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

Paul A. Kirchman, Sangkyu Kim, Chi-Yung Lai and S. Michal Jazwinski

Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, New Orleans, Louisiana 70112

Manuscript received June 5, 1998
Accepted for publication February 19, 1999

ABSTRACT

Replicative 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 life span (Khazaeli *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 dispersal 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 Pahlavani 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

Corresponding author: S. Michal Jazwinski, Department of Biochemistry and Molecular Biology, Louisiana State University Medical Center, 1901 Perdido St., Box P7-2, New Orleans, LA 70112.
E-mail: sjazwi@lsumc.edu

insulin (reviewed in Masoro 1995), and they display increased insulin receptor levels and increased activity of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (Pahlavani *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; Miquel 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; Tengan *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 (*MAT***a**, *ade2-101*^{ochre} *his3-*Δ*200 leu2-*Δ*1 lys2-801*^{amber} *trp1-*Δ*63 ura3-52*), a haploid derivative of YPH501 (supplied by P. Hieter, The Johns Hopkins University, Baltimore, MD); YSK365 (*MAT***a**, *ade2-101*^{ochre} *his3-*Δ*200 leu2-*Δ*1 lys2-801*^{amber} *trp1-*Δ*63 ura3-52* [ρ^0]), an ethidium bromide-induced petite derived from YPK9; SP1-1 (*MAT***a** *leu2 ura3 trp1 ade8 can1 his3 gal2*), a derivative of SP1 (from M. Wigler, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY); W303-1A (*MAT***a** *can1-100 ade2*^{ochre} *his3-11,-15 leu2-3,-112 trp1-1 ura3-1*), from R. Fuller (Stanford University Medical Center, Stanford, CA); A364A (*MAT***a** ade1 *ade2 ura1 his7 lys2 tyr1 gal1 SUC mal*), from T. Weinert (University of Arizona, Tucson, AZ); YPK25 (*MAT*α *kar1 ade2-1 his4-*Δ*15 can*^{r}) was generated by mating strain JC25 (*MAT*α *kar1 ade2-1 his4-*Δ*15 can*^{r} [ρ^-]), 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% glycerol). For life-span analyses and preparation of RNA, yeast cells were cultured at 30° in YPD (2% peptone, 1% yeast extract, 2% glucose) or YPR (2% peptone, 1% yeast extract, 2% raffinose). 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 (Egilmez 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 *CTT1*, 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° during the day and at 12° overnight. Growth at low 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 *CTT1* 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 *Bam*HI 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 *Eco*RI fragment containing *SOD1* was then cloned into the *Eco*RI site of pBM150-ADH. This plasmid was then cut with *Xba*I (an *Xba*I site is contained upstream of the *ADH2* terminator) and ligated to a *CTT1 Bsa*AI/*Bam*HI fragment from pBR322-7309 (Spevak *et al.* 1983), to which *Xba*I linkers had been added.

To delete *RTG2*, the oligonucleotide primer pairs 5′-GGGATCCGATATAGAGTTTGAATG-3′, 5′-TGGCACGCCTACACTTTTCG-3′ and 5′-GCAAGCTATCTAGAGGAAGTG-3′, 5′-AAGATGGATCCGGTGCTGGTGC-3′ were used to amplify regions flanking *RTG2*. The amplified flanking regions were then cloned in inverse orientation into pRS403 (Sikorski and Hieter 1989). This plasmid was linearized with *Bam*HI 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′-ATACTCTAGATGTAGGAGAAGAACTACCAG-3′, 5′-CATTAAGCTTGTTATCTATTTGTATGGCAAC-3′ and 5′-GACCGAGCTCACTAATCTTATCATTCAAGTTGCC-3′, 5′-ACAGTCTAGAATCTTTTGGAAG-3′ were used to amplify flanking regions, which were cloned into pRS406 (Sikorski and Hieter 1989). This plasmid was linearized with *Xba*I before transformation of yeast. For *CIT2*, the primer pairs 5′-CCCCGGCGCGCCTCCCTTGGGTCATTCAATCAATGG-3′, 5′-GAGAACCTGTTATGATATGTGTTG-3′ and 5′-CCTACTTTTACACCCCTCTGC-3′, 5′-GGGAGGCGCGCCGGGGAATAGTGCAAATTGTATGAATCG-3′ were used to amplify flanking regions, which were cloned into pRS406. This plasmid was linearized with *Asc*I before transformation of yeast.

The plasmid used to disrupt *RAS2*, pRa530 (Tatchell *et al.* 1984), contains a 3.8-kb *Pst*I restriction fragment encoding *LEU2* inserted in a unique *Pst*I site of the *RAS2* coding sequence such that a null mutant is generated. This plasmid was cut with *Xba*I and *Hin*dIII 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° 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′-GCGAAATCTACCCCATCC-3′ and 5′-TAGTGCTGAGCCCACAAG-3′. Twenty nanograms of the *CIT2* PCR product was labeled with [α-^{32}P]dCTP by random oligonucleotide priming using the Rediprime kit (Amersham, Arlington Heights, IL). The membrane was washed two times 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°. 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

