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Role of *RAS2* in Recovery from Chronic Stress: Effect on Yeast Life Span

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The replicative life span of *Saccharomyces cerevisiae* was previously shown to be modulated by the homologous signal transducers Ras1p and Ras2p in a reciprocal manner. We have used thermal stress as a life span modulator in order to uncover functional differences between the *RAS* genes that may contribute to their divergent effects on life span. Chronic exposure of cells throughout life to recurring heat shocks at sublethal temperatures decreased their replicative life span. *ras2* mutants, however, suffered the largest decrease compared to wild-type and *ras1* mutant cells. The decrease was correlated with a substantial delay in resumption of budding upon recovery from these heat shocks, indicating an impaired renewal of cell cycling. Detailed analysis of gene expression showed that, during recovery, *ras2* mutants were selectively impaired in down-regulation of stress-responsive genes and up-regulation of growth-promoting genes. Our results suggest that one of the functions of *RAS2* in maintaining life span, for which *RAS1* does not substitute, is to ensure renewal of growth and cell division after bouts of stress that cells encounter during their life. This activity of *RAS2* is effected by the cyclic AMP pathway. Overexpression of *RAS2,* but not *RAS2*^{ser42} which is deficient in the activation of adenylate cyclase, completely reversed the effect of chronic stress on life span. Thus, *RAS2* is limiting for longevity in the face of chronic stress. Since *RAS2* is known to down-regulate stress responses, this demonstrates that for longevity the ability to recover from stress is at least as important as the ability to mount a stress response. © 1998 Academic Press

Key Words: **cell cycle; life span; *RAS; Saccharomyces cerevisiae;* stress; heat shock.**

INTRODUCTION

The yeast *Saccharomyces cerevisiae* possesses a finite replicative capacity as measured by the total number of divisions an individual cell undergoes [reviewed in 1]. The mean replicative life span of a strain, under given conditions, is a characteristic feature. As the cells age, they display morphological and physiological changes, such as increased cell size and generation time, decreased protein synthesis, and altered expression of some genes [reviewed in 1, 2]. The aging process is also accompanied by loss of transcriptional silencing at telomeric loci [3] and at silent mating type loci [4], and changes in resistance to ultraviolet (UV) irradiation [5]. Among the genes that are differentially expressed are the highly homologous genes *RAS1* and *RAS2,* which share a similar profile of age-dependent expression, peaking in young cells and decreasing toward the end of life [6]. They encode small G-proteins that are activated by the exchange factor Cdc25P and inactivated by the GTPase-activating proteins Ira1 and Ira2. The Ras proteins stimulate adenylate cyclase which in turn activates cAMP-dependent protein kinase (PK-A). Either *RAS1* or *RAS2* is sufficient for cell viability, but a double deletion is lethal [reviewed in 7]. Nevertheless, the *RAS* genes have reciprocal effects on life span, since *RAS2* has a life extending effect, while *RAS1* shortens life span [6].

PK-A is known to modulate activation of transcription of a variety of genes in response to various stress conditions [8]. The heat shock response is typical in this regard [9]. The divergent effects of the *RAS* genes on life span may be related to differences in their effects on the response to stress, such as heat shock. Thermal stress induces a heat shock response that is characterized by a temporary arrest in the G_1 phase of the cell cycle, at the regulatory step Start, as indicated by the accumulation of unbudded cells. The arrest is accompanied by a sharp, but temporary, decline in the abundance of most cellular RNAs, alterations in the pattern of protein synthesis, alteration of membrane fluidity, and ultrastructural changes [reviewed in 10]. Simultaneously, heat shock selectively induces expression of stress-responsive genes that encode repair and defense proteins, such as heat shock protein Hsp104 which has been implicated in resolubilization of proteins [11] and reactivation of mRNA splicing [12] after

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heat inactivation. Hsp104p is both necessary and largely sufficient for cell survival at high temperatures after prior exposure to sublethal temperatures [13, 14]. Another gene induced by heat shock is *CTT1* that codes for cytoplasmic catalase, which is required for protection against the oxidative stress associated with heat-induced cell death [15]. *HSP104* and *CTT1* are also induced by other types of stress [16, 17]. The transcriptional induction of *HSP104* and *CTT1* is mediated by the stress-responsive element (STRE) [18, 19], which is negatively regulated by the Ras-cAMP pathway [8, 20]. Consistently, *ras2* and *cyr1* (adenylate cyclase) mutant cells had a higher survival rate after exposure to a single lethal heat shock [9, 16].

In this report, we show that repeated heat shocks, at sublethal temperatures, decrease yeast replicative life span. The heat-induced decrease in life span is much larger in *ras2* mutants compared to *ras1* and wild-type strains, despite more robust stress responses in *ras2.* This effect is associated with an impaired recovery at the levels of budding and growth-promoting gene expression and the inability to down-regulate stress genes. Thus, Ras2p, not Ras1p, is a mediator of growth resumption upon relief from heat shock. This may reflect a role for *RAS2* in maintenance of replicative life span during recurring episodes of stress. We also show that the adenylate cyclase-stimulatory activity of Ras2p is essential for it to perform this role.

MATERIALS AND METHODS

Strains, growth conditions, and transformations. *S. cerevisiae* YPK9 (*MAT***a**, *leu2-Δ1, ura3-52, trp1-Δ63, ade2-101*^{ochre}, lys2-801^{amber}, *his3-Δ200*) is a haploid derivative of YPH501 (supplied by P. Hieter, The Johns Hopkins University). The following strains were generated in the YPK9 background: A *RAS2*-null mutant (*ras2*) was generated with pRa530 and a *RAS1*-null mutant (*ras1*) was essentially generated as previously described [6], except that the *RAS1* sequences were cloned in pRS405 [21]. Deletion and disruption events were confirmed by Southern blot analyses (not shown). Overexpression of *RAS2* and *RAS2*^{ser42} was obtained from the galactose-inducible *GAL10* promoter in plasmid pBM150, which carries the *URA3* gene [6]. Media were YPD (2% peptone, 1% yeast extract, 2% glucose), YPRG (same as YPD, but with 2% raffinose and 2% galactose instead of glucose), SC [0.67% yeast nitrogen base without amino acids and supplemented with uracil (0.012 mg/ml), adenine (0.041 mg/ml), leucine (0.051 mg/ml), lysine (0.029 mg/ml), histidine (0.0198 mg/ml), tryptophan (0.04 mg/ml), and 2% glucose]. Yeast cells were transformed with 1–2 μg DNA using the lithium acetate procedure [22], without carrier DNA. Transformants were selected on SC medium lacking the appropriate nutrients. Cells were cultured at 30°C. Heat shock treatments of batch cultures are described in the relevant figure legends.

