Rtg2 Protein Links Metabolism and Genome Stability in Yeast Longevity

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ABSTRACT

Mitochondrial dysfunction induces a signaling pathway, which culminates in changes in the expression of many nuclear genes. This retrograde response, as it is called, extends yeast replicative life span. It also results in a marked increase in the cellular content of extrachromsomal ribosomal DNA circles (ERCs), which can cause the demise of the cell. We have resolved the conundrum of how these two molecular mechanisms of yeast longevity operate in tandem. About 50% of the life-span extension elicited by the retrograde response involves processes other than those that counteract the deleterious effects of ERCs. Deletion of *RTG2*, a gene that plays a central role in relaying the retrograde response signal to the nucleus, enhances the generation of ERCs in cells with (grande) or in cells without (petite) fully functional mitochondria, and it curtails the life span of each. In contrast, overexpression of *RTG2* diminishes ERC formation in both grandes and petites. The excess Rtg2p did not augment the retrograde response, indicating that it was not engaged in retrograde signaling. *FOB1*, which is known to be required for ERC formation, and *RTG2* were found to be in converging pathways for ERC production. *RTG2* did not affect silencing of ribosomal DNA in either grandes or petites, which were similar to each other in the extent of silencing at this locus. Silencing of ribosomal DNA increased with replicative age in either the presence or the absence of Rtg2p, distinguishing silencing and ERC accumulation. Our results indicate that the suppression of ERC production by Rtg2p requires that it not be in the process of transducing the retrograde signal from the mitochondrion. Thus, *RTG2* lies at the nexus of cellular metabolism and genome stability, coordinating two pathways that have opposite effects on yeast longevity.

AGING is a complicated process (Finch 1990; Jazwinski 1996). Considerable progress has been made in the dissection of the aging process of the yeast *Saccharomyces cerevisiae* over the past 10 years (Jazwinski 2002). The yeast replicative life span is measured by the number of times an individual cell divides and not by chronological age (Mortimer and Johnston 1959; Müller *et al.* 1980). More than 30 genes, involved in stress resistance, metabolism, chromatin-dependent gene regulation, and genetic stability, have been implicated in yeast aging (reviewed in Jazwinski 2001). Homologs from *Caenorhabditis elegans* and humans of at least three yeast genes (*LAG1*, *SIR2*, *SGS1*) have a similar effect on life span or are able to complement effects on longevity caused by a mutation in the yeast gene, suggesting that some of the underlying mechanisms may be evolutionarily conserved (Jiang *et al.* 1998; Heo *et al.* 1999; Tissenbaum and Guarente 2001).

Investigations of yeast aging have focused especially on genetic stability. *SGS1* encodes a DNA helicase of the highly conserved RecQ family (Gangloff *et al.* 1994; Watt *et al.* 1995). Deletion of *SGS1* leads to hyperrecombination and chromosomal instability and also to a decrease in life span (Watt *et al.* 1996; Sinclair *et al.* 1997). Mutations in one of the human homologs of this gene, *WRN*, cause the disease Werner syndrome, which leads to premature aging (Watt *et al.* 1996; Yu *et al.* 1996), a phenotype that has also been contrived in yeast cells (Sinclair *et al.* 1997). Cells from Werner syndrome patients display genomic translocations and deletions (Scappaticci *et al.* 1982; Fukuchi *et al.* 1989). Interestingly, Sgs1p as well as Wrn protein is localized in the nucleolus, suggesting that their role in promoting longevity may be linked to nucleolar function (Sinclair *et al.* 1997; Gray *et al.* 1998; Marciniak *et al.* 1998).

The rDNA genes are arranged in tandem copies, which make this region highly susceptible to recombination. The rDNA is associated with yeast aging (Kennedy *et al.* 1997; Sinclair and Guarente 1997; Kim *et al.* 1999a; Benguria *et al.* 2003). *RAD52*-dependent recombination within the tandem repeats leads to circle formation (Clark-Walker and Azad 1980; Larionov *et al.* 1980; Park *et al.* 1999; Johzuka and Horiuchi 2002). Because each unit contains an autonomously replicating sequence (*ARS*), these rDNA circles can be maintained as episomes (Szostak and Wu 1979). The level of these extrachromosomal rDNA circles (ERCs) is higher in

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old yeast cells (Sinclair and Guarente 1997). The formation of ERCs in yeast results from the unidirectional replication of the rDNA repeats. This unique manner of replication is facilitated by Fob1p, which blocks the replication fork at the 3′-end of the 35S rRNA gene (Kobayashi and Horiuchi 1996). Stalling of these structures increases the risk of double-strand breaks (Brewer and Fangman 1988; Michel *et al.* 1997). Consequently, deletion of the *FOB1* gene prevents ERC formation and coincidentally increases yeast replicative life span (Defossez *et al.* 1999).

It has been reported that daughter cells produced by old mothers have a reduced life-span potential compared to daughter cells produced by young mothers (Högel and Müller, cited in Jazwinski 1993; Kennedy *et al.* 1994). This implies that a dominant senescence factor asymmetrically accumulates in old mothers and that this factor can leak into the daughter cell, a phenomenon for which experimental evidence had been provided earlier (Egilmez and Jazwinski 1989). It has been suggested that the accumulation of ERCs could be the senescence factor. In young cells, ERCs segregate asymmetrically to mother cells and are not inherited by daughter cells. If the amount of ERCs reaches a certain threshold, these elements might "leak" from old mothers into daughters, reducing their life spans (Sinclair and Guarente 1997). There is no direct evidence for this theory, however. Furthermore, recent data have revealed that accumulation of ERCs is not an obligatory feature but rather parallels aging (Ashrafi *et al.* 1999; Heo *et al.* 1999; Kim *et al.* 1999a).

Yeast cells accumulate mitochondrial defects with age. The membrane potential decreases, whereas mitochondrial mass increases. As a result, the number of functional mitochondria decreases with age (Lai *et al.* 2002). Furthermore, old mothers segregate defective mitochondria to their daughters at a higher frequency than do young mothers. These results have led to the conclusion that defective mitochondria may be the cytoplasmic senescence factor (Lai *et al.* 2002).

Apart from genetic instability, the effect of metabolic activity on life span has received considerable attention. It is well documented that altering metabolism by calorie restriction extends the life span of many organisms (Masoro 1995). The characteristics of metabolic activity are dependent on the mitochondria. Apart from producing energy, mitochondria are also the major source of reactive oxygen species in the cell. The role of oxidative stress in aging has been supported in many studies (reviewed in Weindruch and Sohal 1997).

We previously showed that petite yeast strains, which lack fully functional mitochondria, display an increase in life span compared to grande parental strains, whose mitochondria are functionally intact (Kirchman *et al.* 1999). To compensate for defects in mitochondria, a specific pathway that causes changes in nuclear gene expression is activated (Parikh *et al.* 1987). This so-called retrograde response pathway is dependent on *RTG1* and *RTG3*, which encode a heterodimeric transcription factor, as well as on *RTG2* (Liao and Butow 1993; Jia *et al.* 1997). Rtg2p relays the signal generated by dysfunctional mitochondria to Rtg3p (Sekito *et al.* 2000). Induction of the retrograde response has profound effects on cell metabolism through changes in the expression of a multitude of genes, whose products function in the cytoplasm, the mitochondria, and the peroxisomes (Epstein *et al.* 2001). Surprisingly, however, it was shown some time ago that the retrograde response is associated with an accumulation of ERCs in the cell (Conrad-Webb and Butow 1995).

In this work we investigate whether there is a connection between loss of active mitochondria and ERC accumulation. Until now, experiments have been performed to show how ERCs accumulate, but the underlying cause of the induction of this process has remained unknown. Our study shows that the mitochondrial theory of aging and the theory that is based on the formation of ERCs as a primary cause of aging are opposite sides of the same coin. We find that the Rtg2 protein plays a central role in this apposition.

MATERIALS AND METHODS

Yeast strains, plasmids, and media: The *S. cerevisiae* strains used in this study are shown in Table 1. To generate the *fob1* deletion strains, an *Eco*RI-*Bam*HI fragment of plasmid pCB6, which contains the kanamycin gene flanked by the 5′-region (−592 to −34) and the 3′-region (+1707 to +1893) of the *FOB1* gene in pUC18 (Amersham, Buckinghamshire, UK), was used for transformation. The kanamycin gene was in a *Sma*I-*Sac*I fragment from pFA-kanMX4 (Wach *et al.* 1994). The *RTG3* gene was deleted in YPK9 and YSK365, as described earlier (Jiang *et al.* 2000).