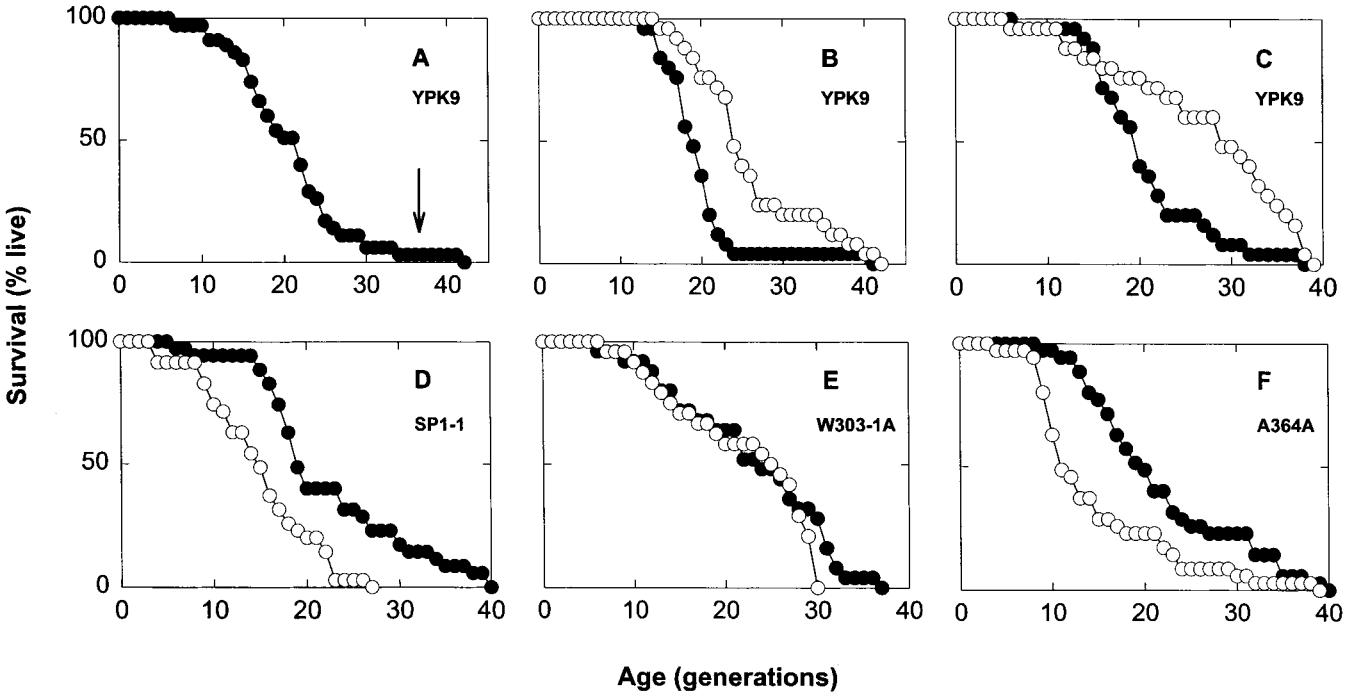

Figure 1.—Life spans of grande and petite strains with glucose as a carbon source. ●, the grande strain; ○, the coisogenic petite. (A) YPK9: the arrow indicates the point at which daughter cells were removed from the long-lived subpopulation to establish the strain used in B. (B) A YPK9 petite strain (mean life span 26.8) derived from the longer-lived subpopulation in A has an extended life span ($P \ll 0.001$) 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 ($P \ll 0.001$) 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 ($P \ll 0.001$) 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 (Conde and Fink 1976). Life-span 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 3, the process of cytoduction, as such, does not affect life span, because it had no effect on the longevity of the parent grande strain.

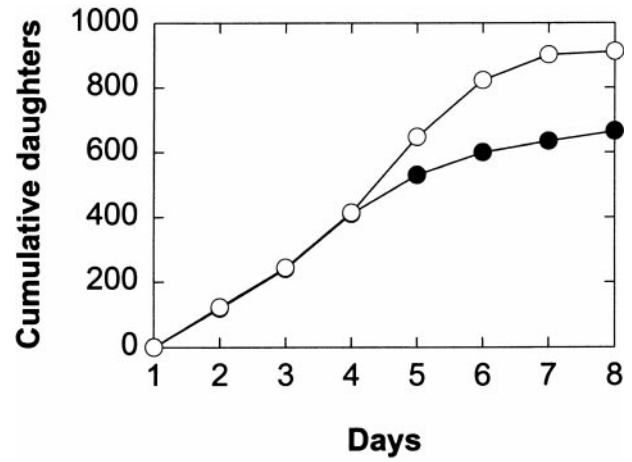

Figure 2.—Senescence is delayed in petites that display extended longevity. The total cumulative number of daughter cells produced by the aging cohort of YPK9 (●) and YSK365 (○) is plotted on each day of the experiment during the entire life span. This is a measure of the rate at which buds are produced or generation time of individual cells.

Loss of mitochondrial DNA prevents cells from respiring by eliminating components of the electron transport chain. Respiration may result in production of reactive

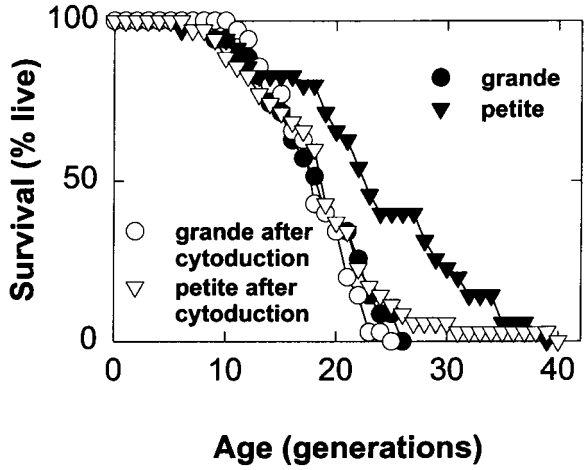

Figure 3.—Replacement of mitochondrial DNA returns the life span of a petite to that of its parent grande strain. Mitochondrial DNA was returned to the petite strain YSK365 (▼; mean 23.7) by cytoduction. The cytoductant's (▽) life span (mean, 19.0) does not differ ($P = 0.4$) from the grande parent strain 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).