Northern blot analysis. Total yeast RNA was prepared by the acidic phenol procedure [23]. Ten micrograms of total RNA were denatured in MOPS buffer (20 mM MOPS, pH 7.0, 5 mM sodium acetate, 1 mM EDTA), 50% formamide, and 2.2 M formaldehyde at 68°C. Samples were electrophoresed in 1% agarose gels containing 20 mM MOPS buffer (pH 7.0) and 2.2 M formaldehyde. RNA was transferred to Nytran membranes (Schleicher & Schuell) by capillary action in 20× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate). Prehybridization and hybridization were carried out at 42°C in 5× SSC, 50 mM sodium phosphate buffer, pH 7.0, 0.1% $Na_2P_4O_7$, 0.5 mg/ml heparin, 0.5% SDS, 50% formamide, and 1.0 mg/ml salmon sperm DNA. mRNA-specific probes were generated by random oligonucleotide-primed DNA synthesis by Klenow enzyme (Boehringer Mannheim), in the presence of [α-^{32}P]dCTP. The labeled probes were purified on Nick columns (Pharmacia Biotech), denatured, and added to the hybridization solution. Membranes were washed 20 h post-hybridization in 0.1× SSC, 0.25% SDS at 55°C, and subjected to autoradiography or were exposed to a phosphor screen in an imaging plate (Molecular Dynamics). Imaging plates were scanned using the Molecular Dynamics 400E PhosphoImager. The molecular probes were: (i) a 0.9-kb *ACT1* fragment PCR-amplified from yeast DNA. One microgram template DNA was diluted in PCR buffer (Promega) in the presence of 1.25 mM $MgCl_2$, 0.1 mM dNTPs, 2.5 units of Taq polymerase (Promega), 1 μM of the upstream and downstream primers (5′-CCCATCTATCGTCGGTAG-3′ and 5′-CCAATCCAGACGGAGTAC-3′, respectively). The reaction was carried out in a Gene Amp PCR system thermocycler (Perkin–Elmer) at: 94°C/1 min, 30 cycles (94°C/30 s, 48°C/30 s, 72°C/30 s), 72°C/5 min; (ii) a 0.4-kb *RPL16A* fragment was PCR-amplified in the presence of 3.8 mM $MgCl_2$. The upstream and downstream primers were 5′-CATCTCCGTTGGTGAATCTG-3′ and 5′-CCTTAGTTGTCTTGTGGGAG-3′, respectively. The reaction was carried out at: 94°C/1 min, 30 cycles (94°C/30 s, 56°C/30 s, 72°C/30 s), 72°C/5 min; (iii) a 1.2-kb *Eco*RI fragment of the *RAS2* gene from pBM150-[*GAL10:RAS2*] [6]; (iv) a 3.5-kb *Bam*HI fragment of the *HSP104* gene from p2HG-*HSP104* (supplied by S. Lindquist, University of Chicago); (v) a 3.9-kb *Hind*III-*Bam*HI fragment of the *CTT1* gene from pBR322-7309 [24]; (vi) a 1.64-kb *Xho*I-*Cla*I fragment of the *CLN2* gene from pHCS39 [25].

Western blot analysis. Total proteins were extracted from cells as previously described [6] and protein concentration was determined using a protein assay kit based on the method of Bradford (Bio-Rad). Ten micrograms were separated on 7.5% polyacrylamide gels in the presence of SDS. Prestained SDS–PAGE protein standards (Bio-Rad) were also included on the gel as size references. After electrophoresis, the proteins were transferred to PVDF membranes (Millipore). Proteins on the blot were reacted with Hsp104p-specific antisera [14], followed by goat anti-mouse secondary antibody (Amersham). Immune complexes were visualized with the ECL reagent (Amersham) and quantitated by soft laser scanning (Biomed Instruments).

Life span determination. A Nikon Labophot-2 microscope with a 20× long working-distance objective and a micromanipulator attachment was used. A fresh colony of yeast cells was grown to logarithmic phase in YPD. For life span analyses with strains containing plasmids, growth was instead in SC (-uracil) medium containing raffinose as the carbon source. One microliter of the culture was spotted onto a YPD plate or onto a YPRG plate in the case of overexpression experiments. Individual cells were pulled aside with the micromanipulator, and were allowed to grow until buds emerged. Thirty-five buds were removed, and were referred to as "virgins" (i.e., cells that have never budded). After they underwent their first cell division, buds were removed from these cells, and the virgin cells, now mothers, were recorded as one generation old. This process was continued until budding ceased. At the end of their life span cells lysed. The number of buds produced prior to lysis is the individual yeast's life span. During the course of the experiment, cells were transferred to 12°C during the night (approximately 14 h) to slow division and allow relief for the investigator. Depending on the age of the cells, 1–2 generations were completed at this temperature per night. This treatment does not alter the replicative life span [26]. Heat-shocked cells were exposed to 37 or 42°C for 1 h, after which they were returned to 30°C. Heat shocks were first applied to virgin cells, and continued daily (i.e., one heat shock per day) throughout the experiment. The nonparametric Mann–Whitney test was used to compare