The *cit2:lacZ* and *fob1:lacZ* strains were generated using either plasmid pCIT-LacZ800 or plasmid pFOB-LacZ800, respectively. These plasmids are based on vector YIp356 (ATCC) containing the *lacZ* and *URA3* genes. Either the *CIT2* promoter (−795 to −1) or the *FOB1* promoter (−809 to −1), obtained by amplification in the polymerase chain reaction (PCR), was cloned between the *Eco*RI and *Sph*I restriction sites of YIp356, upstream of *lacZ*. The pCIT-LacZ800 and pFOB-LacZ800 constructs were linearized using *Stu*I, cutting into the *URA3* gene. Transformation of this fragment resulted in insertion of the *cit2:lacZ* or *fob1:lacZ* cassette into the *ura3-52* locus, restoring *URA3* function.

For the analysis of silencing, an *URA3-LEU2* reporter cassette was constructed from vector pJSS51-9 (Smith and Boeke 1997). pJSS51-9 was digested with *Xho*I to remove the *HIS3* gene and an *Sfo*I-*Msp*A1I fragment of plasmid pRS425 (ATCC) containing the *LEU2* gene was inserted using blunt ends. The *URA3-LEU2* cassette with flanking rDNA sequences was amplified from this plasmid (pJSS-*LEU2*) by the PCR and inserted into the *Sma*I site of pUC18, resulting in plasmid pCB2-*LEU2*. The sequence of the PCR fragment was verified. To insert the *URA3-LEU2* cassette into the rDNA, as shown in Figure 8A, a *Pvu*II-*Sph*I fragment was used for transformation of the different YPK9 and YSK365 strains. The insertion of a single copy in the rDNA was verified using Southern blot analysis.

To construct the pBEVY-*RTG2* plasmid, the *RTG2* gene was amplified, introducing a *MYC* epitope tag at the 3′-end. Added

TABLE 1

Yeast strains used in this study

Strain	Genotype	Reference
YPK9	*MAT***a** *ade2-101^{ochre} his3-Δ200 leu2-Δ1 lys2-801^{amber} trp1-Δ63 ura3-52*	Kirchman *et al.* (1999)
YPK9 *rtg2*Δ	*MAT***a** *ade2-101^{ochre} his3-Δ200 leu2-Δ1 lys2-801^{amber} trp1-Δ63 ura3-52 rtg2*Δ*::HIS3*	Kirchman *et al.* (1999)
YPK9 *fob1*Δ*::URA3*	*MAT***a** *ade2-101^{ochre} his3-Δ200 leu2-Δ1 lys2-801^{amber} trp1-*Δ63 *ura3-52 fob1*Δ*::URA3*	This study
YPK9 *fob1*Δ	*MAT***a** *ade2-101^{ochre} his3-Δ200 leu2-Δ1 lys2-801^{amber} trp1-Δ63 ura3-52 fob1*Δ*::KAN^{r}*	This study
YPK9 *rtg2*Δ *fob1*Δ	*MAT***a** *ade2-101^{ochre} his3-Δ200 leu2-Δ1 lys2-801^{amber} trp1-Δ63 ura3-52 rtg2*Δ*::HIS3 fob1*Δ*::KAN^{r}*	This study
YPK9 *rtg3*Δ	*MAT***a** *ade2-101^{ochre} his3-Δ200 leu2-Δ1 lys2-801^{amber} trp1-Δ63 ura3-52 rtg3*Δ*::URA3*	Jiang *et al.* (2000)
YPK9 *ade2::URA3:LEU2*	*MAT***a** *ade2-101^{ochre} his3-Δ200 leu2-Δ1 lys2-801^{amber} trp1-Δ63 ura3-52 ade2*Δ*::URA3:LEU2*	This study
YSK365	*MAT***a** *ade2-101^{ochre} his3-Δ200 leu2-Δ1 lys2-801^{amber} trp1-Δ63 ura3-52* [ρ^0]	Kirchman *et al.* (1999)
YSK365 *rtg2*Δ	*MAT***a**, *ade2-101^{ochre} his3-Δ200 leu2-Δ1 lys2-801^{amber} trp1-Δ63 ura3-52 rtg2*Δ*::HIS3* [ρ^0]	Kirchman *et al.* (1999)
YSK365 *fob1*Δ	*MAT***a** *ade2-101^{ochre} his3-Δ200 leu2-Δ1 lys2-801^{amber} trp1-Δ63 ura3-52 fob1*Δ*::KAN^{r}* [ρ^0]	This study
YSK365 *rtg2*Δ *fob1*Δ	*MAT***a** *ade2-101^{ochre} his3-Δ200 leu2-Δ1 lys2-801^{amber} trp1-Δ63 ura3-52 rtg2*Δ*::HIS3 fob1*Δ*::KAN^{r}* [ρ^0]	This study
YSK365 *rtg3*Δ	*MAT***a** *ade2-101^{ochre} his3-Δ200 leu2-Δ1 lys2-801^{amber} trp1-Δ63 ura3-52 rtg3*Δ*::URA3* [ρ^0]	Jiang *et al.* (2000)
YPK9 *cit2:lacZ*	*MAT***a** *ade2-101^{ochre} his3-Δ200 leu2-Δ1 lys2-801^{amber} trp1-Δ63 ura3-52::cit2:lacZ:URA3*	This study
YSK365 *cit2:lacZ*	*MAT***a** *ade2-101^{ochre} his3-Δ200 leu2-Δ1 lys2-801^{amber} trp1-Δ63 ura3-52::cit2:lacZ:URA3* [ρ^0]	This study
YPK9 *fob1:lacZ*	*MAT***a** *ade2-101^{ochre} his3-Δ200 leu2-Δ1 lys2-801^{amber} trp1-Δ63 ura3-52::fob1:lacZ:URA3*	This study
YPK9 *rtg2*Δ *fob1:lacZ*	*MAT***a** *ade2-101^{ochre} his3-Δ200 leu2-Δ1 lys2-801^{amber} trp1-Δ63 rtg2*Δ*::HIS3 ura3-52::fob1:lacZ:URA3*	This study
YSK365 *fob1:lacZ*	*MAT***a** *ade2-101^{ochre} his3-Δ200 leu2-Δ1 lys2-801^{amber} trp1-Δ63 ura3-52::fob1:lacZ:URA3* [ρ^0]	This study
YSK365 *rtg2*Δ *fob1:lacZ*	*MAT***a** *ade2-101^{ochre} his3-Δ200 leu2-Δ1 lys2-801^{amber} trp1-Δ63 rtg2*Δ*::HIS3 ura3-52::fob1:lacZ:URA3* [ρ^0]	This study
Sp1-1	*MAT***a** *ura3 leu2 trp1 ade8 can1 his3 gal2*	Kirchman *et al.* (1999)
Sp1-1 ρ^0	*MAT***a** *ura3 leu2 trp1 ade8 can1 his3 gal2* [ρ^0]	Kirchman *et al.* (1999)

*Kpn*I and *Sac*I restrictions sites were used to clone the PCR product downstream of the *ADH2* promoter between the *Kpn*I and *Sac*I restriction sites of the episomal pBEVY-U plasmid (Miller *et al.* 1998), which contains the *URA3* marker. This construct was verified by sequencing.

Yeast cells were grown at 30° in YPD (2% peptone, 1% yeast extract, 2% glucose, pH 6.5). For life-span analysis, YPK9 strains were pregrown in YPG (2% peptone, 1% yeast extract, 2% glycerol) to suppress the growth of petites. Cells were grown in YPAD (YPD containing 120 μg/ml adenine) for preparation of old cells by rate zonal sedimentation. For detection of ERCs in Figure 1, YPK9 and SP1-1 strains were grown in YPR (YPD containing 2% raffinose instead of glucose). The selection of *URA3* transformants was performed on synthetic complete (SC)-uracil (-ura) medium (Guthrie and Fink 1991). For silencing experiments, SC medium containing 2 mg/ml 5-fluoroorotic acid (5-FOA) was used. Transformants obtained using the kanamycin marker were selected on YPD-kan (YPD medium containing 200 μg/ml geneticin). Solid medium contained 2% agar.

Life-span analysis: Life-span analysis was performed as described by Kim *et al.* (1999b). Briefly, 35–40 virgin cells (new buds) from an overnight culture were used for each life-span measurement. These cells were deposited in a row in isolated spots on a YPD plate. The number of buds removed by microdissection prior to cell death is the cell's life span in generations. The Mann-Whitney test was used to assess statistical significance of differences between the life spans of different strains in an experiment. All life spans were repeated at least two times. Figures 3 and 5 show the results of representative experiments.

