Interorganelle Signaling Is a Determinant of Longevity in *Saccharomyces cerevisiae*

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ABSTRACT

Reproductive capacity, which is the number of times an individual cell divides, is the measure of longevity in the yeast *Saccharomyces cerevisiae*. In this study, a process that involves signaling from the mitochondrion to the nucleus, called retrograde regulation, is shown to determine yeast longevity, and its induction resulted in postponed senescence. Activation of retrograde regulation, by genetic and environmental means, correlated with increased replicative capacity in four different *S. cerevisiae* strains. Deletion of a gene required for the retrograde response, RTG2, eliminated the increased replicative capacity. RAS2, a gene previously shown to influence longevity in yeast, interacts with retrograde regulation in setting yeast longevity. The molecular mechanism of aging elucidated here parallels the results of genetic studies of aging in nematodes and fruit flies, as well as the caloric restriction paradigm in mammals, and it underscores the importance of metabolic regulation in aging, suggesting a general applicability.

**AGING** is characterized by loss of function and an exponential increase in mortality rate (Finch 1990). Many model systems are used to study the phenomenon of organismal aging (Jazwinski 1996). There are four broad themes that emerge from studies in these model systems. These themes relate metabolic activity, resistance to stress, gene dysregulation, and genetic stability to determination of longevity (Jazwinski 1996). The first two of these physiological responses, metabolic activity and stress resistance, may be intertwined. Aerobic metabolism carries with it the risk of reactive oxygen species production, which elicits a stress response. The response to oxidative stress involves some of the same effectors as other stress responses, for example the heat shock response (Sanchez et al. 1992; Davidson et al. 1996). The life-span-extending effect of transient exposure to sublethal heat stress is well known. In the fruit fly *Drosophila* melanogaster, brief exposure to heat shock to induce thermotolerance extends lifespan (Khazaie et al. 1997). A similar effect is seen in the nematode *Caenorhabditis elegans* (Lithgow et al. 1995). The induction of the stress response appears to provide benefits that extend beyond survival of the initial insult, suggesting a beneficial effect on longevity of enhanced stress resistance.

In contrast to the involvement of stress resistance, the role of metabolic activity in determining longevity has been less clear. This has been changing recently. Many *C. elegans* mutants that affect life span have been isolated (reviewed in Jazwinski 1996). Most of these mutants are in genes of the daf pathway (Kenyon et al. 1993; Larsen et al. 1995; Morris et al. 1996; Kimura et al. 1997; Lin et al. 1997; Ogg et al. 1997), and even the first *C. elegans* longevity gene identified by mutation, age-1 (Klass 1983; Friedman and Johnson 1988), has been found to reside in this pathway (Dorman et al. 1995; Larsen et al. 1995). The daf pathway is involved in the formation of a dispersed form of *C. elegans*, called the dauer larva, in response to starvation and stress. Many of the longer-living daf mutants also have an increased ability to survive stress as adults (Lithgow et al. 1995; Murakami and Johnson 1996). The daf-2, daf-23, and daf-16 genes form a genetic pathway for longevity (Kenyon et al. 1993; Larsen et al. 1995). The daf-2 gene, at its head, encodes a homologue of the insulin/IGF-1 receptor (Kimura et al. 1997). This suggests a link of nutritional responses and metabolic activity to aging. Extension of life span and increased stress resistance may result from partial activation of the daf pathway, which appears to be involved in metabolic regulation.

The correlation between metabolic activity, stress response mechanisms, and longevity also extends to mammals. Caloric restriction is a mechanism by which the life span of rodents can be extended up to 50% (reviewed in Richardson and Pahlavan 1994; Masoro 1995). Calorie-restricted rats have an increased ability to survive heat stress in old age (Heydari et al. 1993). The increased survival may result from the capacity of these animals to respond to stress by more efficiently activating heat-shock transcription factor 1 (Heydari et al. 1996). Calorie-restricted animals also display important metabolic changes, such as reduced blood glucose and...
insulin (reviewed in Masoro 1995), and they display increased insulin receptor levels and increased activity of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (Pahlavan et al. 1994; Van Remmen et al. 1994).