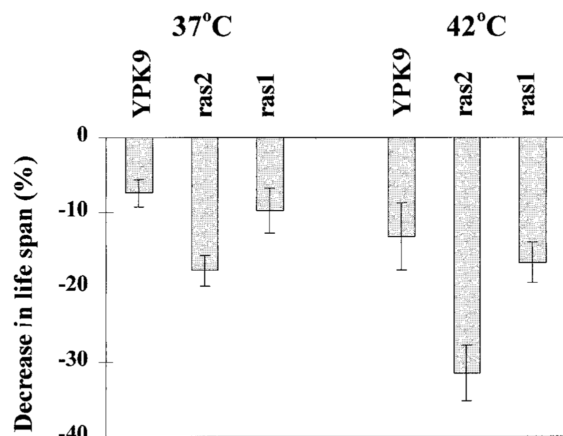


FIG. 1. The effect of recurring heat shocks on replicative life span. Life spans of parental YPK9, *ras2* mutant, and *ras1* mutant cells were determined on YPD plates as explained under Materials and Methods. Cells were maintained at 30°C during the day. Heat-shocked cells were transferred to 37 or 42°C for 1 h per day, starting with virgin cells (i.e., cells that have never budded), throughout the experiment. (The time required for a one-generation old YPK9 cell to complete a cell cycle is 2 h. Cell generation time increases with age [27].) The percentage change in mean life span relative to the non-heat-shocked cells of the same strain was calculated for YPK9, *ras2,* and *ras1* cells. The results are presented as the means of three to five experiments ± SEM (35 cells per experiment), for each strain at each temperature. The mean life span of the non-heat-shocked wild-type control was 19.2 generations. The differences between *ras2* and the wild-type and *ras1* strains were significant when the cells were heat-shocked at 37 or 42°C ($P < 0.05$). There was no significant difference between the wild-type and the *ras1* strains with treatment at either temperature ($P > 0.5$).

survival curves. Life spans were considered to be different if $P < 0.05$. The significance of differences between changes in mean life spans on heat shock was determined by deriving the means from several experiments and performing ANOVA using the Tukey test. The same procedure was used for analysis of bud emergence, which is described in the legend to Fig. 2.

RESULTS

Recurring Heat Shocks Decrease Yeast Replicative Capacity, Particularly in the Absence of RAS2

Understanding the reciprocal effects of the homologous signal transducers Ras1p and Ras2p on life span in the absence of environmental manipulations [6] is a difficult task. We hypothesized that stress-generating environmental changes might influence life span and be useful for uncovering functional differences between the *RAS* genes that contribute to their effects on life span. We focused on thermal stress. Wild-type, *ras1,* and *ras2* strains were subjected daily to transient heat shocks at either 37 or 42°C while their life spans were determined (Fig. 1). Repeated heat shocks at 37°C resulted in slight shortening of the mean life spans of wild-type and *ras1* strains by 10 and 14%, respectively, and a larger shortening of the mean life span of the coisogenic *ras2* strain by 20%. Increasing the severity of heat shocks (42°C) exacerbated the effect. The mean life spans of wild-type and *ras1* strains were identically

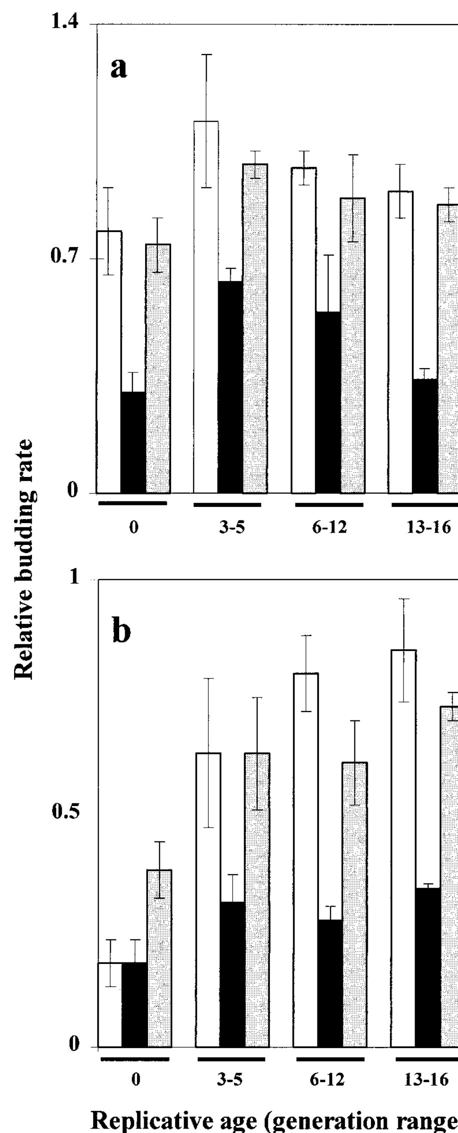


FIG. 2. The effect of recurring heat shocks on bud emergence throughout life. During life span determination, the total number of new buds produced by the mother cells was counted daily during a 1-h heat shock and for 1 h during recovery (i.e., a 2-h interval) and was divided by the number of new buds in the same strain in the absence of heat stress during that time. The results are expressed, for each strain (YPK9, open bars; *ras2* mutant, closed bars; *ras1* mutant, stippled bars), as the average ratio ± SEM of three experiments (35 cells per experiment), at 37°C (a) or at 42°C (b). The age groups to which each set of bars refer are indicated at the bottom. These are not synchronously dividing cells. The relative budding rate from one experiment to the next can vary substantially. Thus, the means of these rates have a significant variance. The range provided by the error bars (SEM) may provide a better estimate of the difference between strains than the *P,* which varied from <0.001 to 0.18, for the comparison between *ras2* and the wild-type and *ras1* strains. There were no differences between *ras1* and the wild-type.

shortened by 18%, while that of the *ras2* strain was shortened by 28%.

RAS2 Is Required for Efficient Renewal of Bud Emergence upon Recovery from Heat Shock

During life span determination, the total number of new buds produced by the mother cells was counted daily 1 h after the heat-shocked cells recovered from a 1-h heat shock, and was compared to the emergence of new buds in the same strain during that time but in the absence of heat shock. Heat shock at 37°C slowed down the emergence of new buds in the wild-type and *ras1* strains only slightly, while in *ras2* cells the process was significantly slowed throughout life (Fig. 2a). Heat shocks at 42°C further slowed down emergence of new buds in all strains, but the effect was again about twofold greater in the *ras2* strain compared to the wild-type and *ras1* strains, which were almost identical (Fig. 2b). It is worth noting that in all the strains virgin cells (Fig. 2, "zero" generations) are most sensitive to heat shock as they go through their first division.

