu\textsuperscript{p} strains contain mitochondria that are partially defective in function due to an as yet unidentified structural alteration of the organelle. I propose that the heritable trait of the Leu\textsuperscript{p} \(\rho^0\) and \(\rho^-\) strains be termed \([\text{Leu}^p]\) (square brackets indicating cytoplasmic inheritance) and that of the normal-growing \(\rho^0\) and \(\rho^-\) strains be termed \([\text{Leu}^+]\).

The role played by the mitochondrial genome in the structure of the organelle is only starting to be understood. Deletion of any one of five proteins disrupts the shape of the yeast mitochondrion and each of the five proteins is required for mtDNA maintenance \([Mgm1p (Jones and Fangman 1992; Shepard and Yaffe 1999; Wong et al. 2000), Mdm10p, Mdm12p (Berger et al. 1997), Mmm1p (Hobbs et al. 2001), and Fzo1p (Rappoport et al. 1998)]\). It is therefore reasonable to expect that the partial or complete loss of mtDNA could cause a mitochondrial structural change, which at some frequency is stably propagated. The nature of the structural difference between the mitochondrion of \([\text{Leu}^p]\) and \([\text{Leu}^+]\) strains is beyond the scope of this initial characterization.

The establishment of \([\text{Leu}^p]\) as a nongenetic heritable change places it in a category previously occupied only by prions and the cortical inheritance phenomena extensively characterized in the ciliates (Grimes and Aufferheide 1991) and more recently in a trypanosome (Moreira-Leite et al. 2001). The elimination of the possibility that nucleic acid is the physical basis of \([\text{Leu}^p]\), however, has a caveat: perhaps a yeast mitochondrial plasmid, the maintenance of which is independent of \(\rho\), can exist in sequence states that specify, alternately, \([\text{Leu}^p]\) and \([\text{Leu}^+]\). Such plasmids were first found in Neurospora (Collins et al. 1981) and later in other filamentous fungi (see Bertrand 2000 for review). Since \(\rho^0\) yeast mitochondria lack gene expression machinery, such a postulated yeast mitochondrial plasmid would have to evade the Leu\textsuperscript{p} and normal-growth phenotypes through the nucleus, a possibility that, although unlikely, cannot be ruled out.

The mitochondrion is intimately involved in leucine biosynthesis:

1. Since the Fe/S cluster required for function of Leu1p, a cytosolic enzyme, is synthesized in the mitochondrion and exported to the cytosol by Atm1p, \(atm\Delta\) confers leucine auxotrophy (Kispal et al. 1999). The requirement of leucine for optimal growth of \([\text{Leu}^p]\) strains could therefore be due to a failure of adequate amounts of Fe/S to reach the cytosol.

2. Leucine is required for optimal growth when a subunit of pyruvate dehydrogenase (Pda1p) is mutated. This enzyme, although not uniquely involved in leucine biosynthesis, is located in the mitochondrial matrix and also influences the stability of mtDNA (Wenzel et al. 1992).

3. The first unique step in leucine biosynthesis is catalyzed by \(\alpha\)-isopropylmalate synthase, an enzyme encoded by LEU4 (Baichwal et al. 1983) and LEU9 (Casalone et al. 2000). leu4 is synthetic with each of leu5 (Baichwal et al. 1983), leu6, leu7, leu8 (Drain and Schimmel 1988), and leu9 (Casalone et al. 2000) for complete leucine auxotrophy. The proteins encoded by leu6, leu7, and leu8 have not yet been identified. The recent identification of Leu5p as the transporter of CoA (or a precursor thereof) into mitochondria (Prohl et al. 2001) explains the absolute requirement of leu5-leu5 strains for leucine since the remaining \(\alpha\)-isopropylmalate synthase, encoded by LEU9 (Casalone et al. 2000) and reported to be localized exclusively to the mitochondrion, requires higher mitochondrial CoA levels for function than can be achieved in leu5 strains (Prohl et al. 2001). Furthermore, the curious pet-like phenotype of leu5 strains (Drain and Schimmel 1986) is also specified by leu6 (Drain and Schimmel 1988), thus implicating leu6 as well in mitochondrial function.

The mitochondria of two yeast mating partners, which form a true diploid zygote, have been convincingly demonstrated to rapidly fuse to form a single reticulum: proteins that reside in the mitochondrial inner membrane (Nunnari et al. 1997; Okamoto et al. 1998), outer membrane (Okamoto et al. 1998), and matrix (Azpiroz and Butow 1993; Okamoto et al. 1998) from one partner of mating pairs were found to be distributed throughout the entire mitochondrion of zygotes well before the emergence of the first bud. My interpretation of the cytoduction data in Figures 2 and 3 assumes that in the heterokaryotic zygotes formed in cytoductive matings, the mitochondria contributed by the two mating partners remain separate. This assumption is difficult to reconcile with such rapid protein homogenization. However, it appears that the true diploid zygotes examined in the protein mixing studies behave differently with respect to mitochondrial mixing than do the heterokaryotic \(kar1 \times KARI\) zygotes used in cytoductions. Indeed, the first detailed study of the use of \(kar1\)
as a tool for shuttling mtDNA into different nuclear backgrounds found that in \( KAR1 \rho^+ \text{cap}^+ \text{oli}^+ \text{par}^+ \times kar1 \rho^- \text{cap}^+ \text{oli}^+ \text{par}^+ \) crosses (cap, oli, and par are mtDNA loci), there was a strong tendency of a bud of a given nucleotype to retain the mitochondrial genotype with which it was originally associated. In addition, that study showed \( kar1 \) to cause a low degree of recombination between mitochondrial markers (Table 6a of Lancashire and Mattoon 1979). A second study yielded similar findings (Sena 1982). In contrast, rapid mixing of parental mitochondrial genomes—allowing substantial formation of nonparental mitochondrial genotypes by recombination—is well established to occur in true diploid zygotes (reviewed by DuJon 1981). The maintenance, in \( kar1 \times KARI \) zygotes, of two distinct mitochondria, which are concluded here to exist in alternative structural states, is therefore plausible.