Old cell preparation: Yeast cells of different ages (generations) were isolated by rate-zonal sedimentation in 10–30% w/v sucrose gradients according to Egilmez *et al.* (1990) for determination of ERC production during the life span. In other experiments, populations of cells of different ages were obtained by sorting cells using the fluorescence-activated cell sorter (FACS) Vantage SE (Becton Dickinson, Franklin Lakes, NJ) with the Enterprise II coherent UV laser (351–364 nm emission). Forward angle light scatter was taken as a measure of cell size and Calcofluor fluorescence was detected in the FL-5 (424/44 nm) channel to estimate bud scar number, as described before (Kim *et al.* 1996). Cells were grown overnight in YPD or selective media. About 1×10^7 cells were stained

for bud scars in 140 mM NaCl, 3.0 mM KCl, 8.0 mM Na_2HPO_4, 3.0 mM KH_2PO_4, pH 7.3 (PBS) containing 0.1 mg/ml Calcofluor (Sigma, St. Louis) for 15 min at room temperature. Before sorting, cells were sonicated for 5 sec and washed two times in PBS. To study silencing, 10,000 young and old cells were collected and plated onto different media as indicated in Figure 8. The young cells were the smallest and least fluorescent cells from the population, while the old cells were the largest and most fluorescent. For analysis of induction of the retrograde response with age, four aliquots of 200,000 cells, from the youngest to the oldest, were selected from the population. These cells were taken to measure *CIT2* promoter activity from the reporter in pCIT-LacZ800 in β-galactosidase assays, as described below.

β-Galactosidase assays: Cells were grown in SC-ura medium. Cells directly from cultures or sorted cells were lysed by freezing in liquid nitrogen and thawing at 30° in 1 ml Z-buffer [0.06 M Na_2HPO_4, 0.04 M NaH_2PO_4, 0.01 M KCl, 1 mM $MgSO_4$, 0.27% (v/v) β-mercaptoethanol]. Assays were performed by adding 200 μl *o*-nitrophenyl β-galactopyranoside at 4 mg/ml. After samples turned yellow or after a maximum of 2 hr, reactions were stopped with 500 μl 1 M Na_2CO_3 and absorbance at 420 nm was measured. This was done so that the measurements were in the linear range of the assay. Activity was calculated in Miller units, as described previously (Miller 1972). To determine the activity of the *FOB1* promoter with the reporter pFOB-LacZ800, 1 ml of late-logarithmic phase cultures was collected for β-galactosidase assays. Protein concentration was measured using the protein assay reagent (Bio-Rad, Richmond, CA) or the NanoOrange protein quantitation kit (Molecular Probes, Eugene, OR) to calculate specific activity for comparing different samples. All assays were performed at least in triplicate and experiments were repeated four times.

ERC detection: YPK9 cells were grown overnight in 5 ml YPAD. DNA was isolated using glass beads as described (Ausubel *et al.*1994). Thirty micrograms of DNA was digested with *Spe*I. This enzyme does not cut in the rDNA circles, but releases a 4.1-kb fragment of the *ACT1* gene, which was used for quantification. The DNA was electrophoresed on a 0.7% agarose gel at 30 V for 18 hr and then transferred to a nylon membrane, which was hybridized with the *ACT1* and 35S-rDNA probes. The *ACT1* and 35S probes were generated by the PCR from genomic DNA. The PCR products were cloned into pUC18 and verified by sequencing. They were excised by restriction enzyme digestion, purified by electrophoresis, and labeled with [α-^{32}P]dCTP, using the RediPrime kit (Amersham). Blots were scanned using the Typhoon (Amersham), and quantification was performed using ImageQuant version 5.2 software (Molecular Dynamics, Sunnyvale, CA). Two-dimensional gel electrophoresis as described by Sinclair and Guarente (1997) was used to verify the circular nature of the detected rDNA monomers and concatamers.

Western blot analysis: Proteins were isolated using the glass-bead method according to Ausubel *et al.* (1994). Proteins were resuspended in disruption buffer [20 mM Tris-Cl, pH 7.9, 10 mM $MgCl_2$, 1 mM EDTA, 5% glycerol, 1 mM dithiothreitol, 0.3 M $(NH_4)_2SO_4$, 1 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor cocktail (Sigma)]. Thirty micrograms of protein was separated on a 0.8% polyacrylamide gel in 25 mM Tris, 0.19 M glycine, and 0.1% SDS, pH 8.3, at 100 V for 4 hr. Proteins were blotted onto a nitrocellulose membrane in ice-cold 25 mM Tris, 192 mM glycine, and 20% methanol, pH 8.2, at 100 V for 1 hr using the Mini-Transblot system (Bio-Rad). The blots were incubated with mouse anti-Myc (1:1000 dilution; Santa Cruz), or anti-Tub2 (β-tubulin, 1:500; Sigma) antibodies for 4 hr. Detection of Rtg2-Myc epitope-tagged protein or Tub2p was performed using an alkaline-phosphatase-coupled secondary anti-mouse antibody and the Western light detection chemiluminescence kit (Applied Biosystems, Foster City, CA), according to instructions of the manufacturer.

Northern blot and reverse transcript (RT)-PCR analysis: RNA was isolated from late-logarithmic-phase cultures using glass beads and hot acidic phenol (Ausubel *et al.* 1994). For Northern blots, typically 10 μg of RNA was electrophoresed on a 1% formaldehyde-agarose gel, which was subsequently blotted overnight onto a nylon membrane. Blots were hybridized overnight in PerfectHyb Plus hybridization buffer (Sigma) with ^{32}P-labeled probes prepared as described above for detection of ERCs. Blots were scanned and quantified, as described above for ERC detection. For the preparation of cDNA from RNA the Superscript kit (Invitrogen, San Diego) was used according to the manufacturer's manual. For amplification of *FOB1* transcripts, primers 5′-CGTAACATCAAGCACTTTAG-3′ and 5′-ATCCGCTTCATTAGCAAGGG-3′ (yielding a 414-bp fragment), and for amplification of *ACT1* (control), primers 5′-CCATCTAT CGTCGGTA-3′ and 5′-CCAATCCAGACGGAGTAC-3′ (yielding a 932-bp fragment), were used in the same PCR reaction. The PCR products were separated by electrophoresis in an agarose gel and detected by staining the gel with ethidium bromide. Gels were analyzed using the Gel Doc 2000 (Bio-Rad).

RESULTS

Accumulation of ERCs during the life span: The rate of recombination and excision events that give rise to ERCs is higher in petite strains than in respiratory-competent, grande strains (Conrad-Webb and Butow 1995), suggesting a higher rate of ERC accumulation during the life span in petites. Yet petites have a longer life span resulting from the activation of the retrograde response pathway (Kirchman *et al.* 1999). A Southern blot of DNA from the grande strains (ρ^+) YPK9 and SP1-1 and the derived long-lived petite strains (ρ^0) (Kirchman *et al.* 1999) is shown in Figure 1. In addition to a strong signal from the genomic rDNA copies (*RDN1* locus on chromosome XII), hybridization with a probe of the 35S region of the rDNA resulted in distinct bands of either lower or higher molecular weight. These bands represent monomers (9.1 kb) and concatamers of rDNA circles, respectively. These data indicate that petite strains live longer, even though their ERC levels are higher.

The yeast cultures analyzed for cellular ERC content mainly contained young cells, because of the asymmetric mode of yeast cell division (Beran *et al.* 1967). It has been suggested that ERCs accumulate exponentially during aging (Sinclair and Guarente 1997). It is possible that the ERC levels observed could change in various ways and at different rates as cells of petite and grande strains progress through their replicative life spans, explaining the longer life span of petite strains despite their higher steady-state ERC content as compared to grandes. To examine this question, we prepared YPK9 (grande) and co-isogeneic YSK365 (petite) cells of specific replicative ages. As can be seen in Figure 2, YPK9 cells gradually accumulated ERCs during their life span. However, in age-matched YSK365 cells the ERC levels, as well as the rate of increase in these levels, were much

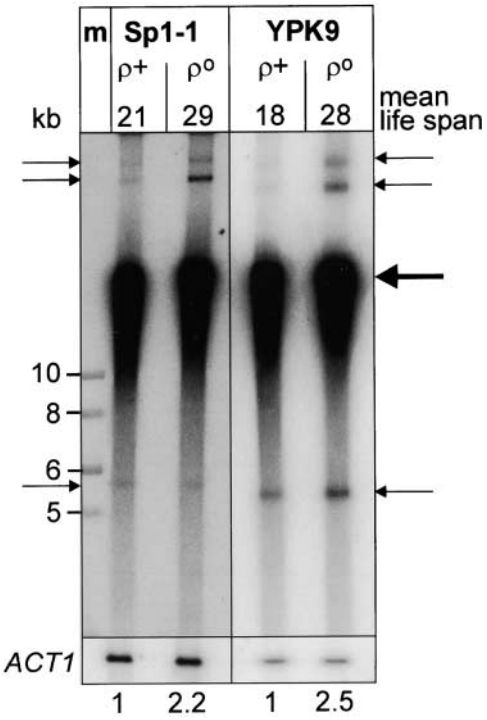

FIGURE 1.—Analysis of ERCs in grande and petite strains. DNA of two different grande (ρ^+) and their derived petite (ρ^0) strains was hybridized on Southern blots with a probe specific to 35S rDNA sequences (top). The mean life spans of the grande and petite strains found previously are indicated above each lane (KIRCHMAN *et al.* 1999). Bands representing ERCs (thin arrows) and the hybridization signal of the genomic rDNA copies (thick arrow) are indicated. For quantification of ERCs, signal intensities were normalized against hybridization signals of *ACT1* (bottom). The increases in ERCs in petite strains compared to their grande counterparts are indicated at the bottom. m, DNA markers.

