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Mutation in the Silencing Gene *SIR4* Can Delay Aging in S. cerevisiae

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Summary

Aging in S. cerevisiae is exemplified by the fixed number of cell divisions that mother cells undergo (termed their life span). We have exploited a correlation between life span and stress resistance to identify mutations in four genes that extend life span. One of these, *SIR4*, encodes a component of the silencing apparatus at *HM* loci and telomeres. The *sir4-42* mutation extends life span by more than 30% and is semidominant. Our findings suggest that *sir4-42* extends life span by preventing recruitment of the SIR proteins to *HM* loci and telomeres, thereby increasing their concentration at other chromosomal regions. Maintaining silencing at these other regions may be critical in preventing aging. Consistent with this view, expression of only the carboxyl terminus of SIR4 interferes with silencing at *HM* loci and telomeres, which also extends life span. Possible links among silencing, telomere maintenance, and aging in other organisms are discussed.

Introduction

Aging is a process in which all individuals of a species undergo a progressive decline in vitality, leading to death. Many ideas on the causes of aging have been proposed, including the accumulation of damage to cellular constituents and the implementation of a developmentally determined state (Finch, 1990). A classical definition of aging, which is based on the statistical analysis of individuals, states that the probability of death increases exponentially with age (Gompertz, 1825). This conclusion, originally deduced from studies of human actuarial tables, applies to many diverse organisms that also display morphological landmarks of aging (Finch, 1990).

Aging has also been studied in the budding yeast Saccharomyces cerevisiae. This yeast divides asymmetrically to give rise to a larger mother cell and a smaller daughter cell. In this organism, life span is defined by the number of cell divisions undergone by mother cells before they stop dividing (Müller et al., 1980). Mortimer and Johnston (1959) followed the fate of mother cells through many rounds of cell division by manipulating the daughter cells away microscopically and showed that mothers had a relatively fixed life span. The probability of these mothers dying increased exponentially with the number of cell divisions that they had undergone (Jazwinski et al., 1989; Pohley, 1987). Therefore aging is thought to occur in yeast, with the metric of age equaling the number of divisions undergone by mother cells.

An early hypothesis proposed to explain yeast aging was that cell death is due to the accumulation of bud scars, which are chitinous rings deposited on the surface of the mother cells to mark each cell division (Johnston, 1966). However, several experiments indicate that bud scars do not cause cell death. First, shifting a *cdc24* temperature-sensitive mutant to the restrictive temperature will cause an accumulation of chitin on the cell surface but does not limit life span when cells are returned to the permissive temperature (Egilmez and Jazwinski, 1989). Second, daughters arising from old mother cells have a longer cell cycling time than daughters arising from young mothers (Egilmez and Jazwinski, 1989). Third, daughters from old mothers have a reduced life span compared with the life span of daughters from young mothers (Kennedy et al., 1994). Since daughters arising from old mothers do not contain bud scars, these latter two observations indicate that bud scars are not a direct cause of aging. These experiments, rather, suggest the possibility of a senescence factor that accumulates in old mother cells and that can be inherited by their daughters.

It has been suggested that the senescence of human diploid fibroblasts (HDFs) in culture is a model for aging (Hayflick, 1965; Goldstein, 1990). This claim is bolstered by the finding that fibroblasts from patients with Werner's syndrome, the premature aging syndrome, divide fewer times in culture before senescing (Salk et al., 1981). Two features of senescent HDFs bear similarity to aging yeast cells. First, in both cases cells undergo enlargement (Egilmez et al., 1990; Mortimer and Johnston, 1959; Sherwood et al., 1988) and a slowing in the rate of cell division as they age (Grove and Cristofalo, 1977; Mortimer and Johnston, 1959). In the case of yeast, the correlation between cell size and aging is not an obligate one (Kennedy et al., 1994). Second, in both cases, senescence is dominant. This was demonstrated by cell fusions between old and young HDFs (Norwood et al., 1974) or by mating between old and young yeast mother cells (Müller, 1985).