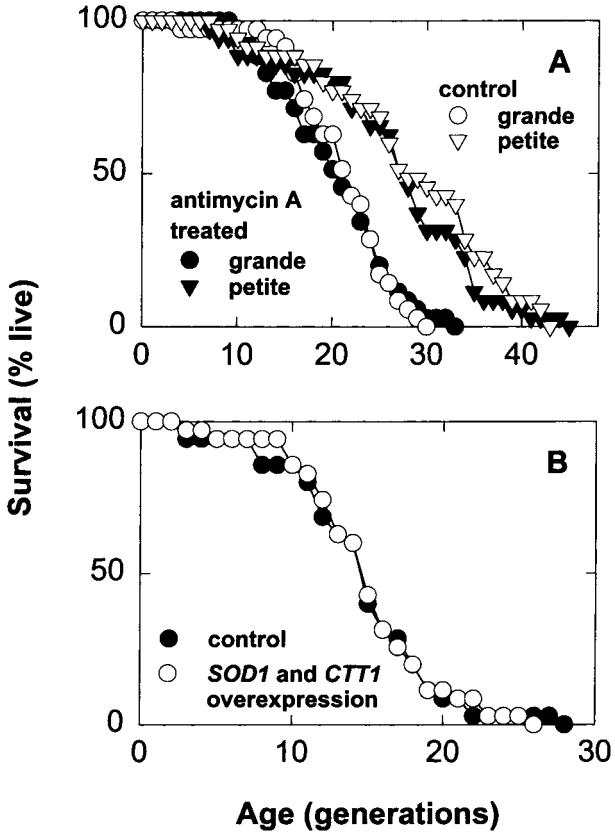

Figure 4.—Loss of respiratory capacity is not responsible for life-span extension. (A) YPK9 cells (●; mean 20.4) grown on antimycin A (2 μg/ml) displayed no difference in life span ($P = 0.25$) from the untreated control (○; mean 21.2). Growth of the petite strain YSK365 (▼; mean 26.8) on the drug also did not affect life span ($P = 0.27$) compared to the untreated control (▽; mean 28.3). (B) Overexpression of the yeast genes for superoxide dismutase (*SOD1*) and cytoplasmic catalase (*CTT1*) in YPK9 (○; mean 14.7) had no effect on life span ($P = 0.4$) compared to the same strain containing the empty vector (●; mean 15.0). Overexpression was obtained on galactose-containing medium, which itself does not affect life span. Overexpression was verified on Northern blots.

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 QH_2-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 (ρ^-) or all (ρ^0) 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 ρ^- or ρ^0 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-

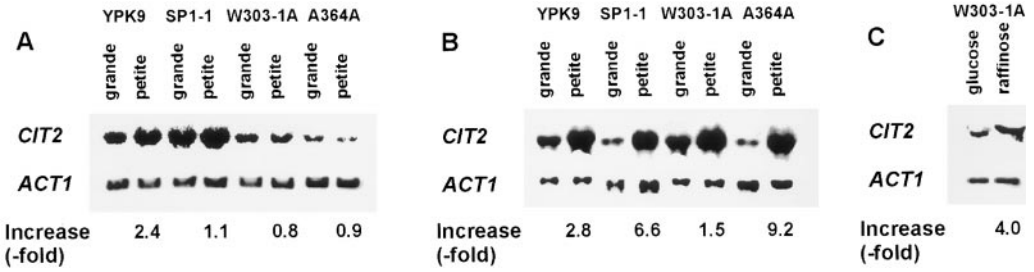

Figure 5.—Expression of the retrograde regulation reporter *CIT2* in petites is dependent on genetic background and environmental conditions. RNA was prepared from the indicated strains grown on (A) glucose or (B) raffinose. (C) A comparison of the W303-1A grande grown on glucose or raffinose. Northern 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).

nose, rather than glucose, as the carbon source. Northern blot analysis revealed that expression of *CIT2* was increased in petites from each of the strains examined (Figure 5B). The increase was still marginal in W303-1A. However, the levels in the W303-1A grande were at least as high as in the YPK9 grande and significantly higher compared to the other grande strains on raffinose. This was not the case on glucose, suggesting that growth on raffinose uncovers constitutive activation of the retrograde response in W303-1A. Indeed, direct comparison of *CIT2* mRNA in W303-1A showed fourfold higher levels on raffinose *vs.* glucose (Figure 5C).

To determine if conditions sufficient to activate retrograde regulation correlated with increased life span in strains other than YPK9, life span analyses were performed on medium containing raffinose. Under these conditions life span was extended in petites from the YPK9 and SP1-1 backgrounds (Figure 6, A and B). In W303-1A there was no significant difference between the grande and the petite (Figure 6C). However, there was a substantial increase (24%) in life span in the W303-1A grande (and a similar one in the petite) on raffinose compared to glucose (Figures 6C and 1E), a condition under which apparent constitutive activation of the retrograde response is evident. In the A364A background, there was also no extension of life span, but rather a maintenance of potential life span. Growth on raffinose has a detrimental effect on the life span of the A364A grande strain compared to growth on glucose (mean of 15.9 *vs.* 21.9 generations, Figures 6D and 1F). However, the petite grown on raffinose attains a life span equal to that attained on glucose (mean of 16.3 generations). Clearly, conditions sufficient to activate retrograde regulation resulted in extension of life span