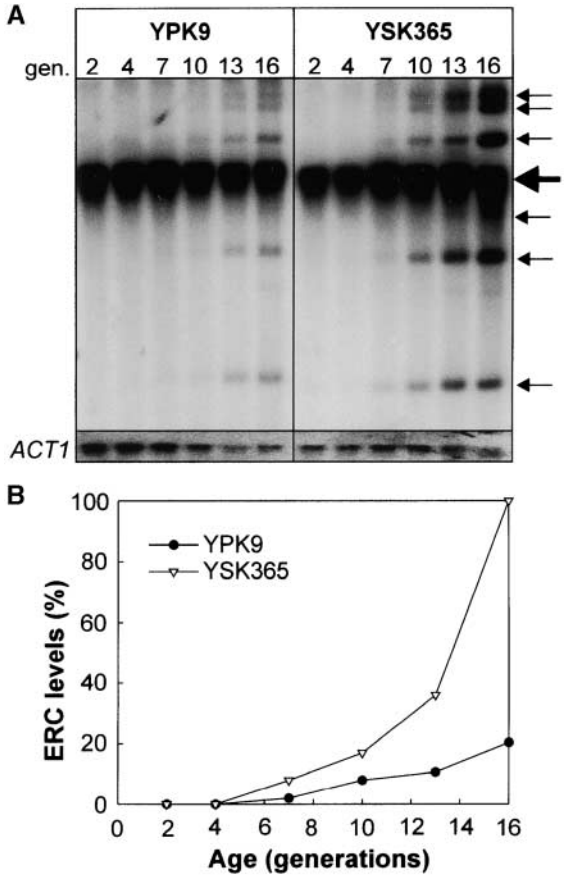

FIGURE 2.—Analysis of ERC accumulation with age. (A) DNA of yeast cells of specific replicative ages (gen., generations) was isolated as described in MATERIALS AND METHODS. After electrophoresis and blotting, the membrane was hybridized with 35S rDNA (top) and *ACT1* (bottom) probes. Thin arrows point to ERCs, whereas the thick arrow indicates the hybridization signal of the genomic rDNA copies. (B) The signal intensities of all ERCs (thin arrows in A) in 16-generations-old YSK365 cells were added up and the total amount was referred to as 100%. Using the *ACT1* signals for normalization, the amounts of ERCs (in percentages) in the other samples were calculated and plotted.

higher. Therefore, we conclude that petite strains have a higher rate of ERC accumulation. Also, these results show directly that ERCs accumulate exponentially during yeast aging. It is important to note that by 16 generations YPK9 cell cohorts nearly exhaust their life expectancy, while, in comparison, YSK365 cell cohorts have nearly 50% of their life expectancy remaining. Thus, the ERC levels may be substantially higher in YSK365 cohorts than in YPK9 cohorts when they become extinct.

Influence of ERC formation on life span: We next asked whether it is possible that ERC formation does not reduce life span in a petite strain. In this case elimination of ERC formation should not further increase the life span of YSK365. Life spans were determined for YPK9 and YSK365 cells in which the *FOB1* gene was deleted. As can be seen in Figure 3A, the mean life span of YSK365 *fob1*Δ cells was increased by 13 generations compared to YSK365, whereas the mean life span of YPK9 *fob1*Δ cells was increased by only 6 generations compared to YPK9. These results show that the ERC-generating pathway influences life span negatively in both grande and petite strains. Nevertheless, the retrograde response appears to partially offset the negative effect of ERCs. The average increase in life span resulting from deletion of *FOB1* in YPK9 was 30% (±4.7% SE), while in YSK365 it was 54% (±9.5% SE) in at least five repetitions. The excess life extension afforded by combining the induction of the retrograde response with elimination of ERC production, which amounted to 24% ($P < 0.001$) or nearly one-half of the life extension observed, provides a rough estimate of the impact on life extension of the retrograde response acting on processes other than those sensitive to ERCs.

To analyze the effect of ERCs and of the retrograde response on the life span of petites more precisely, a YSK365 *fob1*Δ *rtg2*Δ double-deletion mutant was examined. As shown in Figure 3B, deletion of *RTG2* significantly shortened the life span of the petite YSK365. This was compensated by deletion of *FOB1*. The double

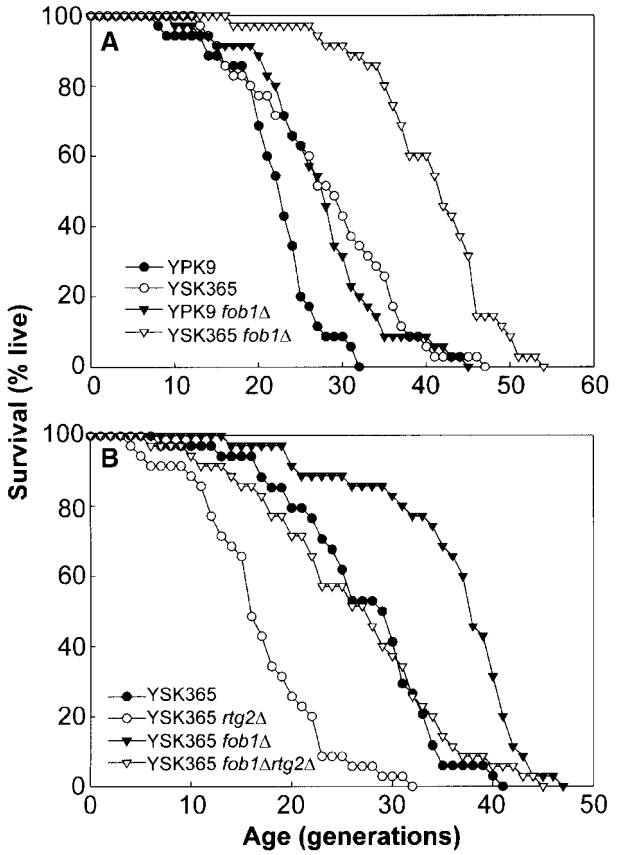

FIGURE 3.—Life-span analysis. All strains were grown on YPD plates. (A) The mean life spans of strains YPK9, YSK365, YPK9 *fob1*Δ, and YSK365 *fob1*Δ shown here were 21.2, 26.5, 27.2, and 39.5 generations, respectively. All the differences between the life spans were significant ($P < 0.002$), except for those of YPK9 *fob1*Δ and YSK365. (B) In a separate experiment, the mean life spans of strains YSK365, YSK365 *rtg2*Δ, YSK365 *fob1*Δ, and YSK365 *fob1*Δ *rtg2*Δ were 26.2, 15.9, 35.1, and 25.4 generations, respectively. All the differences between the life spans were significant ($P < 0.0001$), except for those of YSK365 and YSK365 *fob1*Δ *rtg2*Δ.

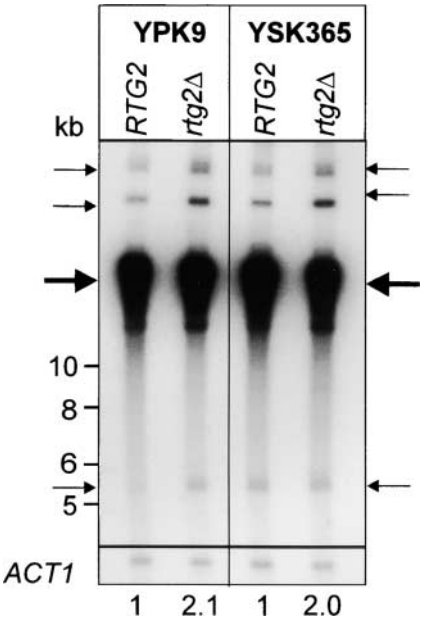

FIGURE 4.—Analysis of ERCs in *rtg2*Δ strains. DNA was isolated from YPK9 and YSK365 strains with (*rtg2*Δ) or without (*RTG2*) a deletion in the *RTG2* gene grown on YPD. The DNA was hybridized on a Southern blot with a 35S rDNA (top) and *ACT1* (bottom) probe for ERC quantification. Indicated bands are as in Figure 1. The increase in ERCs in petite strains compared to their grande counterparts is indicated at the bottom.

mutant displayed the same life span as YSK365. The extra life span observed when only *FOB1* was deleted provides an estimate (38%) of the effect of the retrograde response on life-span functioning by mechanisms other than the counteraction of the negative influences of ERCs on longevity.