Growth and budding properties during heat shock and recovery were also examined in liquid cultures. Liquid cultures served later for a molecular analysis of gene expression. Both *ras* mutant strains showed the same growth rate as the wild-type strain, with a doubling time of 1.5 h (not shown). Cells proliferating at 30°C were heat-shocked at 42°C for 1 h and were returned to 30°C. This protocol did not decrease cell viability (not shown). During recovery, the number of wild-type or *ras1* mutant cells doubled within 2.5 h and tripled within 3.5 h, while that of the *ras2* mutants doubled only after 3.5 h and tripled within 4.5 h (Fig. 3a). All strains eventually reached stationary phase (not shown). The budding index, which dropped dramatically upon heat shock in all strains, increased in wild-type and *ras1* strains during the recovery period within 2 h to its level prior to the heat shock, while the *ras2* strain resumed budding much more slowly (Fig. 3b). Bud emergence is an indicator of passage through the G_1/S boundary [27]. Thus, the prolongation of the first doubling in the *ras2* strain indicated a delayed passage through the G_1/S boundary. These results demonstrate for the first time that *RAS2* is required for rapid renewal of cell cycling upon recovery from heat shock. We have shown previously that the budding rate decreases as a function of replicative age [28]. This could be due to the decrease in *RAS2* expression during aging [6] and to the presence of stress.

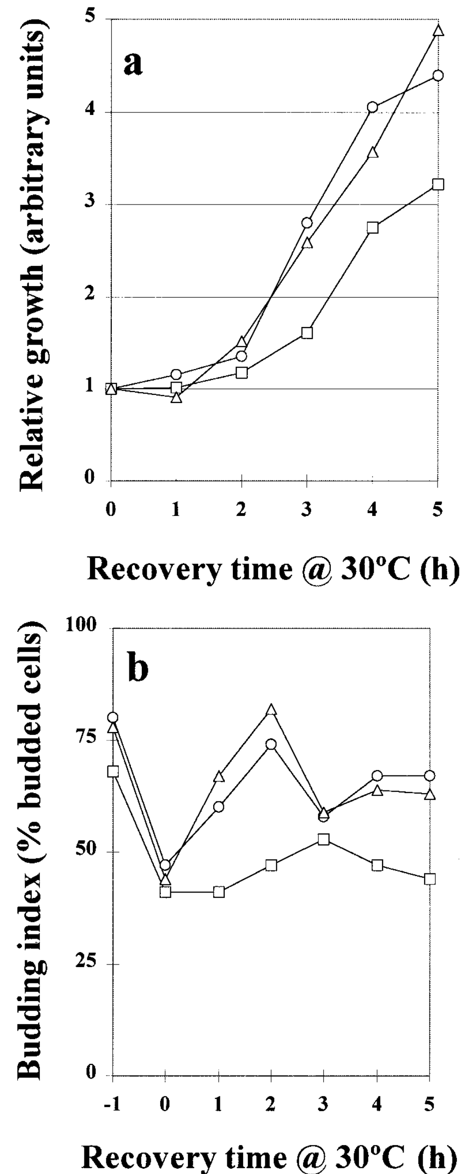


FIG. 3. Growth and budding properties of recovering cultures. YPK9 (circles), *ras2* (squares), and *ras1* (triangles) cells were grown in liquid cultures in YPD at 30°C to logarithmic phase. Cells were transferred to 42°C (−1 h time point) for 1 h after which they were allowed to recover at 30°C (zero time point). Aliquoted cells, at the indicated time points, were sonicated and cell concentrations were determined in a hemacytometer. Cell concentrations at the beginning of recovery were: YPK9, 11×10^6 cells/ml; *ras2,* 15.7×10^6 cells/ml; and *ras1,* 11.3×10^6 cells/ml. The relative growth of the cultures (a) is expressed as the ratio between cell concentration at the indicated recovery time points and that at the beginning of the recovery (zero time point). Simultaneously, the budding index (percentage of cells with buds) was determined (b).

RAS2 Is Required for Efficient Down-Regulation of Stress-Responsive Genes and Up-Regulation of Growth-Promoting Genes upon Recovery from Heat Shock

The impaired recovery of the *ras2* strain from sublethal heat stress might possibly be the result of impaired stress responses and/or growth renewal. To address this question, we carried out Northern blot analyses of several stress-responsive genes and growth-promoting genes. Analyses were performed