A limitation to studying aging in any of these systems is the difficulty in identifying genes that could be important in the process. In mammalian cells, cell fusion experiments have identified four chromosomal loci that appear to be important in promoting senescence (Pereira-Smith and Smith, 1988). While chromosomal locations of these loci have been narrowed (Hensler et al., 1994; Ning et al., 1991; Sugawara et al., 1990), they have not yet been cloned. In Caenorhabditis elegans, a single mutation, *age-1*, extends the life span of the worm by 50% (Friedman and Johnson, 1988; Johnson, 1990). Both *age-1* and the gene for Werner's syndrome have been mapped (Goto et al., 1992) but not yet cloned.

The potential for isolating mutants with altered life spans makes the yeast system attractive for studying aging. However, a barrier to attaining this goal is the difficulty in applying genetic strategies to a phenomenon that can only be measured at the level of individual cells. Here, we describe an approach that circumvents this difficulty, resting

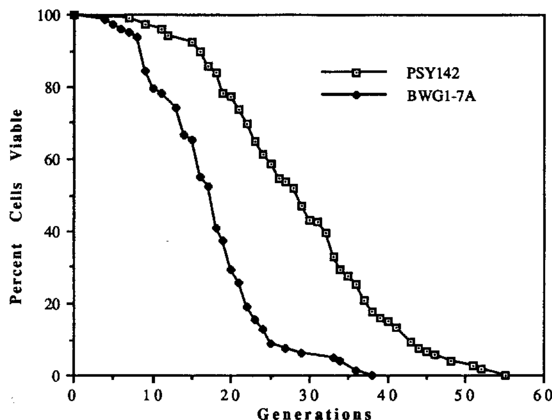

Figure 1. Mortality Curves for PSY142 and BWG1-7A

Mortality curves are shown for BWG1-7A and PSY142 from several experiments totaling 78 cells for BWG1-7A and 106 cells for PSY142. The relative difference between the two strains was consistently observed. The mean life spans for these two strains are 18 generations for BWG1-7A and 29 generations for PSY142.

on the observation that certain yeast strains with longer life spans are more stress resistant than strains with shorter life spans. We describe mutations in four genetic loci that increase the life span of the parental strain 20%–55%. We did not seek mutants with shorter life spans, because we imagined that their effects could be less specific. One of the loci that promoted longevity corresponds to a known gene involved in silencing of chromosomal domains. The nature of this mutation and its interaction with other genes involved in silencing provides a framework upon which to build models of aging in yeast.

Results

Strategy for the Isolation of Longer-Lived Mutants

The strategy we adopted to isolate mutants with longer life spans results from two observations. First, we determined the life spans of several unrelated lab strains and found that they were all different. A typical mortality curve is shown in Figure 1, in which the life spans of two strains are determined by plotting the percentage of cells from the initial population (chosen to be generation 1 daughter cells) that remain viable after an increasing number of divisions. The average number of divisions of the initial population is the mean life span, which is 18 and 29 for these two strains, BWG1-7A and PSY142 (Table 1). Similar life spans were obtained in assays in which cells were kept at 30°C, or in assays in which cells were refrigerated overnight. For most of the life span assays described in the paper, cells were stored at 4°C overnight for convenience. When the above strains were crossed, segregants displayed a spectrum of mean life spans that varied over a 3-fold range. The variation was due to the segregation of more than one gene. Figure 2A shows a typical tetrad, in which life spans vary from 13 (14c) to 37 (14d).

A second important observation for our approach was a correlation between life span and stress resistance in segregants of the BWG1-7A × PSY142 cross. This observation was initially made when we tried to recover cells from plates that had resided at 4°C for an extended period of time. Strains with longer life spans contained a greater percentage of viable cells after this period. Thus, the ability of these strains to remain viable upon storage mimicked their life spans. Figure 2B shows the viable count of each segregant of tetrad 14 after storage at 4°C for 4.5 months. Next, we tested whether a simple starvation protocol would elicit similar differences in survival. Cells were starved for nitrogen and then plated on complete media after intervals of time. Figure 2C shows that the ability of these strains to withstand 7 days of starvation again correlates with the length of their life spans. Other assays of stress tolerance, such as heat shock resistance, also showed this same pattern (data not shown). This finding suggested that genes affecting life span could be identified by isolating mutations of segregant 14c that caused an increase in resistance to starvation.