Effect of Rtg2p on ERC formation: Because ERCs increase when the retrograde response is turned on, we expected ERCs to decrease when the retrograde response is prevented. We found that ERC levels were increased in the YPK9 *rtg2*Δ and YSK365 *rtg2*Δ strains, compared to those in YPK9 and YSK365, respectively (Figure 4). From this observation, we conclude that the high levels of ERCs are not caused by the retrograde response as such, because elimination of the retrograde-signaling protein Rtg2 increases ERC levels in both the grande and the petite strains. We conjectured that ERC formation could have something to do with Rtg2p itself.

We therefore investigated the expression of *RTG2* in the YPK9 and YSK365 strains. Under a variety of growth conditions (logarithmic, late logarithmic, and stationary phase), the expression of *RTG2* was always the same in both strains (Figure 5A). Therefore, the expression of this gene does not depend on mitochondrial defects or growth phase. We also conclude that Rtg2p stability is the same in strains with or without mitochondrial defects, because the amount of tagged Rtg2p detected was the same in grande and petite strains (see Figure 6A).

Rtg3p lies downstream of Rtg2p in the retrograde response pathway. We focused on *RTG3* to further examine the effects of retrograde signaling on ERC production. We found an increase in ERCs in the YSK365 *rtg3*Δ cells (about fourfold) but not in the YPK9 *rtg3*Δ cells (Figure 5B), in contrast to the effect of *RTG2* deletion. The life-span analysis of the *rtg3* mutants also reflects these results. In agreement with the unaffected ERC levels in the YPK9 *rtg3*Δ cells, the life span of this mutant was unchanged (Figure 5C). On the other hand, the life span of the YSK365 *rtg3*Δ cells was shortened, correlating with higher ERC levels (Figure 5D). In the absence of Rtg3p, Rtg2p apparently cannot further transmit the signal it receives from dysfunctional mitochondria, leaving it in a state that favors ERC production. We conclude that Rtg2p lies at a branch point for ERC production in the retrograde response pathway.

To examine the nature of this branch point, a Myc-epitope-tagged Rtg2p was expressed in cells. The level of Rtg2-Myc protein was the same in grande and petite

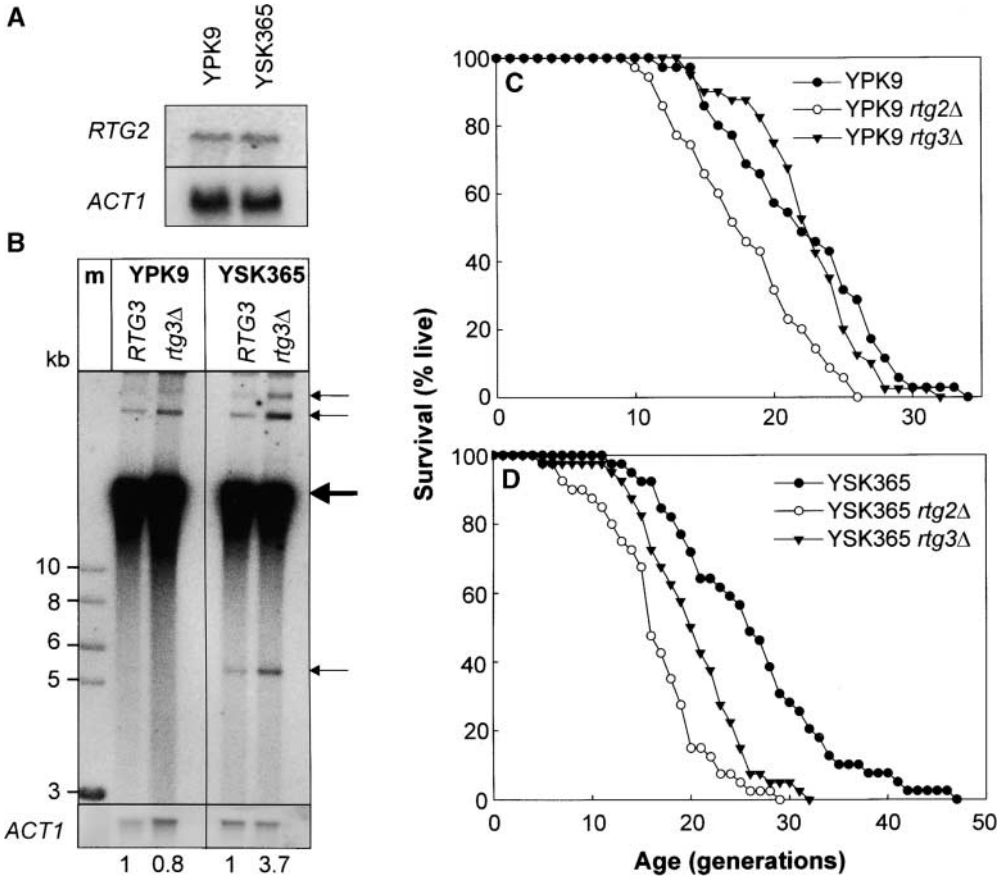

FIGURE 5.—Effect of *RTG3* deletion on ERCs and life span. (A) Northern blot analysis of *RTG2* gene expression. RNA was isolated from YPK9 and YSK365 cultures in stationary phase grown in YPD medium. Blots were hybridized with an *RTG2* probe. For quantification, the same blot was hybridized with an *ACT1* probe. (B) Southern blot of DNA of YPK9 and YSK365 with (*rtg3*Δ) or without (*RTG3*) a deletion in the *RTG3* gene. The blot was probed and the indicated bands are as in Figure 1. The increase in ERCs is indicated at the bottom. (C) Life-span analysis of different YPK9 strains. Cells were grown in YPD. Mean life spans of YPK9, YPK9 *rtg2*Δ, and YPK9 *rtg3*Δ were 21.2, 16.8, and 21.6 generations, respectively. The differences in life spans between YPK9 and YPK9 *rtg2*Δ and between YPK9 *rtg2*Δ and YPK9 *rtg3*Δ were significant ($P < 0.002$). (D) Life-span analysis of different YSK365 strains. Cells were grown in YPD. Mean life spans of YSK365, YSK365 *rtg2*Δ, and YSK365 *rtg3*Δ were 25.4, 15.6, and 19.3 generations, respectively. The differences in life spans between YSK365 and its two deletion strains were significant ($P < 0.001$). The difference between YSK365 *rtg2*Δ and YSK365 *rtg3*Δ was also significant ($P = 0.003$). The life spans shown in C and D were from the same experiment. There was no significant difference between the life spans of YPK9 *rtg2*Δ and YSK365 *rtg2*Δ ($P = 0.27$). The maximum life spans of these strains also did not differ significantly on the basis of the ninetieth percentile ($\chi^2_{(1)} = 0.33$, $P > 0.5$).

strains, showing that the stability of Rtg2p is not influenced by mitochondrial dysfunction (Figure 6A). Furthermore, the protein was functional, because *CIT2* expression was induced in YSK365 *rtg2*Δ cells expressing Rtg2-Myc protein (Figure 6B). Also, the level of *CIT2* expression was found to be the same in YSK365 *rtg2*Δ cells expressing the tagged Rtg2p compared to YSK365 cells expressing endogenous *RTG2* (Figure 6B). This shows that activation of the retrograde response is dependent on the mitochondrial signal and not on limiting amounts of Rtg2p. Overexpression of *RTG2-MYC* in YPK9 and YSK365 resulted in a net increase (1.5-fold) in *RTG2* transcripts (Figure 6C). In contrast, the expression of the target gene *CIT2* was not increased, supporting the conclusion that Rtg2p was not limiting for the retrograde response. As shown in Figure 6D, ERC accumulation was lowered in both YPK9 and YSK365 cells by overexpression of Rtg2p. These results point to the fact that Rtg2p, when it is not engaged in transmitting the retrograde signal from the mitochondrion to Rtg3p, has an additional function that is linked to prevention of ERC formation.