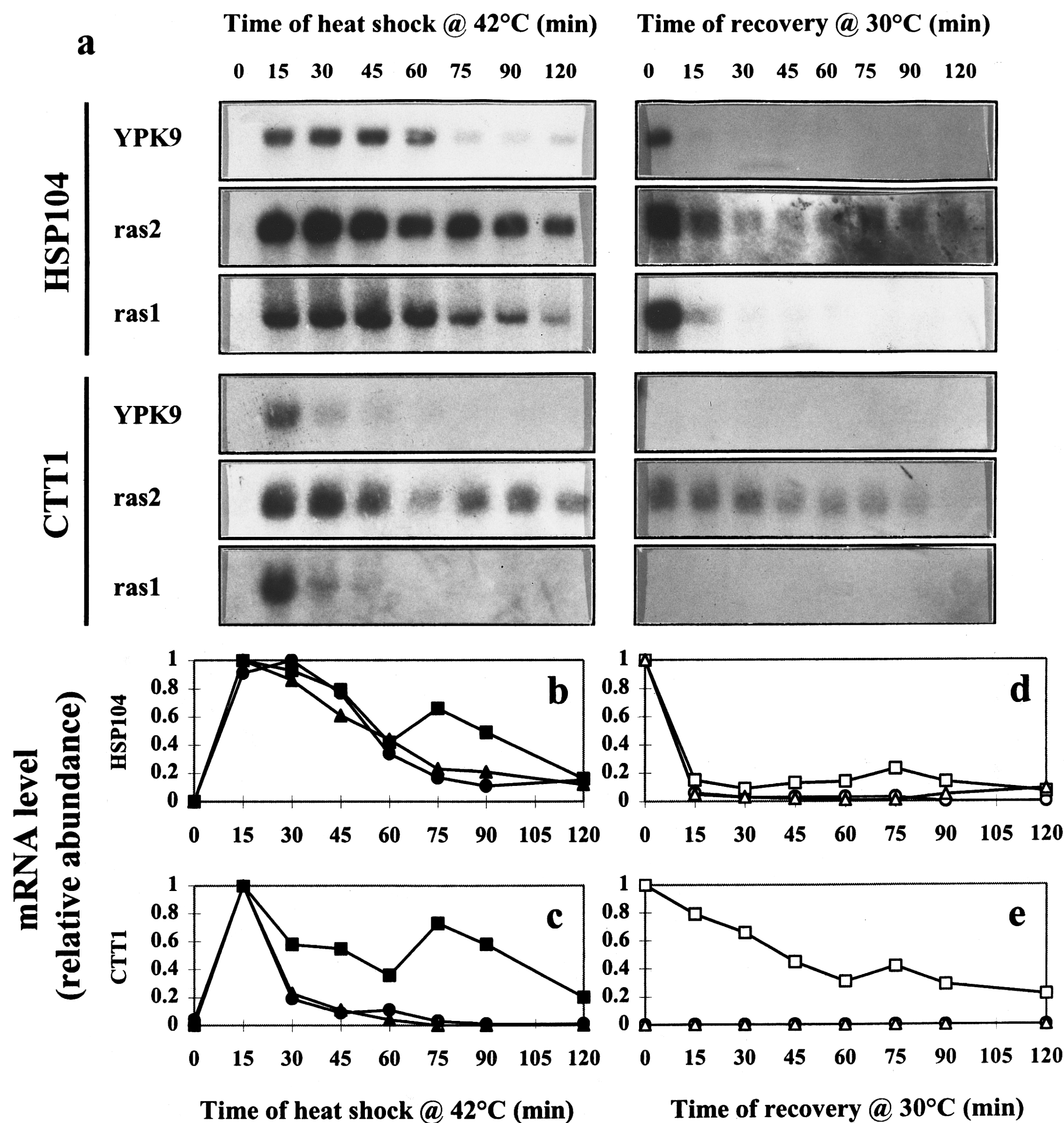


FIG. 4. Changes in the abundance of stress-responsive mRNAs during heat shock and recovery. YPK9, *ras2,* and *ras1* batch cultures were grown in YPD medium to early logarithmic phase. Cells were then transferred from 30 to 42°C (left panels, "heat shock") or were heat-shocked for 1 h at 42°C, after which they were transferred back to 30°C (right panels, "recovery"). Ten micrograms of RNA isolated from these cells at the indicated times were analyzed by Northern blot hybridization with gene-specific probes as indicated at the left (a). The ethidium bromide-stained 18S rRNA indicated even RNA loading in each lane (not shown). Note that different exposures were chosen for each probe. Northern blots were exposed to a phosphor screen in an imaging plate and quantitated. Changes in gene-specific mRNA abundance in each strain (YPK9, circles; *ras2,* squares; *ras1,* triangles) were related to their zero time points during heat shock (b, c) or recovery (d, e). Similar profiles were obtained in several experiments. Representative results are shown.

with cultures that were either heat-shocked up to 2 h (Figs. 4a and 6a, left panels, "heat shock") or were recovering at 30°C from a 1-h heat shock at 42°C (Figs. 4a and 6a, right panels, "recovery"). Quantitative analysis of representative Northern blots revealed that *HSP104* (Fig. 4b) and *CTT1* (Fig. 4c) mRNAs were transiently induced upon heat shock in all three strains. *ras2* cells, however, displayed a sustained elevation of these mRNAs, which was particularly evident for *CTT1.* In *ras2* cells, *HSP104* mRNA, although decreasing, was still maintained at a detectable level after 120 min of recovery at 30°C (Fig. 4d). *CTT1* mRNA, which became undetectable in wild-type or *ras1* strains soon after initiation of heat shock, remained relatively high in *ras2* cells throughout recovery (Fig. 4e). Elevated basal Ctt1p activity in *ras2* mutants was previously shown in another strain [29], and the delayed down-regulation of induced activity

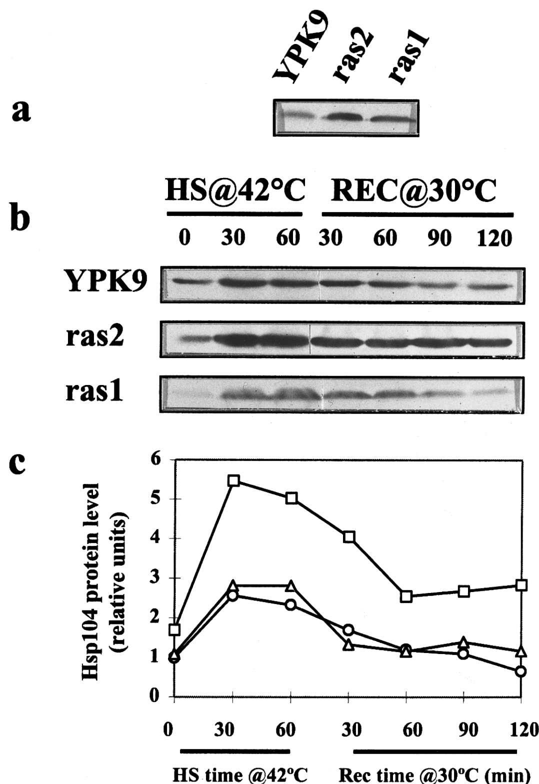


FIG. 5. The abundance of Hsp104 protein during heat shock and recovery. (a) Basal levels. YPK9, *ras2,* and *ras1* cells were grown in YPD medium at 30°C to early logarithmic phase. Ten micrograms of protein extracted from each culture were analyzed on Western blots with Hsp104p-specific antibodies. (b) Transient induction. The above cultures were heat-shocked at 42°C, after which they were allowed to recover at 30°C. Protein was extracted at the indicated time points (in min) of heat shock (HS) and recovery (REC), and 10 μg were subjected to Western blot analysis. Note that different exposures were chosen for each strain. (c) Quantitation of Hsp104p induction. Hsp104p signals were quantitated by soft laser scanning. The basal Hsp104p amount in YPK9 cells (a) was arbitrarily chosen as one and all signals shown in (b) were related to it. YPK9, circles; *ras2,* squares; *ras1,* triangles.