Central in the control of metabolic activity are mitochondria, because they are the major source of energy during aerobic metabolism. They are also a potential internal source of stress to cells. Mitochondria have been implicated in mammalian aging in many studies, mainly in their capacity to generate oxidative stress (reviewed in Sohal and Weindruch 1996; Miguel 1998). Mitochondria are the site where respiration occurs and therefore are the major source of reactive oxygen species in cells. Antioxidant defenses are not always sufficient to completely protect cells from oxidative damage. Protein, lipid, and DNA damage caused by free radicals have been detected at higher frequency in old compared to young. However, it is unclear whether the amount of damage is large enough to account for the decline in mitochondrial function associated with aging. Although loss of respiratory function during aging has been documented (Linnane 1992; Muller-Hocker et al. 1992), it is not certain how it impinges on tissue function (Brierley et al. 1997; Hagen et al. 1997; Tengen et al. 1997). It is possible that alterations in mitochondrial activity have effects on aging that are more subtle than simply the loss of respiratory capacity.

We are using the budding yeast Saccharomyces cerevisiae as a model to study aging. The life span of yeast is measured not by time but by the number of daughter buds a cell produces (Mortimer and Johnston 1959; Muller et al. 1980). Yeasts display an array of changes during their life span, some of which are clearly decremental (reviewed in Jazwinski 1993). This and the exponential increase in mortality as they proceed through their life span (Pohley 1987; Jazwinski et al. 1989) indicates that they age. Application of measured heat shock can extend the yeast life span, similar to the effect in fruit flies and nematodes (Shama et al. 1998). Furthermore, stress and starvation resistance has been used as a means to select for mutants with longer life spans in yeast (Kennedy et al. 1995). Extension of life span in yeast is inextricably tied to increased metabolic activity, because it entails marked increase in the expenditure of energy to produce the additional daughter cells that are the measure of longevity. Several genes that affect life span in yeast have been characterized (reviewed in Jazwinski 1996). Of these genes, one of the most thoroughly characterized, RAS2, is involved in the response to nutritional status and in the modulation of stress responses (Marchler et al. 1993; Tatchell 1993). Disruption of RAS2 shortens yeast life span, while overexpression extends it (Sun et al. 1994). The product of the RAS2 gene is a G protein known to be involved in signal transduction.

Here, we present evidence that a signal from the mitochondrion to the nucleus, termed retrograde regulation, influences longevity in S. cerevisiae. We show that activation of this signal extends replicative capacity and delays senescence. Furthermore, we show that this signal and the corresponding extension in life span is dependent on RAS2.

**Materials and Methods**

**Yeast strains and media:** The strains used in this study were as follows: YPK9 (MATa ade2-101 ORI his3-Δ200 leu2-Δ1 lys2-801 ORI trpl-Δ63 ura3-52), a haploid derivative of YPH501 (supplied by P. Hieter, The Johns Hopkins University, Baltimore, MD); YSK365 (MATa ade2-101 ORI his3-Δ200 leu2-Δ1 lys2-801 ORI trpl-Δ63 ura3-52 Δp′)), an ethidium bromide-induced petite derived from YPK9; SP1-1 (MATa leu2 trpl ade8 can1 Δhis3 gal2), a derivative of SP1 (from M. Wigler, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY); W303-1A (MATa can1-100 ade2-200 his3-Δ11-Δ15 leu2-3-112 trpl-1 ura3-1), from R. Fuller (Stanford University Medical Center, Stanford, CA); A364A (MATa ade1 ade2 ura1 his7 lys2 tyr1 gal1 SUC mal), from T. Weinert (University of Arizona, Tucson, AZ); YPK25 (MATα kar1 ade2-1 his4-Δ15 can′) was generated by mating strain JC25 (MATα kar1 ade2-1 his4-Δ15 can′ Δp′), from the Yeast Genetic Stock Center (Berkeley, CA) with YPK9, removing buds, and selecting for uracil prototrophy and respiratory function. Respiratory function was assayed by the ability to grow on media containing glycerol as the carbon source (YPG: 2% peptone, 1% yeast extract, 2% galactose) or YPR (2% peptone, 1% yeast extract, 2% glucose) or YPR (2% peptone, 1% yeast extract, 2% raf®nose). For selection of transformants or cytoductants, yeast cells were cultured at 30°C in YPD (2% peptone, 1% yeast extract, 2% glucose) or YPR (2% peptone, 1% yeast extract, 2% raf®nose). For selection of transformants or cytoductants, yeast cells were cultured on SC medium (0.67% yeast nitrogen base with ammonium sulfate, 2% glucose, and all required nutrients except those needed for selection).

**Life-span analysis:** Life-span analyses were performed (Egli-Mezei and Jazwinski 1989) by spotting 1 μl of logarithmically growing cells from liquid YPD or YPR onto YPD or YPR plates (2% agar). (For the experiment involving overexpression of SOD1 and CCT1, cells were grown in YPR and then spotted onto YPR plates containing 2% galactose.) Individual unbudded cells were then separated from the population by micromanipulation and allowed to produce buds. These buds were removed and used as the starting population for life-span analysis. For each successive bud removed from these cells, they were counted one generation older. Cells were grown at 30°C during the day and at 12°C overnight. Growth at lower temperature does not affect replicative life span (Muller et al. 1980). Statistical analyses of life spans were performed using the nonparametric Mann-Whitney test.