Isolation and Characterization of Longer-Lived Mutants

BKy1-14c was mutagenized and grown into colonies that were then replica-plated to media without nitrogen or carbon sources. After incubating under these starvation conditions for 8 days, colonies were replica-plated back to rich plates; 39 mutants were recovered that survived this treatment, thereby displaying an increased stress resistance. Of these, 8 exhibited life spans that were elongated compared with the life span of the parent (by 20%–55%). The mutants all exhibited other phenotypes consistent with an increase in stress tolerance. They were more resistant to heat shock and showed an enhanced ability to grow on ethanol (a carbon source that induces the heat shock response in S. cerevisiae [Plesset et al., 1982]). The link between starvation and heat shock was not surprising, because starvation is known to induce heat shock proteins (Kurtz et al., 1986). Further, the mutants all grew to a higher saturation density than the parent, probably because of their enhanced ability to utilize the ethanol in the media after the diauxic shift. One mutant, designated *uth2-42* (for youth), displayed two additional phenotypes: it mated poorly and exhibited a bipolar budding pattern characteristic of diploids.

Eight mutants were crossed to BKy5 (isogenic to 14c, but with the opposite mating type) and sporulated, and seven were shown to segregate 2:2 for stress-related phenotypes in more than ten tetrads each. The *uth3-335* mutant may have an additional mutation that contributes to stress resistance. Genetic analysis indicated that seven mutants were recessive and one was dominant for stress phenotypes. The recessive mutations fell into three complementation groups. The dominant mutation was not linked to representatives of any of these groups, and representatives of each group were not linked to each other. These genes were designated *UTH1–UTH4*; mortality curves for each complementation group are shown in Figures 3A–3D. The differences in life span in this and subsequent experiments presented in this paper were deemed significant by a Wilcoxen signed rank test (see Experimental Procedures).

Table 1. Yeast Strains Used in This Study

Strain	Genotype
BWG1-7A	*MATa ade1-100 his4-519 leu2-3,2-112 ura3-52*
PSY142	*MATα leu2-3,2-112 lys2-801 ura3-52*
CKy20	*MATα arg1 tsm11*
CKy21	*MATa arg1 tsm11*
UCC1001	*MATa ade2-101 his3-Δ200 leu2-Δ1 lys2-801 trp1-Δ1 ura3-52 adh4::URA3*-TEL
BKy1	*MATa ade1-100 his4-519 leu2-3,2-112 LYS2 ura3-52* *MATα ADE1 HIS4 leu2-3,2-112 lys2-801 ura3-52*
BKy1-14a	*MATa ade1-100 leu2-3,2-112 lys2-801 ura3-52*
BKy1-14b	*MATα leu2-3,2-112 ura3-52*
BKy1-14c	*MATα ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52*
BKy1-14d	*MATa his4-519 leu2-3,2-112 ura3-52*
BKy5	*MATa ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52*
BKy6	*MATa ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52* *MATα ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52*
BKy17	*MATa ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 SIR4* *MATα ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 sir4-42*
BKy21	*MATa ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 sir4-42*
BKy28	*MATa ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 sir4-42* *MATα ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 sir4-42*
BKy30	*MATa ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 SIR4/LEU2*
BKy100	*MATα ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 ste4::URA3*
BKy101	*MATα ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 ste12::URA3*
BKy102	*MATα ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 sir1::LEU2*
BKy103	*MATα ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 sir3::URA3*
BKy104	*MATα ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 sir4::URA3*
BKy105	*MATα ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 sir4-42 sir1::LEU2*
BKy106	*MATα ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 sir4-42 sir3::URA3*
BKy107	*MATα ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 sir4::URA3 LEU2/sir4-42*
BKy108	*MATα ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 sir4-42 URA3/SIR4*
BKy109	*MATa ade1-100 his4-519 leu2-3,2-112 lys2-801 ura3-52 adh4::URA3*-TEL sir4::LEU2

All strains were generated in this study except BWG1-7A, which is described in Guarente and Mason, 1983; UCC1001, which was a gift of D. Gottschling; and the mating testers CKy20 and CKy21, which were gifts of C. Kaiser. The terminology *LEU2/sir4-42* in strain BKy107 indicates that the *sir4-42* allele has been integrated at the *LEU2* locus, for example.