found here confirms and extends that study. To determine whether the differences in *HSP104* mRNA were also evident at the protein level, we performed a Western blot analysis. Upon heat shock, Hsp104 protein was induced in all strains approximately threefold (Figs. 5b and 5c), as also shown recently in a wild-type strain [30]. However, the *ras2* strain had almost a twofold increase in basal levels of Hsp104p compared to wild-type and *ras1* strains (Fig. 5a). Thus, relative to the wild-type strain, the *ras2* strain had a sixfold increase in Hsp104p, and its level remained significantly higher even when the cells were placed at 30°C to recover (Figs. 5b and 5c). As shown here, the effects of *ras2* mutation on expression of *HSP104* are evident at the protein as well as the mRNA level.

The growth-promoting genes we monitored were *CLN2* and *RPL16A,* which are clear markers of growth renewal [31, 32], and *ACT1.* In comparison to the stress-responsive genes, growth-promoting genes exhibited the opposite pattern of change in their expression. *ACT1* (Fig. 6b), *RPL16A* (Fig. 6c), and *CLN2* (Fig. 6d) mRNA levels declined dramatically upon heat shock. As cells were allowed to recover, *ACT1* (Fig. 6e), *RPL16A* (Fig. 6g), and *CLN2* (Fig. 6f) mRNA levels in wild-type and *ras1* strains started accumulating after 30 min. The increase in *ACT1* mRNA in *ras2* cells started only after 60 min (Fig. 6e). The increase in *RPL16A* and *CLN2* mRNAs started only after 90 min, and pre-heat-shock levels were not fully reestablished even after 2 h (Figs. 6f and 6g). This analysis provides an explanation, at the molecular level, for the *ras2* defect in growth resumption.

The Northern blot analyses clearly show that upon recovery the *ras2* strain is impaired in the kinetics of down-regulation of stress-responsive genes and in up-regulation of growth-promoting genes. Thus Ras2p, not Ras1p, acts as the molecular switch that is required for concerted modulation of gene expression upon resumption of proliferation. This is one of only a few instances in which differences between *RAS1* and *RAS2* have been examined and identified.

Overexpression of RAS2 in ras2 Mutants Abolishes the Heat Shock-Induced Decrease in Replicative Life Span, in Association with Improved Recovery from Heat Shock

The results presented thus far show that in *ras2* mutants the exaggerated attenuation of replicative life span due to heat stress coincides with an impaired resumption of growth upon recovery from heat shock. Therefore, shortening of life span could, at least in part, be attributed to the accumulation of impaired growth resumption events, due to inefficient renewal of cell cycling. Given the pivotal role of *RAS2* as the mediator of efficient growth resumption, we hypothesized that its overexpression in the *ras2* mutant should not only improve the ability to recover, but also should alleviate the heat-induced decrease in replicative life span. To test this hypothesis, *GAL10* promoter-driven *RAS2* was overexpressed in the *ras2* background, and the effects of recurring heat shocks on both life span and growth resumption were examined. Overexpression of *RAS2* in these cells completely abolished the decrease in longevity. In fact, the mean life span of the heat-shocked cells was similar to that of cells that did not experience heat stress (Fig. 7a).

Analysis of bud emergence during the experiment showed that, almost at any given stage in life, resumption of budding upon recovery was faster in the presence of the exogenous *RAS2* (Fig. 7b). Thus, overexpression of *RAS2* in *ras2* mutants abolishes the heat

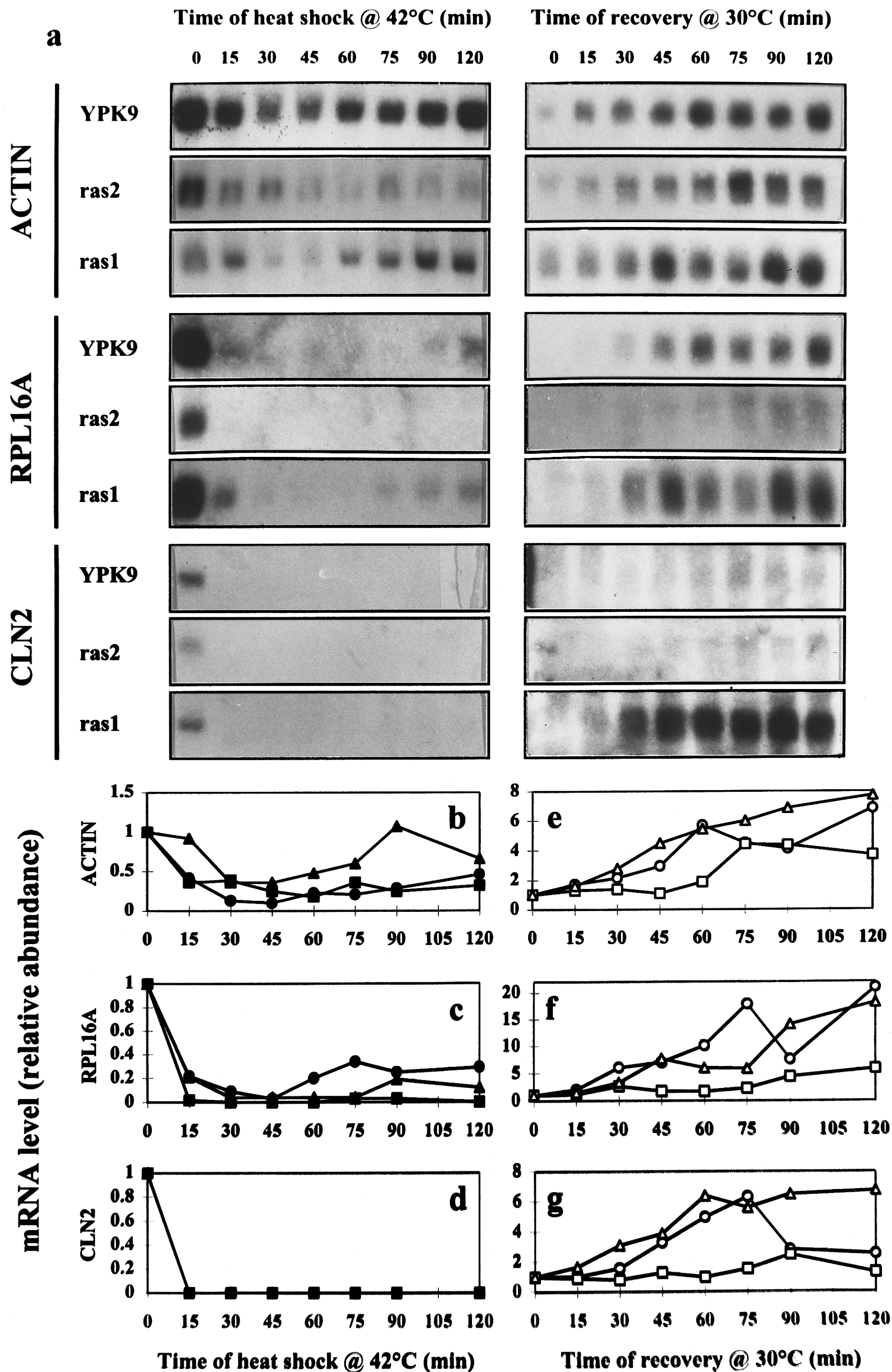
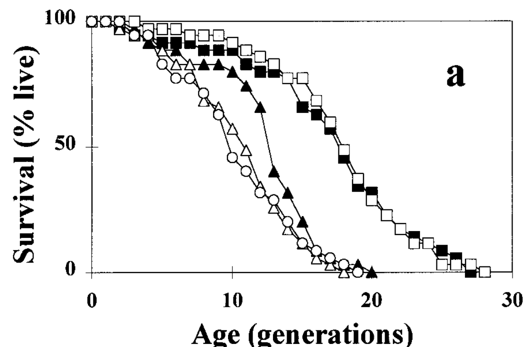