**Cytoduction:** YSK365 (an ethidium bromide-induced petite coisogenic to YPK9) was mated with strain YPK25, which contains a nuclear mutation in the Kar1 gene (Conde and Fink 1976). Kar1 mutant strains are able to form zygotes with cells of the opposite mating type, but are dominant negative for karyogamy. This results in a zygote with two separate nuclei. Buds produced from these fused cells receive the nucleus of only one of the parent strains and a mixture of cytoplasm from both and are called cytoductants. The cytoductants were screened for the auxotrophic markers present in YPK9 and YSK365 and also for the ability to grow on glycerol. All progeny produced in this way were able to grow on medium containing glycerol as the carbon source (YPG), indicating replacement of the mitochondrial DNA.

**Plasmids and yeast transformation:** Overexpression of SOD1
and CT1 was accomplished by cloning these genes into plasmid pBM150-ADH to place them under the control of the galactose-inducible promoters GAL1 and GAL10. pBM150-ADH is a derivative of pBM150 (Johnston and Davis 1984) containing the transcription terminator of the yeast ADH2 gene (provided by L. Hyman, Tulane Medical Center, New Orleans) cloned into the BamHI site. SOD1 was amplified from total yeast genomic DNA, cloned into the vector pCR2.1 (Invitrogen, San Diego), and sequenced to insure no mutations were introduced during amplification. An EcoRI fragment containing SOD1 was then cloned into the EcoRI site of pBM150-ADH. This plasmid was then cut with XbaI (an XbaI site is contained upstream of the ADH2 terminator) and ligated to a CT1 BsaAI/BamHI fragment from pBR322-7309 (Sperck et al. 1983), to which XbaI linkers had been added. To delete RTG2, the oligonucleotide primer pairs 5′-GGGATCCGATATAGTTGAATG-3′ and 5′-GACGAGCTCACTAATCTTATCATTCAAG-3′ were used to amplify regions flanking RTG2. The amplified flanking regions were then cloned in inverse orientation into prS406 (Sikorski and Hieter 1989). This plasmid was linearized with BamHI and used to replace RTG2 by gamma deletion by transforming YPK9 and YSK365 to histidine prototrophy.

Similar strategies were used to delete COX4 and CIT2. For COX4, the primer pairs 5′-ATACTCTAGATGAGAAAAGAAGAAG AACTACCAG-3′, 5′-CTTAAAGCTTTTCTCATATGGCAAC-3′ and 5′-GACGAGCTCAGTCTTATCATTCAAGTGCTGCC-3′ were used to amplify flanking regions, which were cloned into prS406 (Sikorski and Hieter 1989). This plasmid was linearized with XbaI before transformation of yeast. For CIT2, the primer pairs 5′-CCCCGCGCCGCCCCTCTCTTTGGGTTCTTTCACTAAAAGTG-3′, 5′-GAGAATCTGTATGATGTTATCCTTCTGGTATTTG-3′ and 5′-CTCTTTTTACACCCCTGTCGCC-3′, 5′-GGGAGGCGGCGCGGGGGAGATG-3′ were used to amplify flanking regions, which were cloned into prS406. This plasmid was linearized with AciI before transformation of yeast.

The plasmid used to disrupt RAS2, pRa530 (Tatchell et al. 1994), contains a 3.8-kb PstI restriction fragment encoding LEU2 inserted in a unique PstI site of the RAS2 coding sequence such that a null mutant is generated. This plasmid was cut with XbaI and HindIII prior to transforming yeast. Yeast transformations were performed using the lithium acetate method (omitting the carrier DNA; Ausubel et al. 1994). Deletions and disruptions were confirmed by Southern blot analysis (Ausubel et al. 1994).