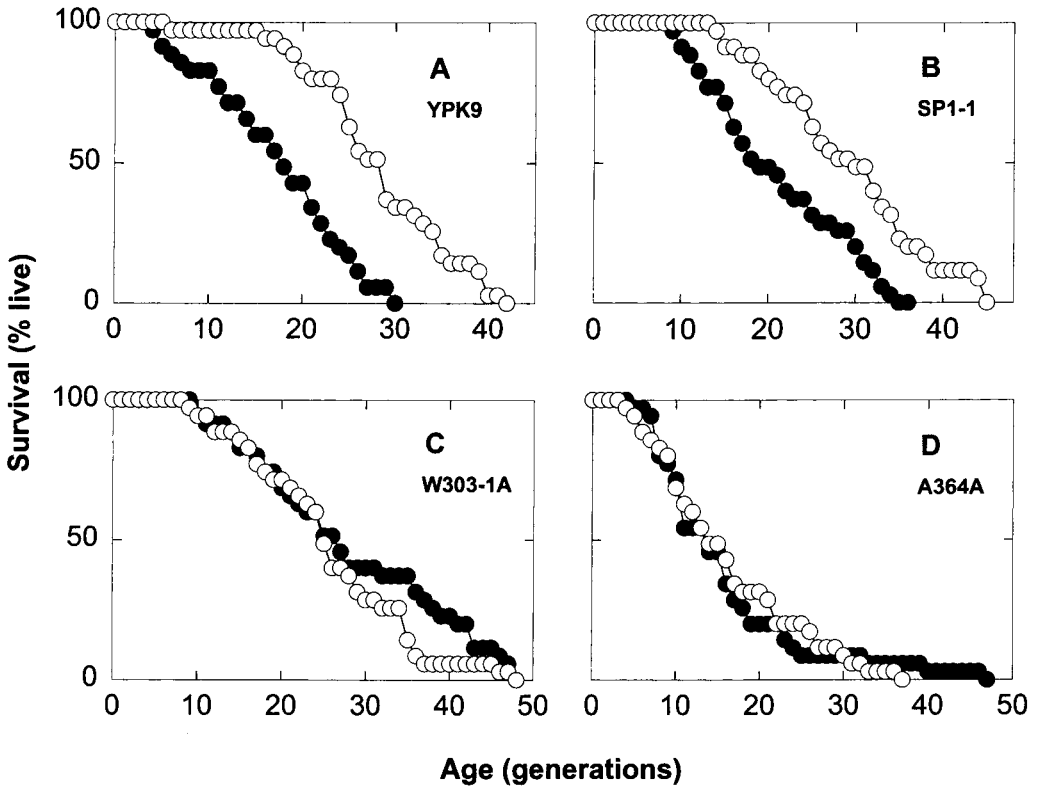

Figure 6.—Life spans of grande and petite strains with raffinose as a carbon source. ●, the grande strain; ○, the coisogenic petite. (A) YPK9 petite, YSK365, (mean 28.3) has an extended life span ($P \ll 0.001$) compared to YPK9 (mean 17.6). (B) An SP1-1 petite (mean 29.4) has an extended life span ($P < 0.01$) 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).

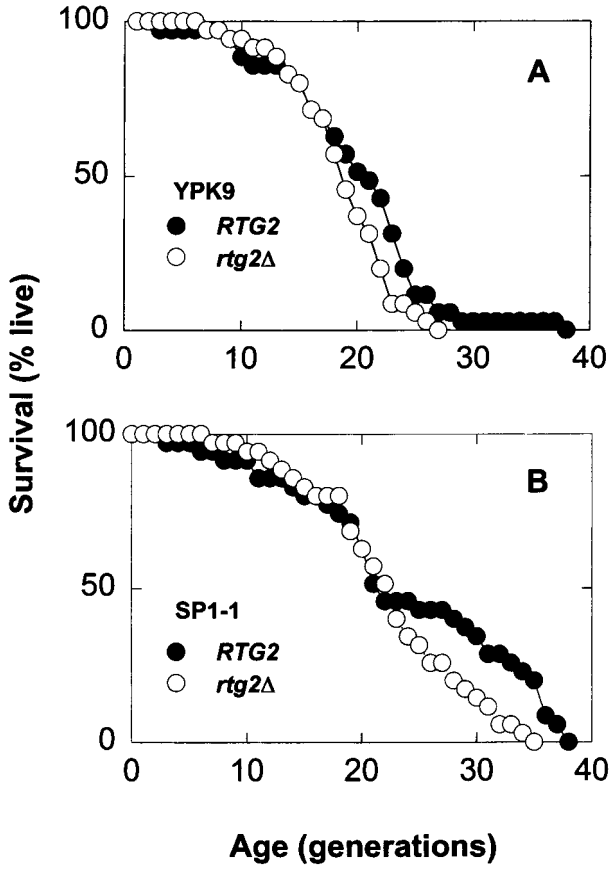

Figure 7.—*RTG2* is required for extension of petite life span. (A) Life span of YPK9 *rtg2*Δ (●; mean 19.0) and its coisogenic petite YSK365 *rtg2*Δ (○; mean 17.7) on glucose. The two *RTG2* deletion strains did not differ in life span (P = 0.2). (B) Life span of SP1-1 *rtg2*Δ grande (●; mean 24.1) and petite (○; mean 22.4) on raffinose. The two *RTG2* deletion strains did not differ in life span (P = 0.2). Similar results were obtained with additional *rtg2*Δ clones in both genetic backgrounds.

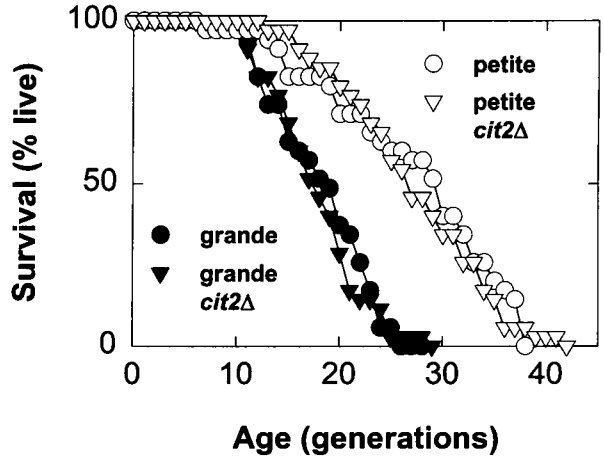

Figure 8.—*CIT2* is not required for extension of life span in petites. The life span of YPK9 (●; mean 18.2) is unchanged (P = 0.34) by the introduction of a *CIT2* deletion (▼; mean 18.0). The life span of the coisogenic petite YSK365 (○; mean 27.3) is also unchanged (P = 0.4) by the introduction of a *CIT2* deletion (▽; mean 27.3).

in three of the strains, or in its maintenance under otherwise life-shortening conditions in A346A.