FIG. 6. Changes in the abundance of growth-promoting mRNAs during heat shock and recovery. Northern blot analysis (a) of the growth-promoting genes indicated at the left was performed as described in the legend to Fig. 4a. Changes in gene-specific mRNA abundance in each strain (YPK9, circles; *ras2*, squares; *ras1*, triangles) were related to their zero time points during heat shock (b, c, d) or recovery (e, f, g). Similar profiles were obtained in several experiments. Representative results are shown.



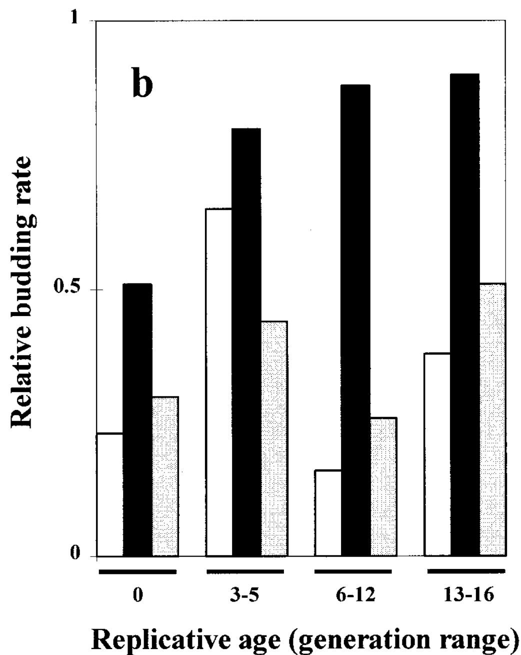


FIG. 7. Life span and bud emergence are dependent on the cAMP stimulatory effects of Ras2p. *ras2* cells were transformed with plasmids containing either *RAS2* or *RAS2*^{ser42}, under the control of the galactose-inducible promoter *GAL10,* or with the empty vector pBM150 as control. Life span analyses were performed on YPRG medium to induce expression. (a) The life span of chronically heat-stressed cells (as described in Fig. 1) expressing *RAS2*^{ser42} (open circles) (mean of 10.5 generations) was significantly shorter ($P \ll 0.001$) than the life span of cells expressing *RAS2* (open squares) (mean of 17.9 generations). *RAS2*-overexpressing cells had a similar life span ($P = 0.75$) whether they were heat stressed (open squares) (mean of 17.9) or not (closed squares) (mean of 17.2), which was significantly longer ($P \ll 0.001$) than that of the non-heat-stressed control (closed triangles) (mean of 12.5). Heat stress shortened ($P < 0.05$) the life span of the control (open triangles) (mean of 10.8), and overexpression of *RAS2*^{ser42} (open circles) (mean of 10.5) did not correct this ($P = 0.74$). (b) Bud emergence was determined for each age group as described in the legend to Fig. 2. The strains are *ras2* pBM150, open bars; *ras2* pBM150-*RAS2,* closed bars; *ras2* pBM150-*RAS2*^{ser42}, stippled bars.

shock-induced decrease in replicative capacity, which is associated with inefficient recovery from heat shock. The same cannot be said of *RAS2*^{ser42} overexpression (Figs. 7a and 7b). This *RAS2* allele is defective in stimulation of adenylate cyclase [33]. The life spans of cells overexpressing the *RAS2*^{ser42} allele did not differ from control cells containing the vector alone that were subjected to heat stress. These results implicate the *RAS*-cAMP pathway in the life span-maintaining role of efficient recovery from stress. This role is distinct from the capability of *RAS2*^{ser42} overexpression to extend life span in the absence of overt stress [6].