**RNA preparation and Northern blot analysis:** RNA was prepared from yeast cells growing logarithmically in either YPD or YPR by extraction with hot acidic phenol, as described by Ausubel et al. (1994). Samples (10 μg) were electrophoresed in 1% agarose gels containing formaldehyde. RNA was transferred to nylon membranes and immobilized by irradiation with UV light. Membranes were prehybridized for 2 hr at 42°C in 50% formamide, 5× SSC (1× SSC = 150 mm NaCl, 15 mm sodium citrate, pH 7.0), 0.5% SDS, 0.1% sodium pyrophosphate, 50 mm sodium phosphate (pH 7.0), 0.5 mg/ml heparin, 0.1 mg/ml single-stranded salmon sperm DNA, and then probed under the same conditions for 16 hr with a fragment of CIT2 obtained by PCR using the primers 5′-GCGAAATCTACCACATGCTC-3′ and 5′-TAGTGTGCGCCGCAACAG-3′. Twenty nanograms of the CIT2 PCR product was labeled with [α-32P]dCTP by random oligonucleotide priming using the Rediprime kit (Amersham, Arlington Heights, IL). The membrane was washed twice in 1× SSC, 0.5% SDS for 30 min at room temperature and two times in 0.2× SSC, 0.1% SDS for 30 min at 42°C. Hybridization and wash conditions were sufficiently stringent to discriminate between CIT2 and the homologous CIT1. Northern blots were analyzed on a PhosphorImager using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

**RESULTS**

**Mitochondrial petites have a longer life span:** In the course of performing life-span analyses, it was noted that a yeast strain, YPK9, often displayed what appeared to be an extremely long-lived subpopulation of cells (Figure 1A). To determine if these long-lived cells represented a separate population or merely random variations in life span, daughter cells of the long-lived cells were removed and allowed to grow to colonies. Upon examination of these colonies it was immediately apparent that they were different. YPK9 contains the ade2 marker, which causes cells to accumulate a red pigment on YPD plates (Roman 1956). The strains generated from the long-lived YPK9 cells formed colonies that did not accumulate this red pigment. We have previously noted and have found reference to the fact (Reaume and Tatum 1949) that respiration-deficient petite strains containing an ade2 mutation do not form the red pigment. The strains derived from the long-lived cells of YPK9 were streaked on medium containing glycerol, a nonfermentable carbon source, rather than glucose. They were unable to grow on glycerol, indicating that they were respiration-deficient petites (hereafter referred to as petites) that lack fully functional mitochondria. The petite strains had longer life spans than did the respiration-competent grande strain YPK9. A representative life-span analysis is shown in Figure 1B for a petite strain that arose spontaneously from YPK9. Growth of cells on glycerol prior to initiation of life-span analysis eliminated the appearance of long-lived petites in the aging cohort, indicating that they preexist in the population and that there is no increase in the generation of petites during aging.

Petites were induced in several unrelated strains, to ascertain whether life extension in petites occurred in other genetic backgrounds. The strains were grown in liquid medium supplemented with ethidium bromide, which causes loss of mitochondrial DNA (Goldring et al. 1970). Single colonies were isolated and then screened for the ability to grow on a nonfermentable carbon source. Two petite isolates in each background were selected for further analysis. The life spans of the petite strains were compared to their parent grande strains. A variety of results ranging from substantial extension to extreme shortening of life span were seen. The petites isolated from YPK9 again showed extended life span (Figure 1C). What is more, the petites continued dividing at a rapid rate, characteristic of young cells (Egilmez and Jazwinski 1989), much longer than the grande (Figure 2). This indicates that their senescence was postponed (Sun et al. 1994). Petites isolated from the SP1-1 and A364A strains
Figure 1.—Life spans of grande and petite strains with glucose as a carbon source. •, the grande strain; ○, the coisogenic petite. (A) YPK9: the arrow indicates the point at which daughter cells were removed from the long-lived subpopulation to establish the strain used in B. (B) A YPK9 petite strain (mean life span 26.8) derived from the longer-lived subpopulation in A has an extended life span ($P < 0.001$) compared to YPK9 (mean, 19.8). (C) An ethidium bromide-induced YPK9 petite (YSK365; mean 27.3) has an extended life span ($P < 0.01$) compared to YPK9 (mean 20.7). (D) An SP1-1 petite (mean 15.0) has a shortened life span ($P < 0.001$) compared to SP1-1 (mean 22.4). (E) W303-1A (mean 23.0) and W303-1A petite (mean 22.2) show no difference in life spans ($P = 0.24$). (F) An A364A petite (mean 14.8) has a shortened life span ($P < 0.001$) compared to A364A (mean 21.9).

showed a shortened life span (Figure 1, D and F), while those isolated from W303-1A showed no change (Figure 1E).

Respiration does not directly affect longevity: The best predictor of mortality is life span itself. Manipulations that extend life span identify processes that are limiting for longevity, unlike life span-shortening treatments that may simply reveal factors that decrease viability. We therefore chose to investigate the mechanism of extension of life span by petites in the YPK9 background.