If activation of retrograde regulation was responsible for the extension of life span, then eliminating the retrograde response should also eliminate life span extension. Three genes are known to be required for retrograde regulation, *RTG1*, *RTG2*, and *RTG3* (Liao and Butow 1993; Jia *et al.* 1997). Rtg2p may promote the formation of an active Rtg1p-Rtg3p heterodimeric transcription factor (Rothermel *et al.* 1997). It has been reported that deletion of *RTG2* completely eliminates expression of *CIT2* in petites (Liao and Butow 1993); we therefore chose to delete *RTG2* from YPK9, SP1-1, and petites from both strains. Life-span analyses were performed using glucose as a carbon source for YPK9-derived strains and raffinose for SP1-1 derivatives. Deletion of *RTG2* completely eliminated the extension of life span seen in petites, confirming that retrograde regulation is necessary for the extension (Figure 7). This result also demonstrates that the extension of life 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 *CIT2* expression by 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 c 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

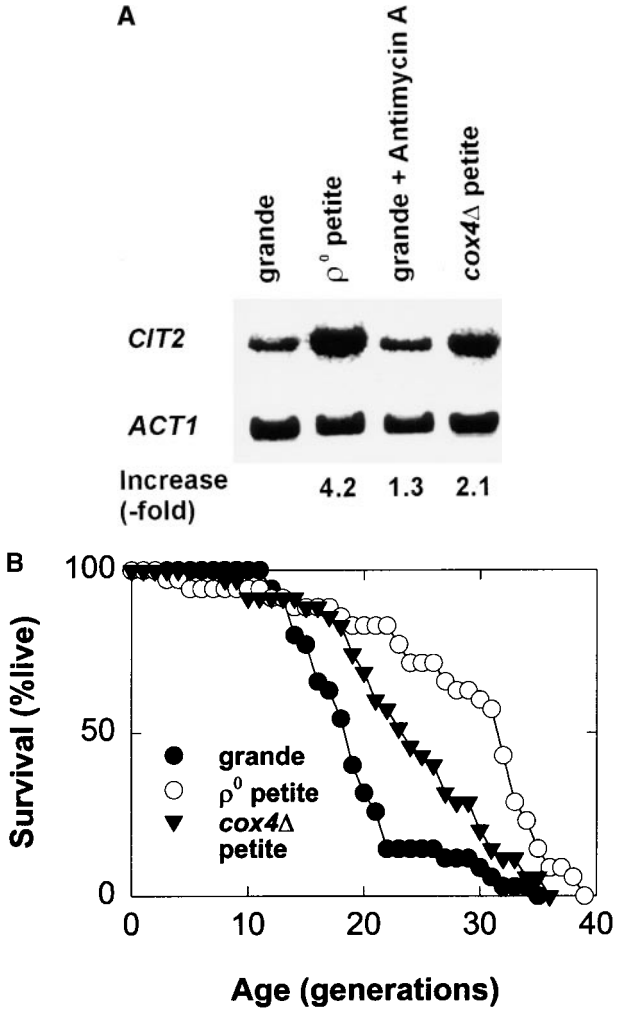

Figure 9.—A nuclear petite exhibits partial activation of the retrograde response and an intermediate extension of life span. (A) Strains were grown on media containing glucose and Northern blot analysis was performed as described in Figure 5. Antimycin A was present at 2 μg/ml in the medium, where indicated. (B) The life span of the *cox4* nuclear petite (▼; mean 23.9) is extended ($P < 0.01$) compared to the coisogenic grande YPK9 (●; mean, 19.5), but also differs ($P < 0.01$) from that of the coisogenic, cytoplasmic petite YSK365 (○; mean 28.4).

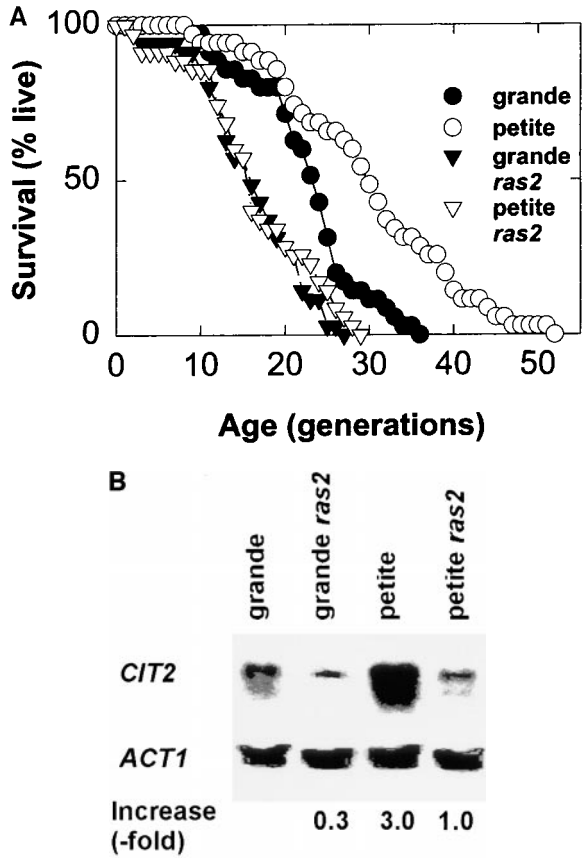

Figure 10.—Extension of life span and retrograde regulation are dependent on *RAS2*. (A) The life span of the petite strain YSK365 (○; mean 29.9) is reduced ($P < 0.001$) when a disruption of *RAS2* is introduced into the strain (▽; mean 16.5). The life span of the *ras2* petite does not differ ($P = 0.8$) from the coisogenic grande strain (YPK9) containing the *RAS2* disruption (▼; mean 16.3). Disruption of *RAS2* shortens life span ($P < 0.01$) relative to the coisogenic control YPK9 (●; mean 22.8). Similar results were obtained with additional *ras2* clones. (B) Strains were grown on media containing glucose and Northern blot analysis was performed as described in Figure 5.