Because of its additional phenotypes, we focused on *uth2-42* and showed that sterility and bipolar budding pattern both cosegregated with stress tolerance. Moreover, in three complete tetrads we found that a lengthened life span also cosegregated with the other mutant phenotypes. Thus, a single mutation is likely to cause all of these phenotypes. Finally, we found that the extension in life span caused by *uth2-42* was observed in a continuous assay in which cells were kept at 30°C for the entire course of the experiment (data not shown).

Cloning *UTH2* and Its Identity to *SIR4*

UTH2 was cloned by its ability to restore mating to the *uth2-42* strain, assayed by replica-plating transformants to a lawn of a tester strain of opposite mating type (CKy21). Positive clones were recovered in Escherichia coli, and

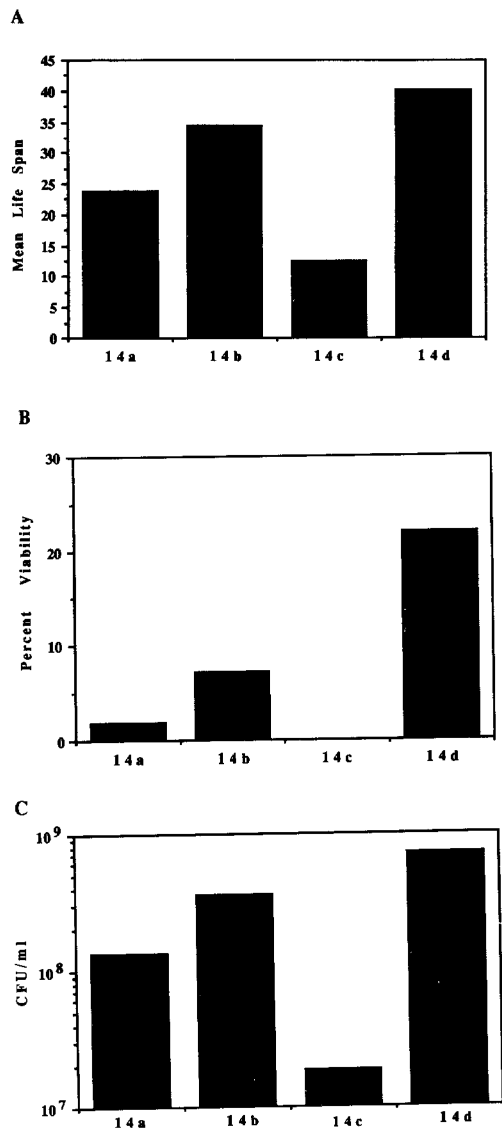

Figure 2. Correlated Phenotypes for Tetrad BKy1-14

(A) Mean life spans of segregants of tetrad BKy1-14 (resulting from BWG1-7A x PSY142) were determined by mortality curves involving 20 cells of each strain except 14d (19 cells).

(B) The viability of BKy1-14 segregants stored for 4.5 months at 4°C. The percent viability is the number of colony-forming units per total microscopic cell count. For strain BKy1-14c, no viable cells were detected.