Several ethidium bromide-induced petites were generated independently in the YPK9 background; they all displayed extended life spans similar to those in Figure 1, B and C. One ethidium bromide-induced petite strain isolated in YPK9, YSK365 was used for further analysis. First, to insure that the extension of life span was due to the loss of mitochondrial DNA and not to chromosomal mutations that might be caused by the ethidium bromide treatment, mitochondrial DNA was added back to YSK365 by cytoduction (Condé and Fink 1976). Lifespan analyses of the cytoductants showed that the mean life span was returned to that of the parent strain YPK9 (Figure 3), confirming that the extension of life span was due to the loss of mitochondrial DNA. As shown in Figure 1, the process of cytoduction, as such, does not affect life span, because it had no effect on the longevity of the parent grande strain.

Loss of mitochondrial DNA prevents cells from respiring by eliminating components of the electron transport chain. Respiration may result in production of reactive...
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Figure 3.—Replacement of mitochondrial DNA returns the life span of a petite to that of its parent grande strain. Mitochondrial DNA was returned to the petite strain YSK365 (▼; mean 23.7) by cytoduction. The cytoductant's (▼) life span (mean, 19.0) does not differ (P = 0.4) from the grande parent strain YPK9 (●; mean 18.3). The procedure used to return mitochondrial DNA has no effect (P = 0.4) on life span when performed on YPK9 (○; mean 18.2).

Oxygen species that cause attenuation of life span. However, life-span analyses were performed on media containing glucose as a carbon source. In the presence of glucose, yeast derive most of their energy through glycolysis, and respiration is repressed (Gancedo and Serrano 1989). Because loss of mitochondrial DNA in YPK9 caused an increase in life span, we wanted to determine if the increase was due to a direct effect on respiration. To inhibit respiration, life-span analyses were performed on medium containing antimycin A, an inhibitor of QH₂-cytochrome c reductase. The concentration of antimycin A used was sufficient to completely inhibit growth on plates containing glycerol as a carbon source. Antimycin A at that concentration had no effect on the mean life spans of YPK9 or YSK365 on glucose (Figure 4A). It might be reasoned that petites would generate less oxidative stress than grandes (Guidot et al. 1993; Longo et al. 1996). To determine whether reactive oxygen species were responsible for the shorter life span of YPK9 (grande) grown on a fermentable carbon source, the genes for both superoxide dismutase (SOD1) and cytoplasmic catalase (CTT1) were overexpressed. This overexpression also had no effect on life span (Figure 4B).

Retrograde regulation is the molecular mechanism for life extension: Because the extension of life span did not appear to be simply due to the inability to respire, we searched for another mechanism to explain our results. Altered patterns of nuclear gene expression have been reported in yeast that lose large portions (p⁻) or all (p₀) of their mitochondrial DNA (Parikh et al. 1987). This apparent communication between the mitochondrial and nuclear genomes has been termed retrograde regulation (Liao and Butow 1993). The gene used as a reporter of retrograde regulation is CIT2, encoding peroxisomal citrate synthase, which can be transcribed as much as 30-fold higher in p⁻ or p₀ petites (Liao et al. 1991).

RNA was isolated from the four grande strains used for life-span analyses and their coisogenic petites. Northern blot analysis revealed that expression of CIT2 was increased only in the petites generated from YPK9 (Figure 5A). Thus, extension of life span correlated with activation of retrograde regulation in the YPK9 background. It has been reported that the induction of retrograde regulation is not always detected on media containing glucose as a carbon source (Liao et al. 1991). To determine if retrograde regulation could be activated in all backgrounds, RNA was isolated from the strains and their petites after growth in medium containing raffi-
Figure 5.—Expression of the retrograde regulation reporter CIT2 in petites is dependent on genetic background and environmental conditions. RNA was prepared from the indicated strains grown on (A) glucose or (B) rafínose. (C) A comparison of the W303-1A grande grown on glucose or rafínose. Northern blots of total RNA were probed with CIT2 and then stripped and reprobed with ACT1. The increase in CIT2 mRNA in petites is calculated from quantitation of phosphorimages corrected for loading errors by normalizing to ACT1, which is unchanged by carbon source or respiratory state of the cell (Szekely and Montgomery 1984; Parikh et al. 1987).