(Figure 4A) that this treatment did not increase life span, 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 ρ^0 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). 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 (mean 21%) was approximately one-half that of the extension in YSK365 (45%).

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* ρ^0 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* ρ^0 strain could grow on media containing glycerol as the sole carbon

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 (*rtg2*Δ) 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 ρ^0 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 (Calleja *et al.* 1993). Furthermore, the *clk-1* gene of the nematode, which when mutated extends life span, is a homologue of the yeast *CAT5*/*COQ7* gene (Ewbank *et al.* 1997). The yeast gene is a regulator of mitochondrial function. Life extension by the nematode *daf* 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 (Vélot *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 petites 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 bHLH/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 Grp78.

This work was supported by grants from the National Institute on Aging of the National Institutes of Health (U.S.P.H.S.) and from the Glenn Foundation for Medical Research, and by donations from Heinz Keller of Huonville, Tasmania, Australia. P. A. Kirchman is a recipient of a postdoctoral fellowship from the National Institute on Aging.

LITERATURE CITED

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, D. D. Seidman *et al.*, 1994 *Current Protocols in Molecular Biology*, Green Publishing Associates/Wiley-Interscience, New York.

Brierley, E. J., M. A. Johnson, O. F. James and D. M. Turnbull,

1997 Mitochondrial involvement in the ageing process. Facts and controversies. Mol. Cell. Biochem. **174:** 325–328.

Calleja, M., P. Peña, C. Ugalde, C. Ferreiro, R. Marco *et al.*, 1993 Mitochondrial DNA remains intact during Drosophila aging, but the levels of mitochondrial transcripts are significantly reduced. J. Biol. Chem. **268:** 18891–18897.

Chelstowska, A., and R. A. Butow, 1995 RTG genes in yeast that function in communication between mitochondria and the nucleus are also required for expression of genes encoding peroxisomal proteins. J. Biol. Chem. **270:** 18141–18146.

Conde, J., and G. R. Fink, 1976 A mutant of *Saccharomyces cerevisiae* defective for nuclear fusion. Proc. Natl. Acad. Sci. USA **73:** 3651–3655.

Conrad-Webb, H., and R. A. Butow, 1995 A polymerase switch in the synthesis of rRNA in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **15:** 2420–2428.

Davidson, J. F., B. Whyte, P. H. Bissinger and R. H. Schiestl, 1996 Oxidative stress is involved in heat-induced cell death in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **93:** 5116–5121.

Dorman, J. B., B. Albinder, T. Shroyer and C. Kenyon, 1995 The *age-1* and *daf-2* genes function in a common pathway to control the lifespan of *Caenorhabditis elegans*. Genetics **141:** 1399–1406.

Dudas, S. P., and R. Arking, 1995 A coordinate upregulation of antioxidant gene activities is associated with the delayed onset of senescence in a long-lived strain of *Drosophila*. J. Gerontol. **50A:** B117–B127.

Egilmez, N. K., and S. M. Jazwinski, 1989 Evidence for the involvement of a cytoplasmic factor in the aging of the yeast *Saccharomyces cerevisiae*. J. Bacteriol. **171:** 37–42.

Ewbank, J. J., T. M. Barnes, B. Lakowski, M. Lussier, H. Bussey *et al.*, 1997 Structural and functional conservation of the *Caenorhabditis elegans* timing gene *clk-1*. Science **275:** 980–983.

Finch, C. E., 1990 *Longevity, Senescence, and the Genome*. University of Chicago Press, Chicago.

Fraenkel, D. G., 1985 On ras gene function in yeast. Proc. Natl. Acad. Sci. USA **82:** 4740–4744.

Friedman, D. B., and T. E. Johnson, 1988 A mutation in the *age-1* gene in *Caenorhabditis elegans* lengthens life and reduces hermaphrodite fertility. Genetics **118:** 75–86.

Gancedo, C., and R. Serrano, 1989 Energy-yielding metabolism, pp. 205–259 in *The Yeasts: Metabolism and Physiology of Yeasts*, Vol. 3, Ed. 2, edited by A. H. Rose and J. S. Harrison. Academic Press, London.

Goldring, E. S., L. I. Grossman, D. Krupnick, D. R. Cryer and J. Marmur, 1970 The petite mutation in yeast. Loss of mitochondrial deoxyribonucleic acid during induction of petites with ethidium bromide. J. Mol. Biol. **52:** 323–335.

Guidot, D. M., J. M. McCord, R. M. Wright and J. E. Repine, 1993 Absence of electron transport (Rho 0 state) restores growth of a manganese-superoxide dismutase-deficient *Saccharomyces cerevisiae* in hyperoxia. Evidence for electron transport as a major source of superoxide generation in vivo. J. Biol. Chem. **268:** 26699–26703.

Hagen, T. M., D. L. Yowe, J. C. Bartholomew, C. M. Wehr, K. L. Do *et al.*, 1997 Mitochondrial decay in hepatocytes from old rats: membrane potential declines, heterogeneity and oxidants increase. Proc. Natl. Acad. Sci. USA **94:** 3064–3069.

Heydari, A. R., B. Wu, R. Takahashi, R. Strong and A. Richardson, 1993 Expression of heat shock protein 70 is altered by age and diet at the level of transcription. Mol. Cell. Biol. **13:** 2909–2918.