DISCUSSION

We have established that recurring, sublethal thermal stress decreases the replicative capacity of yeast. This observation enabled us to show that efficient recovery from this stress, mediated primarily by *RAS2,* plays a significant role in life span maintenance. *ras2* mutants, although maintaining higher levels of stress-responsive functions (Figs. 4 and 5), suffered the largest heat-induced decrease in life span compared to wild-type and *ras1* strains (Fig. 1). This result appears to contradict previous data showing that *ras2* and *cyr1* (adenylate cyclase) mutant cells had a higher survival rate after exposure to a single lethal heat shock [9, 16]. *ras2* mutants indeed have an advantage in surviving a lethal heat shock, since they sustain their stress responses and delay the renewal of cell cycling. This probably allows a longer period of damage repair prior to growth renewal. However, maintenance of replicative life span, or in other words completion of many successive cell cycles, during bouts of nonlethal stress that cells might face during their life span, appears to require rapid renewal of cell cycling as soon as damage has been repaired. In other words, the individual yeast pays a price to have the ability to rapidly resume growth following repeated episodes of nonlethal heat stress during its lifetime. This price is the attenuation of the capacity to resist a lethal heat shock. It is *RAS2* that allocates resources between these distinct capabilities.

Our analysis clearly indicates that a significant problem in *ras2* mutants was the delayed up-regulation of growth-promoting genes (*ACT1, RPL16A,* and *CLN2*) (Fig. 6). The inhibition in *CLN2* mRNA accumulation may be sufficient to postpone passage through Start in cells recovering from heat stress, if not in normally cycling cells. Our results are consistent with the Ras-cAMP-mediated induction of *CLN2* [31] and *RPL16A* [32] upon release from nutrient-induced arrest. The coordinated expression of several G_1 cyclins, including *CLN2* [34], and some ribosomal protein genes [35] has been recently shown to be mediated by the TFIID auxiliary factor, $TAF_{II}145$, which is essential for progress through the G_1/S boundary. An interaction of *$TAF_{II}145$* with the *RAS* genes has not been addressed in these papers, but may be worthwhile in

view of our findings. On the other hand, it is known that *RAP1* is involved in the Ras-cAMP-modulated induction of *RPL16A* [32, 36] and some other ribosomal protein genes by nutrients [37]. The effect of *RAS2* on the expression of *ACT1* has not been previously described. To our knowledge, neither the *ACT1* nor the *CLN2* promoters contain Rap1p-binding sites. There are other reasons to suspect *RAP1*-independent mechanisms. The induction of several ribosomal protein genes upon nutritional shift-up does not require *RAP1* nor does it require the cAMP pathway [38], alluding to the participation of a cAMP-independent pathway(s) in recovery from stressful conditions as well. Interestingly, life extension by *RAS2* in the absence of overt stress involves a cAMP-independent pathway [6].

The N-termini of yeast Ras1p and Ras2p are 91% homologous, yet the Ras2p N-terminus is a more potent activator of adenylate cyclase than the N-terminus of Ras1p. Overexpression of exogenous Ras1p or its N-terminal domain elevated cAMP levels in *ras2* cells, but did not complement several *ras2* phenotypes [39], suggesting that the hypervariable domains are responsible for these differences, possibly through interaction with other regulatory pathways. *RAS2* has been shown to (i) participate in the induction of filamentous growth, upon nitrogen starvation, via the Ste20/MAP kinase module [40]; (ii) genetically interact with *PBS2,* a MAP kinase kinase that is involved in adaptation to osmotic shock [41]; and (iii) participate in completion of mitosis via a cAMP-independent pathway [42]. A putative cAMP-independent *RAS2* pathway and its involvement in life span determination was first documented by Sun *et al.* [6]. Based on the insufficiency of *RAS1* for mediating efficient recovery in the absence of *RAS2* and the fact that *RAS1* is dispensable for the recovery process in the presence of *RAS2* (Figs. 2 and 3), we conclude that Ras2p, and not Ras1p, acts as the molecular switch that is required for the efficient resumption of proliferation. Recent published articles, in which other growth renewal conditions have been examined, support our conclusion. First, *RAS2* and a functional cAMP pathway are required for the early steps of spore germination, in which cells reenter the cell cycle [43]. Second, *RAS2,* but not *RAS1,* suppressed the synchronous growth arrest of cells, induced by overexpression of the protein–tyrosine phosphatase gene, *PPS1,* in a cAMP-independent manner [44].

RAS2 appears to operate as a homeostatic device in yeast longevity. It utilizes a cAMP-dependent, as well as a cAMP-independent, pathway in this capacity. We propose that these pathways function to establish a balance between promotion of longevity [6] in the absence of significant stress and life maintenance in the presence of chronic, sublethal stress (described here). The latter function requires *RAS2* for recovery from stress. The attenuation of stress responses in the presence of Ras2p activity is the price yeasts pay to possess this capability. We suggest that this allows them to "run away" from stress by resumption of cell division and production of large numbers of progeny, at least some of which can survive stress. In stationary phase, yeasts must be able to "stay and fight" by full and sustained induction of stress responses, if necessary. How this is achieved is not entirely clear at present.

The impaired recovery in *ras2* mutants is associated with a heat-induced decrease in life span. We expect that maintenance of replicative capacity under other forms of stress would also require *RAS2*-mediated recovery. UV irradiation and the UV-mimetic agent 4-nitroquinoline-1-oxide (4NQO) induce a delay in G_1 progression [45]. *RAS2* is required for both the UV response [46] and survival in the presence of 4NQO (S. Kim and S.M. Jazwinski, unpublished data), for which *RAS1* is expendable. In addition, we have previously shown that resistance to UV stress during the life span correlates with *RAS2* expression [5]. We therefore predict that exposure of yeast to repeated, sublethal UV irradiation will result in a severe shortening of life span in *ras2* mutants.

In summary, our results indicate that efficient recovery from stress plays an important role in maintaining replicative capacity during recurring stress. The recovery process is primarily mediated by Ras2p, and not Ras1p. These results lead us to propose that the shorter life span of *ras2* mutants in the absence of external stress [6] may, at least in part, be due to impaired recovery from bouts of stress generated intracellularly or to cryptic environmental stress. Furthermore, assuming that *RAS1* and *RAS2* compete for downstream effectors, elimination of *RAS1* would facilitate *RAS2* action. In fact, life span is extended in *ras1* mutants, as such a model would predict [6].

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