Figure 6.—Life spans of grande and petite strains with rafínose as a carbon source. (A) YPK9 petite, YSK365, (mean 28.3) has an extended life span (P < 0.001) compared to YPK9 (mean 17.6). (B) An SP1-1 petite (mean 29.4) has an extended life span (P < 0.01) compared to SP1-1 (mean 21.1). (C) A W303-1A petite (mean 25.8) does not differ in life span (P = 0.4) from W303-1A (mean 28.5). (D) An A364A petite (mean 16.3) does not differ in life span (P = 0.38) from A364A (mean 15.9).
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Figure 8.—CIT2 is not required for extension of life span in petites. The life span of YPK9 (●; mean 18.2) is unchanged (P = 0.34) by the introduction of a CIT2 deletion (▲; mean 18.0). The life span of the coisogenic petite YSK365 (○; mean 27.3) is also unchanged (P = 0.4) by the introduction of a CIT2 deletion (▽; mean 27.3).

span seen with SP1-1 petites when raffinose is used as a carbon source is dependent on the activation of the retrograde response and not simply on the alternate carbon source. Furthermore, carbon source itself (glucose vs. raffinose) does not affect the life span of YPK9 (grande) or its coisogenic petite (YSK365; Figures 1C and 6A).

Retrograde regulation is defined as a signaling pathway from the mitochondrion to the nucleus that results in the Rtg1p/Rtg2p/Rtg3p-dependent activation of genes. CIT2 is one of these genes, and it is used routinely as the diagnostic. One simple explanation for the increased longevity of strains in which the retrograde response has been activated is that before activation the levels of CIT2 expression are limiting. If this were the case, activation of retrograde regulation would allow the extension of life span. To determine whether CIT2 is required for the extension of life span, the CIT2 gene was deleted from both YPK9 and the coisogenic petite YSK365, and life-span analyses were performed. The results show no change in life span of YPK9 or YSK365 on deletion of CIT2 (Figure 8). Thus, CIT2 is dispensable for extended longevity in the petite.

Nuclear petites also activate retrograde regulation and increase replicative capacity: To ascertain whether loss of mitochondrial DNA is essential for life extension, a nuclear petite mutation was derived by deleting COX4. Loss of the COX4 gene product, subunit IV of cytochrome oxidase, results in complete loss of respiratory capacity (Poyton and McEwen 1996). COX4 deletion mutants were isolated from YPK9, and the ability to activate the retrograde response was assayed. Another treatment previously reported to activate the retrograde response is inhibition of respiration by antimycin A (Liao et al. 1991). Because we had previously shown...
The life span of the petite strain YSK365 (mean 29.9) is reduced (P < 0.001) when a disruption of RAS2 is introduced into the strain (mean 16.5). The life span of the ras2 petite does not differ (P = 0.8) from the coisogenic grande strain (YPK9).

A nuclear petite exhibits partial activation of RAS2 disruption (mean 16.3). Disruption of RAS2 shortens the retrograde response and an intermediate extension of life span (P < 0.01) relative to the coisogenic control YPK9 span.

Strains were grown on media containing glucose (mean 22.8). Similar results were obtained with additional mitotic petite YSK365 petites. (B) Strains were grown on media containing glucose and Northern blot analysis was performed as described in Figure 5.

To ensure that deletion of COX4 did not cause destabilization and loss of mitochondrial DNA and, consequently, activation of the retrograde response, a cox4 strain was assayed for the presence of a complete mitochondrial genome. To do this, 30 independent colonies of YPK9 cox4 were mated with a COX4 petite strain. These mixtures were then plated on media containing glycerol as a carbon source to assay for respiratory competence. While neither YPK9 cox4 or the COX4 petite strain could grow on media containing glycerol as the sole carbon source, the cox4 petite strain grew on glycerol as the sole carbon source.

The extension of life span was proportional to the extent to which the retrograde response was induced. In fact, the magnitude of the extension of life span in two independent cox4 strains, we also isolated RNA from YPK9 cells grown in the presence of antimycin A. Northern blot analysis demonstrated that treatment with antimycin A induced CIT2 expression to a very small degree (1.3-fold), in close agreement with previous findings (Liao et al. 1991). Deletion of COX4 showed a more substantial 2.1-fold activation of the response. However, this was only half the level of CIT2 expression in the petite, YSK365, which was induced 4.2-fold in this experiment (Figure 9A).

Because deletion of COX4 did activate the retrograde response, its influence on life span was also assayed. The life spans of the cox4 strains were significantly extended, but not to the magnitude of the cytoplasmic petite YSK365 (Figure 9B).
source, all of the strains resulting from the mating were able to grow. This result verifies that induction of the retrograde response in YPK9 cox4 was not due to loss of mitochondrial DNA.