Heydari, A. R., S. You, R. Takahashi, A. Gutsmann, K. D. Sarge *et al.*, 1996 Effect of caloric restriction on the expression of heat shock protein 70 and the activation of heat shock transcription factor 1. Dev. Genet. **18:** 114–124.

Jazwinski, S. M., 1993 The genetics of aging in the yeast *Saccharomyces cerevisiae*. Genetica **91:** 35–51.

Jazwinski, S. M., 1996 Longevity, genes, and aging. Science **273:** 54–59.

Jazwinski, S. M., J. B. Chen and N. K. Egilmez, 1989 Replication control and cellular life span. Exp. Gerontol. **24:** 423–436.

Jia, Y., B. Rothermel, J. Thornton and R. A. Butow, 1997 A basic helix-loop-helix-leucine zipper transcription complex in yeast functions in a signaling pathway from mitochondria to the nucleus. Mol. Cell. Biol. **17:** 1110–1117.

Johnston, M., and R. W. Davis, 1984 Sequences that regulate the divergent GAL1-GAL10 promoter in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **4:** 1440–1448.

Kennedy, B. K., N. R. Austriaco, J. Zhang and L. Guarente, 1995 Mutation in the silencing gene *SIR4* can delay aging in *S. cerevisiae*. Cell **80:** 485–496.

Kenyon, C., J. Chang, E. Gensch, A. Rudner and R. Tabtiang, 1993 A *C. elegans* mutant that lives twice as long as wild type. Nature **366:** 461–464.

Khazaeli, A. A., M. Tatar, S. D. Pletcher and J. W. Curtsinger, 1997 Heat-induced longevity extension in Drosophila. I. Heat treatment, mortality, and thermotolerance. J. Gerontol. **52A:** B48–B52.

Kimura, K. D., H. A. Tissenbaum, Y. Liu and G. Ruvkun, 1997 *daf-2*, an insulin receptor-like gene that regulates longevity and diapause in *Caenorhabditis elegans*. Science **277:** 942–946.

Klass, M. R., 1983 A method for the isolation of longevity mutants in the nematode *Caenorhabditis elegans* and initial results. Mech. Ageing Dev. **22:** 279–286.

Knight, S. A., S. Labbé, L. F. Kwon, D. J. Kosman and D. J. Thiele, 1996 A widespread transposable element masks expression of a yeast copper transport gene. Genes Dev. **10:**, 1917–1929.

Koonin, E. V., 1994 Yeast protein controlling inter-organelle communication is related to bacterial phosphatases containing the Hsp 70-type ATP-binding domain. Trends Biochem. Sci. **19:** 156–157.

Larsen, P. L., 1993 Aging and resistance to oxidative damage in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA **90:** 8905–8909.

Larsen, P. L., P. S. Albert and D. L. Riddle, 1995 Genes that regulate both development and longevity in *Caenorhabditis elegans*. Genetics **139:** 1567–1583.

Liao, X., and R. A. Butow, 1993 *RTG1* and *RTG2*: two yeast genes required for a novel path of communication from mitochondria to the nucleus. Cell **72:** 61–71.

Liao, X., W. C. Small, P. A. Srere and R. A. Butow, 1991 Intramitochondrial functions regulate nonmitochondrial citrate synthase (*CIT2*) expression in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11:** 38–46.

Lin, K., J. B. Dorman, A. Rodan and C. Kenyon, 1997 *Daf-16*: an HNF-3/forkhead family member that can function to double the life-span of *Caenorhaabditis elegans*. Science **278:** 1319–1322.

Linnane, A. W., 1992 Mitochondria and aging: the universality of bioenergetic disease. Aging **4:** 267–271.

Lithgow, G. J., T. M. White, S. Melov and T. E. Johnson, 1995 Thermotolerance and extended life-span conferred by single-gene mutations and induced by thermal stress. Proc. Natl. Acad. Sci. USA **92:** 7540–7544.

Liu, H., C. A. Styles and G. R. Fink, 1996 *Saccharomyces cerevisiae* S288C has a mutation in *FLO8*, a gene required for filamentous growth. Genetics **144:** 967–978.

Longo, V. D., E. B. Gralla and J. S. Valentine, 1996 Superoxide dismutase activity is essential for stationary phase survival in *Saccharomyces cerevisiae*. Mitochondrial production of toxic oxygen species in vivo. J. Biol. Chem. **271:** 12275–12280.

Marchler, G., C. Schuller, G. Adam and H. Ruis, 1993 A *Saccharomyces cerevisiae* UAS element controlled by protein kinase A activates transcription in response to a variety of stress conditions. EMBO J. **12:**, 1997–2003.

Masoro, E., 1995 Dietary restriction. Exp. Gerontol. **30:** 291–298.

Miquel, J., 1998 An update on the oxygen stress-mitochondrial mutation theory of aging: genetic and evolutionary implications. Exp. Gerontol. **33:** 113–126.

Morris, J. Z., H. A. Tissenbaum and G. Ruvkun, 1996 A phosphatidylinositol-3-OH kinase family member regulating longevity and diapause in *Caenorhabditis elegans*. Nature **382:** 536–539.

Mortimer, R. K., and J. R. Johnston, 1959 Life span of individual yeast cells. Nature **183:** 1751–1752.

Muller, I., M. Zimmermann, D. Becker and M. Flomer, 1980 Calendar life span versus budding life span of *Saccharomyces cerevisiae*. Mech. Ageing Dev. **12:** 47–52.

Muller-Hocker, J., K. Schneiderbanger, F. H. Stefani and B. Kadenbach, 1992 Progressive loss of cytochrome c oxidase in the human extraocular muscles in ageing—a cytochemical-immunohistochemical study. Mutat. Res. **275:** 115–124.

Murakami, S., and T. E. Johnson, 1996 A genetic pathway conferring life extension and resistance to UV stress in *Caenorhabditis elegans*. Genetics **143:** 1207–1218.