***RTG2* affects the *FOB1* pathway:** Both Rtg2p and Fob1p affect ERC production. Because the retrograde response is mediated by the transcription factor Rtg1p-Rtg3p, this transcription factor or one of the products of its target genes could influence the expression of *FOB1*. The expression of this gene could not be readily analyzed on Northern blots, because of the low level of *FOB1* transcripts. Therefore, it was analyzed by using semiquantitative RT-PCR. Different numbers of PCR cycles were performed to determine the logarithmic phase of the reaction. We found no difference in the expression level of *FOB1* in YPK9 compared to that in YPK9 *rtg2*Δ or in YSK365 compared to YSK365 *rtg2*Δ strains (Figure 7A). Additionally, an expression assay was used to study *FOB1* promoter activity in these strains. The promoter region examined contains the TATTAA box at position −300, as predicted using SIGSCAN (PRESTRIDGE 1991). β-Galactosidase assays showed no difference in the promoter activity among the various strains (Figure 7B). This result indicates that the retrograde response does not directly or indirectly regulate *FOB1* gene expression. Northern blot analysis showed that *RTG2* is normally expressed in YPK9 *fob1*Δ and YSK365 *fob1*Δ strains (not shown).

The fact that an *RTG2* deletion increases ERCs and that Rtg2p does not influence the expression level of

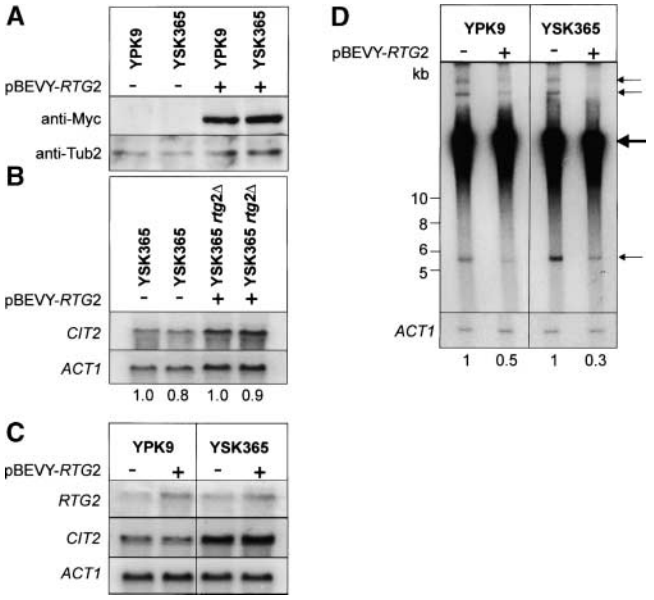

FIGURE 6.—Effect of overexpression of *RTG2* on retrograde response and on ERC formation. All control strains carried the empty pBEVY vector. (A) Strains YPK9 and YSK365 were transformed with the pBEVY-*RTG2* plasmid in which the *RTG2* gene is tagged with a sequence encoding a Myc epitope. Expression of the Rtg2-Myc fusion protein was verified using an anti-Myc antibody (anti-Myc). According to the size of the band, the signal represents the Rtg2-Myc fusion protein. To quantify protein amounts in the transformed strains, the anti-β-tubulin antibody (anti-Tub2) was used for normalization. The relative amounts of Rtg2-Myc protein were virtually identical in YPK9 (1.0) and in YSK365 (0.9). (B) Northern blot analysis of *CIT2* expression in two separate RNA preparations from strains YSK365 and YSK365 *rtg2*Δ, transformed with the pBEVY-*RTG2* plasmid. Blots were hybridized with a *CIT2* probe. Blots were also hybridized with an *ACT1* probe for normalization. *CIT2* transcript levels are expressed at the bottom relative to the hybridization signal of the first YSK365 sample on the left. No expression of *CIT2* was detected in parallel lanes containing RNA from YSK365 *rtg2*Δ cells lacking the pBEVY-*RTG2* plasmid (not shown). (C) Overexpression of the *RTG2* gene was verified by Northern blot analysis. The increase in transcripts in both strains was ~1.5-fold (top). The same blot was hybridized with a *CIT2* probe (middle) and an *ACT1* probe (bottom) for normalization. The relative *CIT2* expression levels were the same in YPK9 transformed (0.9) and untransformed strains (1.0) by pBEVY-*RTG2* and in YSK365 transformed (1.1) and untransformed (1.0) by the same plasmid. (D) Southern blot analysis of ERCs in YPK9 and YSK365 strains untransformed or transformed by pBEVY-*RTG2*. Quantification of ERCs and the indicated bands are as in Figure 1. The decrease in the amount of ERCs in the cells overexpressing the Rtg2-Myc protein is indicated at the bottom.

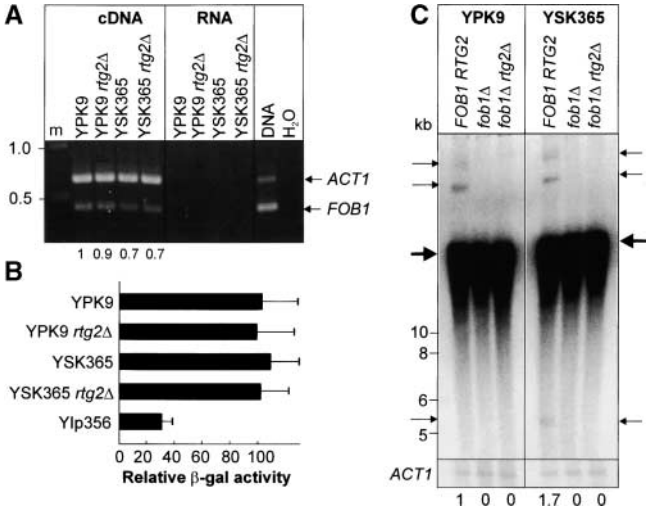

FIGURE 7.—The effect of *RTG2* deletion on *FOB1* expression and of *RTG2* and *FOB1* deletion on ERC accumulation. (A) RT-PCR analysis of samples isolated from the indicated strains. PCR amplification of *FOB1* transcripts was performed using either cDNA or RNA (negative control). The PCR was also performed using genomic DNA (positive control). For quantification of transcripts, *ACT1* transcripts were amplified in the same reaction. The relative expression of *FOB1* in the strains compared to YPK9 is indicated at the bottom. (B) The indicated strains were transformed with the pFOB1-LacZ800 plasmid, which was integrated at the *ura3-52* locus. As a control, YPK9 cells were also transformed with the (empty) *lacZ* vector (YIp356) without addition of any promoter sequences before the *lacZ* gene. β-Galactosidase assays were performed as described in MATERIALS AND METHODS. Enzyme activity is expressed relative to YPK9 (100%). In this experiment, 100% activity represents a specific activity of 12.5 nmol/min/mg protein. (C) Southern blot of DNA from grande (YPK9) or petite (YSK365) strains with *FOB1* or *FOB1* and *RTG2* deletions. Quantification of ERCs was as in Figure 1, and the indicated bands are as in Figure 1. The decrease in ERCs is indicated at the bottom.

the *FOB1* gene allowed us to determine whether Rtg2p converges with the *FOB1* pathway leading to ERC formation or affects ERC production through a novel pathway independent of *FOB1*. We found no formation of ERCs in the YPK9 *rtg2*Δ *fob1*Δ and the YSK365 *rtg2*Δ *fob1*Δ strains (Figure 7C). These results show that the increase in ERCs observed in YPK9 *rtg2*Δ and YSK365 *rtg2*Δ strains is dependent on the *FOB1* pathway. Either the retrograde response and *FOB1* pathways converge or Rtg2p interacts either directly or indirectly with Fob1p preventing ERCs, so that deletion of *RTG2* or recruitment of Rtg2p in retrograde signaling increases the amount of Fob1p available for ERC formation.

Analysis of silencing: It has been shown that the level of silencing at the rDNA locus correlates with the rate of recombination in the rDNA (GOTTLIEB and ESPOSITO 1989). A loss of silencing increases, in a dosage-dependent manner, the recombination rate of marker genes inserted into the rDNA (FRITZE *et al.* 1997). We examined the possibility that silencing of rDNA is lower in YSK365 than in YPK9, causing the higher levels of ERCs in the petite strain. A reporter gene cassette was inserted into the rDNA locus of various strains (Figure 8A). This cassette contains a small fragment of the *TRP1* promoter, which is silenced at the rDNA locus (SMITH and BOEKE 1997), followed by the *URA3* gene. With this cassette, it is possible to screen for low silencing (good growth on SC-ura and no growth on SC+FOA medium) or high silencing in the rDNA (no growth on SC-ura and good growth on SC+FOA). This cassette was also