**Retrograde regulation is dependent on RAS2:** We have previously shown that disruption of the RAS2 gene causes a decrease in life span (Sun et al. 1994). RAS2 is involved in the response of the yeast cell to nutrient availability (Tatchell 1993). Furthermore, null mutants of RAS2 grow very poorly on nonfermentable carbon sources (Fraenkel 1985; Tatchell et al. 1985), indicating a connection between RAS2 and mitochondrial function. With the associations between life span, mitochondria, and RAS2 in mind, we decided to investigate whether RAS2 influenced the retrograde regulation-dependent extension of life span. Disruption of RAS2 in the petite strain YSK365 resulted in complete abrogation of life span extension (Figure 10A). Not only was the extension eliminated, life spans of petite strains containing disruptions of RAS2 were reduced to those of the ras2 grande strain. Northern blot analysis of the CIT2 transcript demonstrates that disruption of RAS2 limits CIT2 expression in the petite strain (YSK365 ras2) to the level in the parent grande (YPK9 RAS2; Figure 10B). In fact, RAS2 may have an effect on the constitutive expression of CIT2, as a comparison of YPK9 and YPK9 ras2 would suggest (Figure 10B). This is the first demonstration of the involvement of RAS2 in retrograde regulation.

**DISCUSSION**

We have shown that a petite yeast, cytoplasmic or nuclear, that lacks fully functional mitochondria has a longer life span than its coisogenic grande parent. The life extension correlates directly with the capacity to activate retrograde regulation. Abrogation of retrograde regulation (rtg2Δ) eliminates the life extension without compromising the processes that establish the basal life span. Thus, activation of the retrograde response is necessary for the life extension, and it is also the factor limiting for longevity. We have also shown that RAS2, a longevity gene (Sun et al. 1994), modulates the retrograde response. These results demonstrate that interorganelle communication of metabolic signals is one of the mechanisms that determines yeast longevity.

The life extension observed is not due to loss of respiratory capacity per se, suggesting that it is not due to an effect on the production of oxidants. However, this does not mean that yeast are immune to the effects of oxidative damage. The effects of such damage on yeast longevity may be possible to detect during growth on a nonfermentable carbon source. Because of their ability to derive energy through fermentation, yeast provide a unique opportunity to study factors influencing longevity independent of the confounding effects of oxidative damage.

The retrograde response is induced in petite yeast, although in some cases this induction requires growth on raffinose rather than glucose for it to be uncovered. The life-span extension associated with this induction can be complicated in certain strains (SP1-1, A364A) by other effects of the carbon source. In addition, the retrograde response appears to be constitutively active in some strains (W303-1A). The variation between strains of S. cerevisiae is not without precedent and has often been found to be the result of single gene mutations, as in the cases of filamentous growth (Liu et al. 1996) and copper transport (Knight et al. 1996), for example. Although the effects in different strains vary in degree, activation of the retrograde response increases replicative capacity in every genetic background tested relative to the nonactivated control. The ability to manipulate the retrograde response independently by both genetic and environmental means and the associated effects this has on longevity provides the most direct causal evidence for a molecular mechanism of aging.

A direct comparison of the increase in life span relative to the magnitude of activation of the retrograde response can be performed within a genetic background. The life-span extensions in YPK9 cox4 and YPK9 ρ0 petites directly correlated with the levels of CIT2 expression in these strains relative to YPK9 grande. Activation of the retrograde response when cells were grown in the presence of the respiratory inhibitor antimycin A was also apparent, but very minor. The fact that we saw no increase in life span when cells were grown in the presence of antimycin A is probably due to the weak activation of the retrograde response, but may also be due to some other effect of the drug. The degree to which the retrograde response is activated may be dependent on the nature of the deficit in the electron transport chain. We are currently examining additional respiration-deficient mutants to determine whether the correlation between the level of activation of the retrograde response and the extent of life-span increase is consistent. The results with cox4 indicate further that loss of mitochondrial DNA is not the only way in which the retrograde response can be elicited. The retrograde response is stimulated fourfold, and replicative capacity is increased 25%, in a W303-1A grande strain simply by growing it on media containing raffinose instead of glucose. The induction of retrograde regulation by growth on raffinose in strains that do not elicit this response on glucose extends or maintains their longevity, indicating that it is the induction of the retrograde response and not simply the petite that is responsible.

Few genes that are affected by the retrograde response have been described. The fact that the commonly used reporter of the retrograde response, CIT2, was not essential for the extension of life span was not necessarily surprising. Undoubtedly, there are other genes influenced by the retrograde response. The promoter of CIT2 contains sequences that have been shown...
to be required for the binding of Rtg1p/Rtg3p to activate the retrograde response (Rothermel et al. 1997). A search of the entire yeast genome for these sequences reveals no fewer than 10 genes that may be influenced by the retrograde response. A relaxation of the stringency of the search criteria reveals a larger number.