Ogg, S., S. Paradis, S. Gottlieb, G. I. Patterson, L. Lee *et al.*, 1997 The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in *C. elegans*. Nature **389:** 994–999.

Pahlavani, M. A., V. Haley-Zitlin and A. Richardson, 1994 Influence of dietary restriction on gene expression: changes in transcription of specific genes, pp. 143–156 in *Modulation of the Aging Process by Dietary Restriction*, edited by B. P. Yu. CRC Press, Boca Raton, FL.

Parikh, V. S., M. M. Morgan, R. Scott, L. S. Clements and R. A. Butow, 1987 The mitochondrial genotype can influence nuclear gene expression in yeast. Science **235:** 576–580.

Pohley, H.-J., 1987 A formal mortality analysis for populations of unicellular organisms (*Saccharomyces cerevisiae*). Mech. Ageing Dev. **38:** 231–243.

Poyton, R. O., and J. E. McEwen, 1996 Crosstalk between nuclear and mitochondrial genomes. Annu. Rev. Biochem. **65:** 563–607.

Reaume, S. E., and E. L. Tatum, 1949 Spontaneous and nitrogen mustard-induced nutritional deficiencies in *Saccharomyces cerevisiae*. Arch. Biochem. **22:** 331–338.

Richardson, A., and M. A. Pahlavani, 1994 Thoughts on the evolutionary basis of dietary restriction, pp. 226–231 in *Genetics and Evolution of Aging*, edited by M. R. Rose and C. E. Finch. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Roman, H., 1956 Studies of gene mutation in Saccharomyces. Cold Spring Harbor Symp. Quant. Biol. **21:** 175–185.

Rothermel, B. A., J. L. Thornton and R. A. Butow, 1997 Rtg3p, a basic helix-loop-helix/leucine zipper protein that functions in mitochondrial-induced changes in gene expression, contains independent activation domains. J. Biol. Chem. **272:** 19801–19807.

Sanchez, Y., J. Taulien, K. A. Borkovich and S. Lindquist, 1992 HSP-104 is required for tolerance to many forms of stress. EMBO J. **11:** 2357–2364.

Shama, S., C.-Y. Lai, J. M. Antoniazzi, J. C. Jiang and S. M. Jazwinski, 1998 Heat shock-induced life span extension in yeast. Exp. Cell Res. **245:** 379–388.

Sikorski, R. S., and P. Hieter, 1989 A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:** 19–27.

Sinclair, D. A., and L. Guarente, 1997 Extrachromosomal rDNA circles—a cause of aging in yeast. Cell **91:** 1033–1042.

Small, W. C., R. D. Brodeur, A. Sandor, N. Fedorova, G. Li *et al.*, 1995 Enzymatic and metabolic studies on retrograde regulation mutants of yeast. Biochemistry **34:** 5569–5576.

Sohal, R. S., and R. Weindruch, 1996 Oxidative stress, caloric restriction, and aging. Science **273:** 59–63.

Spevak, W., F. Fessl, J. Rytka, A. Traczyk, M. Skoneczny *et al.*, 1983 Isolation of the catalase T structural gene of *Saccharomyces cerevisiae* by functional complementation. Mol. Cell. Biol. **3:** 1545–1551.

Sun, J., S. P. Kale, A. M. Childress, C. Pinswasdi and S. M. Jazwinski, 1994 Divergent roles of *RAS1* and *RAS2* in yeast longevity. J. Biol. Chem. **269:** 18638–18645.

Szekely, E., and D. L. Montgomery, 1984 Glucose represses transcription of *Saccharomyces cerevisiae* nuclear genes that encode mitochondrial components. Mol. Cell. Biol. **4:** 939–946.

Tatchell, K., 1993 *RAS* genes in the budding yeast *Saccharomyces cerevisiae*, pp. 147–188 in *Signal Transduction: Prokaryotic and Simple Eukaryotic Systems*, edited by T. Kurjan. Academic Press, San Diego.

Tatchell, K., D. T. Chaleff, D. DeFeo-Jones and E. M. Scolnick, 1984 Requirement of either of a pair of ras-related genes of *Saccharomyces cerevisiae* for spore viability. Nature **309:** 523–527.

Tatchell, K., L. C. Robinson and M. Breitenbach, 1985 *RAS2* of *Saccharomyces cerevisiae* is required for gluconeogenic growth and proper response to nutrient limitation. Proc. Natl. Acad. Sci. USA **82:** 3785–3789.

Tengan, C. H., A. A. Gabbai, S. Shanske, M. Zeviani and C. T. Moraes, 1997 Oxidative phosphorylation dysfunction does not increase the rate of accumulation of age-related mtDNA deletions in skeletal muscle. Mutat. Res. **379:** 1–11.

Vanfleteren, J. R., 1993 Oxidative stress and ageing in *Caenorhabditis elegans*. Biochem. J. **292:** 605–608.

Vanfleteren, J. R., and A. De Vreese, 1995 The gerontogenes *age-1* and *daf-2* determine metabolic rate potential in aging *Caenorhabditis elegans*. FASEB J. **9:** 1355–1361.

Van Remmen, H., W. Ward, R. V. Sabia and A. Richardson, 1994 Effect of age on gene expression and protein degradation, pp. 171–234 in *The Handbook of Physiology of Aging*, edited by E. Masoro. Oxford University Press, New York.

Vélot, C., P. Haviernik and G. J.-M. Lauquin, 1996 The *Saccharomyces cerevisiae RTG2* gene is a regulator of aconitase expression under catabolite repression conditions. Genetics **144:** 893–903.

Yan, L.-J., R. L. Levine and R. S. Sohal, 1997 Oxidative damage during aging targets mitochondrial aconitase. Proc. Natl. Acad. Sci. USA **94:** 11168–11172.

Communicating editor: A. P. Mitchell