(C) The ability of BKy1-14 segregants to maintain colony-forming potential during starvation. An equal number of cells of each strain was inoculated from rich YEPD media to minimal sporulation media lacking nitrogen and carbon sources and incubated for 7 days before plating on YEPD. The number of colony-forming units per milliliter is shown.

one, pBK40, was able to confer efficient mating ability when retransformed back into yeast. This clone also restored starvation and heat shock sensitivity to the *uth2* mutant. pBK40 contained an insert of approximately 8 kb. An internal fragment was labeled and used to probe a panel of λ clones containing yeast DNA (Riles et al., 1993). The λ clone that hybridized contained *SIR4* (for silent information regulator), a component of the yeast silencing complex that represses copies of *MATα* and *MAT**a*** information at *HML* and *HMR* (Hartwell, 1980; Laurenson and Rine, 1992; Rine and Herskowitz, 1987). Restriction enzyme mapping of pBK40 indicated that it contained *SIR4* and at least 1 kb of flanking DNA to either side. A loss-of-function mutation in *SIR4* would explain the sterile and bipolar budding phenotypes of the *uth2* mutant. To determine linkage, the insert was transferred to a *LEU2*-containing integrating vector and targeted to the *SIR4* locus in BKy5. This integrant (BKy30) was mated with *uth2-42* (containing pBK40 to allow mating), and after eviction of pBK40, the diploid sporulated; 13 of 13 tetrads contained 2 Leu^+, fertile: 2 Leu^-, sterile segregants, showing that *SIR4* is tightly linked to the *uth2-42* mutation. We concluded that *uth2-42* was probably an allele of *SIR4* (now designated as *sir4-42*).

Specificity of Elongation of Life Span

The *sir4* mutant is sterile, because it expresses **a** and α information simultaneously. We carried out two experiments to show that the lengthening of life span was not because of the **a**/α cell type. First, we determined the life span of the isogenic BKy1-14c/BKy5 diploid, designated BKy6 (see Experimental Procedures), and found that, as expected (Müller, 1971), it was not significantly different from the haploid parents (Table 2). Second, we introduced into BKy5 a plasmid that expressed *MATα* and found that this transformant also did not have a lengthened life span (Table 2).

In C. elegans, it has been shown that certain mutations that cause sterility also confer a lengthened life span (Van Voorhies, 1992). To determine whether sterility, more generally, could be related to life span in S. cerevisiae, we disrupted *STE4* or *STE12* (see Experimental Procedures), genes involved in the mating pheromone-response pathway. Again, life span was not affected in either of these sterile strains (Table 2).

Finally, because the stress and mating phenotypes of *sir4-42* were recessive, we surmised that the phenotype of a *SIR4* null mutation would mimic that of *sir4-42*. The entire *SIR4* gene was deleted in BKy1-14c (see Experimental Procedures), and the strain with the deletion (BKy104), indeed, was stress tolerant, sterile, and exhibited bipolar budding (data not shown). Importantly, however, it did not have a lengthened life span (Figure 4A). In fact, because of the large number of cells studied in this experiment, we can conclude that the deletion shortened life span by a small, but statistically significant, degree.

The above finding suggested that the effect of *sir4-42* on life span, unlike its effects on stress and mating, might be due to a gain of function. To test this, we determined whether the *sir4-42* allele was dominant to *SIR4* for the phenotype of lengthened life span. The wild-type *SIR4* was transferred to an integrating vector and targeted to *URA3* in the *sir4-42* mutant. The resulting *SIR4/sir4-42* haploid (BKy109) was stress sensitive and mated efficiently, as expected. However, the life span of this strain was intermediate between the *SIR4* parent, BKy1-14c, and the *sir4-42* mutant (Figure 4B), indicating that the *sir4-42* mutation is semidominant with respect to life span. As a second test