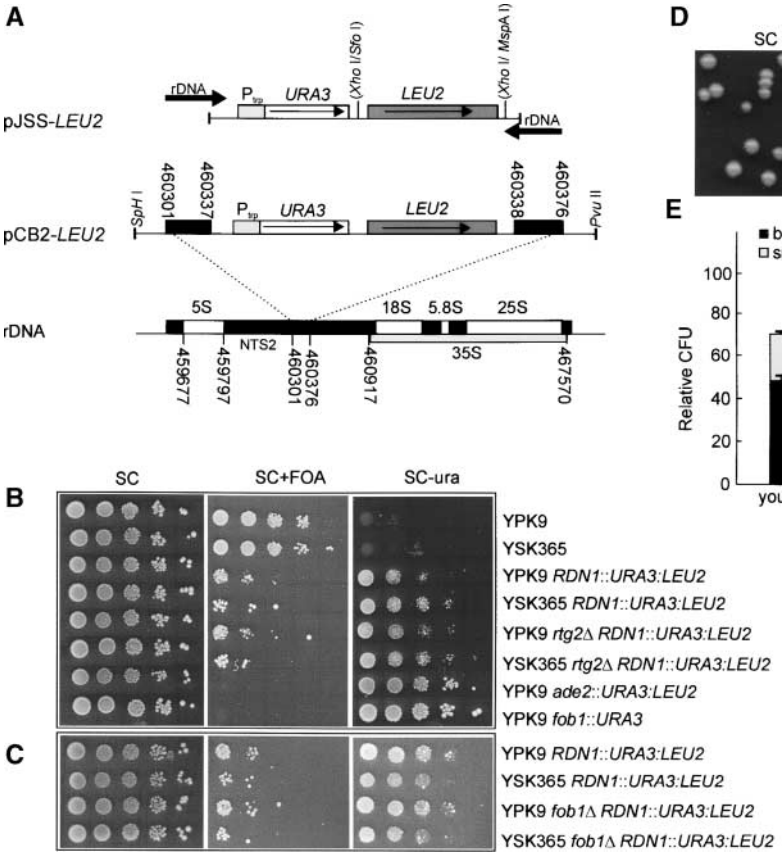

FIGURE 8.—Analysis of silencing. (A) Schematic of the cloning and insertion strategy for the *URA3:LEU2* reporter cassette in the rDNA locus between the 5S and 35S rRNA genes. Addition of rDNA sequences to the *URA3:LEU2* cassette was done by using PCR (top) with primers (arrows) containing rDNA sequences. The exact position according to the *Saccharomyces* Genome Database is indicated (middle). The integration site is indicated at the bottom. See MATERIALS AND METHODS for further description. (B) Plating assay of the different strains transformed with the *URA3:LEU2* cassette. Cells from a liquid culture were plated in 10-fold serial dilutions (from left to right) on SC, SC-ura, and SC+FOA medium. A *URA3* positive strain (YPK9 *fob1*Δ) with normal *URA3* promoter activity and a strain in which the *ADE2* gene was replaced by the *URA3:LEU2* cassette were used as positive controls. (C) Analysis of pop-out events. The *fob1*Δ strains were included to check whether results in B are influenced by pop-out of the *URA3:LEU2* cassette. Pop-outs require *FOB1*. (D) Plating of FACS-sorted young YPK9 cells resulted in the formation of small colonies (arrows) on SC+FOA plates. These colonies were not observed on SC. (E) The small and big colonies (expressed as colony-forming units, or CFUs) growing from sorted young and old cells of strains YPK9 and YPK9 *rtg2*Δ on SC+FOA plates were counted, and the CFUs were compared to those obtained on SC medium (set at 100%), which are reported as relative CFUs here. The results for small colonies are indicated by shaded bars. Error bars indicate SD.

inserted into the *ade2* locus of YPK9 as a control for expression of *URA3*.

First, the level of silencing was compared between YPK9 and YSK365 on SC-ura and SC+FOA medium. As can be seen in Figure 8B (rows 3 and 4), no difference was found between the silencing levels. Both strains grew equally, and they grew on both media. To determine whether *RTG2* affects the silencing process, the cassette was also inserted into the rDNA of the YPK9 *rtg2*Δ and YSK365 *rtg2*Δ strains. No difference was found in the growth of these strains (rows 5 and 6) compared to the parental strains (rows 3 and 4). Also, a deletion of the *FOB1* gene in YPK9 and YSK365 did not change the amount of growth on the different plates (Figure 8C). This result indicates that in this experiment the growth of cells was not influenced by excessive pop-out of ERCs containing nonsilenced reporter gene cassettes. We conclude that the high levels of ERCs in YSK365 are not caused by a lower level of silencing in this strain.

In the past it was reported that silencing at the telomeres and silent mating-type loci decreases with age (KIM *et al.* 1996; SMEAL *et al.* 1996). A movement of Sir3p and Sir4p to the nucleolus was reported (KENNEDY *et al.* 1997), but the silencing level of the rDNA in old cells has not yet been investigated. Because we observed a strong increase of ERCs with age, we thought that perhaps silencing at the rDNA locus also decreases with age. YPK9 cells containing the reporter gene cassette at the rDNA locus were sorted according to age and plated onto SC and SC+FOA to score for silencing. As can be seen in Figure 8, D and E, a smaller proportion of the young cells was able to grow on FOA plates, compared to that of the older cells. Additionally, some colonies were much smaller in size, indicating a lower silencing level in young cells compared to that in older cells. Similar results were obtained with the YPK9 *rtg2*Δ strain (Figure 8E), indicating that the increased ERC production with age in that strain does not result from changes in chromatin structure associated with a decrease in silencing. The results with YSK365 and YSK365 *rtg2*Δ strains prompted the same conclusion (not shown). The data indicate that silencing at the rDNA locus increases in older cells. The concomitant increase in silencing of rDNA and accumulation of ERCs during aging indicates that the latter is not dependent on loss of silencing.

The increase in ERCs with age correlates with increased induction of the retrograde response: The underlying cause of ERC accumulation with age has not

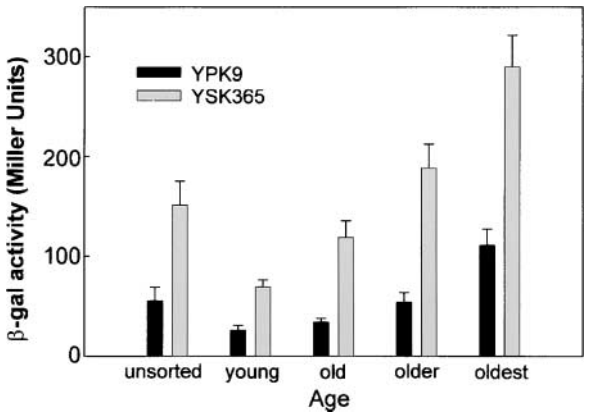

FIGURE 9.—Induction of the retrograde response in old cells. The pCIT2-LacZ800 vector was transformed into YPK9 and YSK365 cells at the *ura3-52* locus. After growing cultures to late-logarithmic phase, the cells were sorted into groups of increasing replicative ages, as described in MATERIALS AND METHODS. The young and oldest groups correspond to the young and old groups in Figure 8E, respectively. The average age of the cells in the young, old, older, and oldest groups was 0, 4, 7–8, and 11–12 generations, respectively, as determined by counting bud scars after staining of cells in corresponding preparations with Calcofluor. β-Galactosidase assays were performed, and the enzyme activity is expressed in Miller units. The activity was also determined in unsorted cells, representing the total population. Lysates contained 50.1 ± 2.4 ng protein/1 × 10^5 cells. Error bars indicate SE.

been reported. Recently, it was shown that in a grande strain mitochondrial function decreases with age (LAI *et al.* 2002). This accumulation of dysfunctional mitochondria suggests that the retrograde response also increases with age to compensate for this defect, because we know that the induction of this response and the life extension it promotes is commensurate with the extent of the mitochondrial defect (JAZWINSKI 2000). If the extent of Rtg2p recruitment in retrograde signaling is higher in old cells, this could explain the high levels of ERCs in these cells, because we have shown that ERC formation is dependent on the status of Rtg2p. Because ERCs increase in YSK365 as well as in YPK9, and even at a higher rate, the retrograde response should then increase in both strains. This is not necessarily expected for YSK365, because these cells are born as petites lacking fully functional mitochondria. To examine the induction of the retrograde response with age, a reporter construct was introduced into YPK9 and YSK365 in which the *CIT2* promoter drives expression of *lacZ*. Cells were sorted according to replicative age, and the activity of the *CIT2* promoter, which is a measure of the retrograde response, was determined in a β-galactosidase reporter assay (Figure 9). As can be seen, the retrograde response was higher in older cells compared to that in young cells. The increase in activity between the youngest and the oldest grande cells was about threefold, whereas the increase for the petite cells was about fourfold, even though the latter start out with higher activity. The rates at which the retrograde response increases correspond to the ERC accumulation rates shown in Figure 2. For YPK9, these data indicate that mitochondrial dysfunction increases with age, due perhaps to mitochondrial DNA damage. For the petite strain, these data indicate that apart from a loss of mitochondrial DNA, other changes to mitochondria that increase the intensity of the retrograde response signal can occur. The retrograde response effectively compensates for mitochondrial dysfunction, because it extends life span (KIRCHMAN *et al.* 1999). However, it apparently does not prevent the accrual of further mitochondrial deficits during aging. ERC production appears to coincide with the advancing recruitment of Rtg2p in retrograde signaling, which occurs due to the progressive induction of the retrograde response during the replicative life span. The data show that the ultimate cause of ERC formation during aging is mitochondrial dysfunction, because the Rtg2p sensor of this dysfunction regulates the production of ERCs.