These earlier studies demonstrating coinheritance of nuclear and mitochondrial genotypes in \( kar1 \) crosses (Lancashire and Mattoon 1979; Sena 1982) imply that in heterokaryotic zygotes each of the haploid nuclei remain tethered to their respective parental mitochondria, which also remain unfused. Such an interpretation explains why in the \( \rho^+ [Leu^+] \times \rho^- [Leu^-] \) crosses discussed here (data not shown), no cytoduction of \( [Leu^-] \) was observed. In contrast, when one of the parents was instead \( \rho^- [Leu^+] \) (Figure 2) or \( \rho^- [Leu^-] \) (Figure 3), the trait could be cytoduced from that strain to a \( \rho^+ \) mating partner. This difference implies that the inheritance advantage of \( \rho^- \) mitochondria compared to \( \rho^+ \) mitochondria in \( kar1 \times KARI \) zygotes overrides the association of the parental mitochondrial with their respective haploid nuclei. The well-established ability for \( \rho^- \) genomes to be cytoduced into \( \rho^+ \) hosts is also consistent with the preferential inheritance of \( \rho^- \) over \( \rho^+ \) mitochondria. Moreover, \( \rho^- [Leu^+] \) mitochondria are apparently far better at displacing \( \rho^+ [Leu^-] \) mitochondria (Figure 2) than \( \rho^- [Leu^-] \) mitochondria are at displacing those that are \( \rho^+ [Leu^+] \) (Figure 3). This asymmetry suggests a lower degree of tethering between the nucleus and \( [Leu^-] \) vs. \( [Leu^+] \) mitochondria. The coinheritance of nucleus and mitochondrion is also suggested by a completely different line of investigation: Fisk and Yaffe (1997) identified three distinct classes of alleles of \( mtdm1 \), which affect the inheritance of the nucleus, the mitochondrion, or both. The segregation of the two types of organelle therefore shares at least one component and more extensive overlap in segregation mechanisms is plausible. Protein mixing experiments using heterokaryotic zygotes would be quite informative.

In the first publication describing \textit{petite}, small colonies were shown to give rise, on rare occasion, to what were termed “revertants” (Ephrussi et al. 1949). Such reversion was the subject of an entire subsequent article (Ephrussi and Hottinguer 1951) and was also discussed in detail in a monograph (Ephrussi 1953; reprinted in Ephrussi 1999). Since \textit{petite} strains are now known to have lost mtDNA, their reversion to respiratory competence is impossible. Indeed, the respiratory capacity of yeast was not measured in those earliest studies of \textit{petite}. In light of the present report, it is likely that two changes occurred in Ephrussi’s strains to cause \textit{petite}: the loss of mtDNA and the appearance of \([Leu^-] \). The reversion described by Ephrussi \textit{et al.} (1949) appears to be analogous to the conversion of strains \( 3Z \) and \( 16kar/0 \) from \([Leu^-] \) to \([Leu^+] \). Finally, the description here, to my knowledge for the first time since 1953, of the existence of \( \rho^+ \) and \( \rho^- \) derivatives that grow as well as do their \( \rho^+ \) parents, is consistent with the preference of yeast for fermentative over respiratory growth (Lagunas 1986).

Two distinct aspects of \([Leu^-] \) warrant examination at the molecular level. First, what accounts for the phenotypic differences between \([Leu^-] \) and \([Leu^+] \) strains? The partial leucine requirement may be the result of a slowing of either the Leu1p-catalyzed or the Leu4p/Leu9p-catalyzed steps of leucine biosynthesis. Both steps involve transport across the mitochondrial membranes, a process that the data of Figure 5 suggest may be defective. Second, why is \([Leu^-] \) heritable? That is, how is the structural difference between \([Leu^-] \) and \([Leu^+] \) mitochondria propagated? The incomplete dominance of \([Leu^-] \) and \([Leu^+] \) and their independence of Hsp104p suggest the prion model not to be applicable. On the other hand, the ability of \([Leu^-] \) strains to be converted at a low frequency to those with normal growth properties is consistent with a prion model, but is also consistent with other models in which two states interconvert. One such model is the positive feedback loop first proposed by Novick and Weiner (1957). Interconversion is inconsistent with models wherein \([Leu^-] \) is caused by the loss of structural information. For example, the activities of at least two enzymes, which function exclusively in the mitochondrial matrix—Hsp60p (Cheng et al. 1990) and Yah1p (Lange et al. 2000)—are required for the production of additional active enzyme. The loss of proper localization of either of these proteins is therefore an irreversible change. Models of higher complexity that involve proteinaceous and/or membraneous supramolecular assemblies capable of adopting alternate conformational states that correspond to \([Leu^-] \) and \([Leu^+] \) can also be envisioned. If a complex situation of this type holds for \([Leu^-] \), it may be necessary to identify new examples of nonprionic structural inheritance that are more amenable to molecular characterization before \([Leu^-] \) is understood at that level.

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