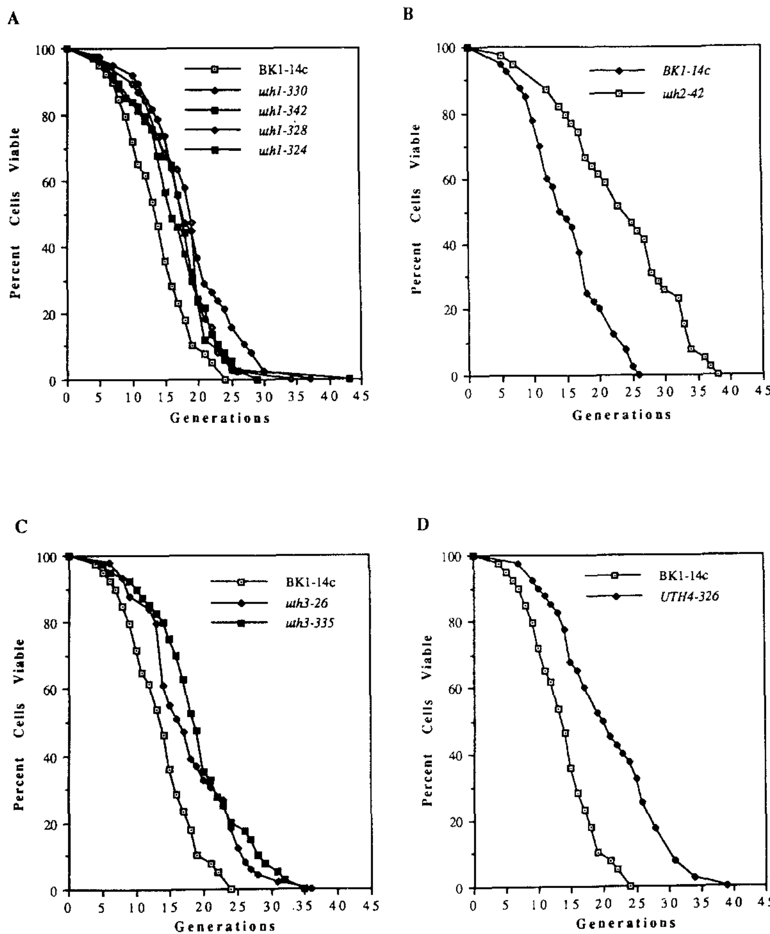

Figure 3. The Mortality Curves of *uth* Mutants

Mortality curves are shown for BKy1-14c and *uth1-4* mutants in (A)–(D), respectively. All curves are derived from two experiments. The data presented in Figure 2B were generated in two experiments unrelated to the experiments performed to generate Figures 2A, 2C, and 2D. The differences in the life spans between each *uth* mutant and BKy1-14c were shown to be statistically significant (see Experimental Procedures). The sample size for strain BK1-14c was 40 cells.

(A) The sample sizes for mutants of *UTH1* were as follows: *uth1-324*, 37 cells; *uth1-328*, 38 cells; *uth1-330*, 38 cells; *uth1-342*, 34 cells.

(B) The sample size for *uth2-42* was 40 cells.

(C) The sample sizes for mutants of *UTH3* were as follows: *uth3-26*, 49 cells; *uth3-335*, 40 cells.

(D) The sample size for *uth-326* was 40 cells.

Table 2. The Effects of Sterility on Mean Life Span

Strain	Sample Size	Mean Life Span	Maximum
BKy1-14c	20	15.6	25
BKy5	20	14.5	20
BKy6	20	15.3	27
BKy100 (*ste4Δ*)	20	15.9	24
BKy101 (*ste12Δ*)	20	16.5	24
BKy5 + *MATa*	20	14.6	26

The column labeled maximum indicates the number of daughters produced by the oldest mother cell.

The data presented derive from one independent experiment. Life span analysis has been repeated at least three times for all of these strains, and similar results have been observed.

for dominance, we used mating to construct isogenic diploids *SIR4/SIR4* (BKy6), *SIR4/sir4-42* (BKy17), and *sir4-42/sir4-42* (BKy28) (using the *SIR4* plasmid to permit mating in *sir4-42* mutants as described in Experimental Procedures). The homozygous diploids had life spans similar to those of their haploid parents, and the heterozygous diploid displayed a life span intermediate between the homozygotes (data not shown). These findings show that the extended life span in the *sir4-42* mutant is semidominant and, therefore, due to a gain-of-function mutation.