DISCUSSION

The life extension afforded by the induction of the retrograde response in petite yeast suggests at first glance that ERCs do not influence life span, because their accumulation with aging is more profound in these strains. However, the induction of the retrograde response masks the effect of ERCs. Consistent with this interpretation, prevention of the induction of the retrograde response in petites by deletion of either *RTG2* or *RTG3* substantially curtails life span, while, in contrast, deletion of *FOB1* extends it well beyond what is seen on induction of the retrograde response alone. The smaller effect of *RTG3* deletion compared to *RTG2* deletion on the life span of petites may be due to the more proximal role of Rtg2p in ERC production and to its function in two separate pathways, ERC generation and the retrograde response. Rtg3p, on the other hand, acts indirectly on ERC formation in its position downstream of Rtg2p in the retrograde response.

The retrograde response and the ERC production pathway, governed by *FOB1*, converge. The increase in ERCs caused by deletion of *RTG2* is completely suppressed by *FOB1* deletion. The question arises whether the sole effect of the retrograde response on life span is the countering of the negative effect of ERCs. This does not appear to be the case. More ERCs are in petites than in grandes, yet petites have a longer life span. The life extension resulting from *FOB1* deletion is greater in petites as compared to grandes, the difference amounting to ~50% of the total life extension provided by simultaneous induction of the retrograde response and elimination of ERCs. Therefore, there is an excess effect of the retrograde response on functions other than counteraction of the effects of ERCs. This conclusion is confirmed by the effect on the life span of petites

of *RTG2* deletion, which abrogates much of the life extension provided by deletion of *FOB1*. Thus, deletion of *RTG2* does not simply negate the life extension in a petite by inducing ERCs, indicating that the increase in life span in petites is due specifically to the retrograde response.

Rtg2p responds to a signal generated by dysfunctional mitochondria and relays this signal to the Rtg1p-Rtg3p transcription factor by mediating the dephosphorylation of Rtg3p. This facilitates the translocation of this transcription factor into the nucleus, where it alters the expression of genes that compensate for the dysfunction. The signal elicited by the dysfunctional mitochondria cannot itself be the trigger for ERC accumulation, because ERCs accumulate in the grande YPK9 *rtg2*Δ strain that has fully functional mitochondria. The trigger also cannot be the dephosphorylation of Rtg3p, or the translocation of the Rtg1p-Rtg3p complex into the nucleus, or the changes in the expression of retrograde responsive genes, because these events do not occur in the petite YSK365 *rtg2*Δ strain, and yet ERCs accumulate. We conclude that it is Rtg2p itself that prevents ERC production. However, Rtg2p must be in a state in which it is not involved in the induction of the retrograde response. It is possible that it is necessary for Rtg2p to interact with other proteins to inhibit ERC accumulation.

Rtg2p has been implicated in various interactions with other proteins in the cell in addition to the interaction with Rtg3p in the retrograde response discussed thus far. It is clear that Rtg2p responds to signaling from the TOR pathway (Komeili *et al.* 2000), which coordinates several distinct nutrient-responsive cellular pathways. Rtg2p is also involved in the nitrogen catabolism regulation pathway, in which it interacts with Mks1p (Pierce *et al.* 2001). The TOR kinases have also been implicated in this pathway. Mks1p is a growth regulator, which acts downstream of the Ras-cAMP pathway as a negative regulator of this pathway (Matsuura and Anraku 1993). It has been shown that Ras2p potentiates the retrograde response (Kirchman *et al.* 1999). The interaction of Rtg2p with Mks1p in the negative regulation of the retrograde response by TOR signaling has been documented recently (Dilova *et al.* 2002), and evidence has been presented for a complex between Rtg2p and Mks1p (Sekito *et al.* 2002). The interactions recounted here all likely occur in the cytoplasm. More recently, it has been found that Rtg2p is a component of the SLIK complex in the nucleus (Pray-Grant *et al.* 2002). The SLIK complex is related in its composition to the SAGA complex, which is a conserved histone acetyltransferase coactivator that regulates gene expression. SAGA and SLIK have multiple, partially overlapping activities in transcription by RNA polymerase II. Rtg2p is necessary for the functional integrity of SLIK, and cells deficient in SLIK display decreased expression of *CIT2*, the diagnostic gene for the retrograde response (Pray-Grant *et al.* 2002). Rtg2p is bound to chromatin at the promoters of the genes it regulates, when they are activated by acetylation (Pray-Grant *et al.* 2002).

The loss of fully functional mitochondria apparently leads to the activation of a recombination pathway that generates ERCs or it in some way enhances their accumulation. We favor the notion that deregulation of an Rtg2p-dependent recombination pathway in petites lies at the root of the increase in ERC accumulation. This hypothesis, however, must be tested. Support for the involvement of Rtg2p in recombination comes from the implication of this protein in trinucleotide-repeat expansion in yeast (Bhattacharyya *et al.* 2002). This activity of Rtg2p, like ERC production, is also independent of Rtg1p and Rtg3p. The homologous recombination that generates ERCs is dependent on *RAD52*, which is required for the repair of the double-strand breaks caused by Fob1p (Park *et al.* 1999). Petites are more resistant than grandes to exogenous oxidative agents (Traven *et al.* 2001; C. Lai and S. Jazwinski, unpublished results). However, they become highly sensitive in the absence of Rad52p (Davermann *et al.* 2002). This suggests the involvement of Rtg2p in recombination and/or repair, but the participation of this protein need not be at the level of gene expression. Instead, it could be a component of the protein complexes that are involved in these processes.

Overexpression of *RTG2* reduced ERC production (Figure 6), but it did not extend life span (not shown). Thus, life extension requires more than simply the elimination of ERC formation among the events controlled by Rtg2p. We have shown already that the retrograde response is among the events under Rtg2p control that increase longevity (Kirchman *et al.* 1999). However, it is evident that excess Rtg2p levels on their own do not enhance the retrograde response (Figure 6), providing an explanation for lack of life extension. It is also becoming apparent that Rtg2p is a component of protein complexes that impinge on several, perhaps competing, pathways, which may have opposite effects on yeast longevity. The involvement of Rtg2p in the retrograde response, the SLIK complex, and ERC formation is particularly evident. Rtg2p acts as a sensor of mitochondrial dysfunction in the retrograde response, whose activation extends life span apart from any effects on ERC production or its consequences. In relaying the retrograde signal, Rtg2p can no longer suppress ERC production, which curtails longevity. However, part of the life-extending effect of the retrograde response is mediated by counteracting the negative effects of ERCs.

What is the signal that changes the state of Rtg2p? We have shown recently that ERC accumulation does not occur in petites that result from mutations in *ATP2*, which encodes the β-subunit of mitochondrial F_1-ATPase (Lai *et al.* 2002). The mutants are deficient in mitochondrial membrane potential. This deficiency is apparently due to the inability of the mitochondrial ADP/ATP translo-

cator to exchange mitochondrial ADP for the ATP derived from glycolysis in the absence of hydrolysis by F_1 ATPase (Buchet and Godinot 1998). This would result in an increase in the cytoplasmic ATP/ADP ratio. Rtg2p possesses a domain that has similarity to the ATP-binding domain of Hsp70 (Koonin 1994). Mitochondrial Hsp70 alternates the protein partners it binds, depending upon whether it is in a complex with ADP or ATP (von Ahsen *et al.* 1995), and it thus senses the ATP/ADP ratio. Rtg2p may also act as an ATP sensor in the cell. In the presence of functional mitochondria, we propose that Rtg2p remains a part of a complex in the nucleus, where it suppresses ERC production. When mitochondria become impaired such as during aging, there may be a shift in the ATP/ADP balance, and Rtg2p becomes engaged in the induction of the retrograde response. However, this leads to the production of ERCs as a consequence. Daughters of young cells receive fully functional mitochondria, while old mothers tend to segregate defective mitochondria to their daughters (Lai *et al.* 2002). Thus, the latter daughters would have the retrograde response induced with attendant ERC production, while the former would be born without ERCs. Even ρ^0 petites appear to accumulate further mitochondrial damage with age, resulting in the increased induction of the retrograde response (Figure 9). Hence, the factors that serve to maintain mitochondrial membrane potential appear to be at risk during aging. The results presented here provide a link between cellular metabolism and genomic stability, which is important in aging, and they indicate that Rtg2p is a focal point of this link.

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