One gene that we examined that had not been previously linked to the retrograde response is RAS2. Disruption of RAS2 eliminates the extension of life span in a petite. In fact, it is epistatic. This result indicates that RAS2 or a RAS2-dependent function modulates the effect of the mitochondrial signal on longevity. Although other interpretations are possible, we suggest that RAS2 converges on the retrograde response in this capacity. The levels of CIT2 expression were reduced in both the grande and petite strains by the introduction of a RAS2 disruption. Although the retrograde response elicited in the petite appears intact in the ras2 strain, the reduction in its magnitude may be sufficient to abrogate any increase in life span. The reduction in CIT2 expression in the ras2 grande may explain the shorter life span compared to the RAS2 grande. However, explanation of the fact that the ras2 petite strain had a life span no greater than that of the ras2 grande but threefold higher expression of CIT2 appears more complex. The effect on life span may be below the level of detection. Another possibility is that RAS2 modulates life span by additional mechanisms.

One other mechanism that has been described to have an effect on life span in yeast is the formation of extrachromosomal rDNA circles, which were shown to curtail life span (Sinclair and Guarente 1997). It is significant that the induction of petites has been shown to result in appearance of extrachromosomal ribosomal DNA (Conrad-Webb and Butow 1995), suggesting that retrograde regulation may dominate in promoting longevity in the face of these circles. In contrast to the effect of extrachromosomal circles, whose amplification and effect on life span requires DNA replication, the molecular mechanism of aging we report here is equally applicable to mitotic and to postmitotic cells.

The little that is known about the role of retrograde regulation in yeast physiology is informative. The downstream effectors of retrograde regulation, Rtg1p, Rtg2p, and Rtg3p, have multiple metabolic effects related to energy metabolism (Small et al. 1995). These regulators modulate the activity of an array of enzymes, including isocitrate dehydrogenase, mitochondrial citrate synthase 1, and the cytoplasmic enzymes acetyl-CoA synthetase and pyruvate carboxylase in addition to citrate synthase 2 (Small et al. 1995). These regulators are also required for the induction of peroxisome biogenesis by regulating expression of at least three genes that encode peroxisomal proteins, including two involved in fatty acid oxidation (Chelstowska and Butow 1995). Retrograde regulation thus provides an example of intracellular signaling involving a three-way path of communication between mitochondria, nuclei, and peroxisomes.

Certain observations suggest that there may exist something akin to the retrograde response in flies and worms. Alterations in mitochondrial activities that suggest signaling from this organelle to the nucleus have been observed during aging in the fruit fly (Callieja et al. 1993). Furthermore, the clk-1 gene of the nematode, which when mutated extends life span, is a homologue of the yeast CAT5/C0Q7 gene (Ewbank et al. 1997). The yeast gene is a regulator of mitochondrial function. Life extension by the nematodedaf mutants is associated with enzyme changes, the metabolic consequences of which overlap those that constitute the retrograde response (Vanfleteren and De Vreese 1995). It is also noteworthy that Rtg2p upregulates expression of the aconitase gene in yeast (Vel et al. 1996). This mitochondrial enzyme is a specific target of oxidative damage during aging (Yan et al. 1997). Thus, the retrograde response may also help to sustain metabolic activity in the face of oxidative stress. Long-lived C. elegans mutants (Larsen 1993; Vanfleteren 1993) and Drosophila lines (Dudas and Arking 1995) show elevated levels of antioxidant enzyme activities and are more resistant to oxidative stress. Oxidative stress plays a role in heat-induced death in yeast (Davidson et al. 1996), and petes are more resistant to heat stress (C.-Y. Lai and S. M. Jazwinski, unpublished results).

The potential relevance of this study to mammalian aging lies not only in the role of mitochondria in human aging recited earlier. The retrograde response bears some similarity to caloric restriction. The regulators of the retrograde response in yeast are involved in adjusting metabolism to allow utilization of acetate (Small et al. 1995), which has a lower caloric content than glucose. Judging by the nature of the downstream effectors of the retrograde response, a similar pathway may exist in mammals. The Rtg1p and Rtg3p retrograde regulators belong to the BH LH/Zip family of transcription factors (Jia et al. 1997), many homologues of which are found in mammals. Rtg2p, in turn, possesses an Hsp70-type ATP-binding domain (Koonin 1994), found in a family of mammalian proteins including stress response proteins and the glucose-regulated protein Gro78.

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LITERATURE CITED


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