The *sir4-42* mutation was cloned by gap repair and mapped to a SmaI fragment spanning codons 743 to the UAA stop at the end of the 1358-residue *SIR4* open reading frame (see Experimental Procedures). The clone was shown to contain the mutation by a functional test in which it was transferred to an integrating vector and targeted to *LEU2* in strain BKy104 (*Δsir4*). The resulting strain had an extended life span, indicating that the integrating vector contained the *sir4-42* allele (data not shown). The SmaI fragments from the mutant or wild-type *SIR4* gene were incorporated into Bluescript and sequenced across the entire *SIR4* coding sequence. A single difference was found in the mutant that generated a stop at codon 1237, removing 121 residues from *SIR4*.

The Lengthening of Life Span by *sir4-42* Requires *SIR3*

How does *sir4-42* extend life span? We initially began by asking whether *sir4-42* acted alone or in concert with other members of the SIR complex. The activities of SIR2, SIR3, and SIR4 are closely coupled in that all are required for silencing at the *HM* loci and at telomeres (Aparicio et al., 1991; Rine and Herskowitz, 1987). The function of SIR1 is different in that it is only required at the *HM* loci (Aparicio et al., 1991), and even there, its requirement is not absolute (Pillus and Rine, 1989). To determine whether *SIR3* and *SIR1* were required for the extention of life span, the genes were disrupted in the *sir4-42* mutant and, as a control, in BKy1-14c (see Experimental Procedures). The *sir1*

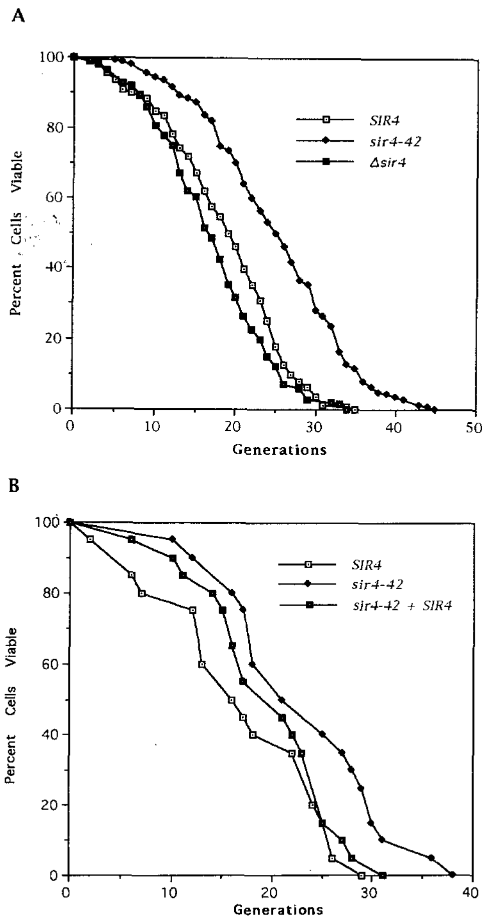

Figure 4. Deletion of the *SIR4* Gene Does Not Extend Life Span: The *sir4-42* Mutation Is Semidominant for Increased Longevity

(A) Mortality curves are shown for BKy1-14c (*SIR4*), *sir4-42* (i.e., BK1-14c *sir4-42*), and BKy104 (*Δsir4*) and are derived from seven experiments. Statistical analysis showed that the mean life span difference between *sir4-42* and *SIR4* was significant, as was the smaller difference between *SIR4* and *Δsir4* (see Experimental Procedures). The sample sizes for the strains in this experiment were as follows: BKy1-14c, 139 cells; *sir4-42*, 139 cells; BKy104, 136 cells.

(B) In strain BKy108 (*sir4-42* + *SIR4*), a wild-type *SIR4* gene has been integrated into the *URA3* locus of *sir4-42*, giving the strain one mutant and one wild-type copy of *SIR4*. Mortality curves for BKy1-14c (*SIR4*), *sir4-42*, and BKy108 (*sir4-42* + *SIR4*) are derived from one experiment. Similar results have been obtained in other experiments. Statistical analysis determined that the mean life span of BKy109 was significantly different from the means of both *sir4-42* and BKy1-14c (see Experimental Procedures). The sample sizes for the strains in this experiment were as follows: BKy1-14c, 20 cells; *sir4-42*, 20 cells; BKy108, 20 cells.

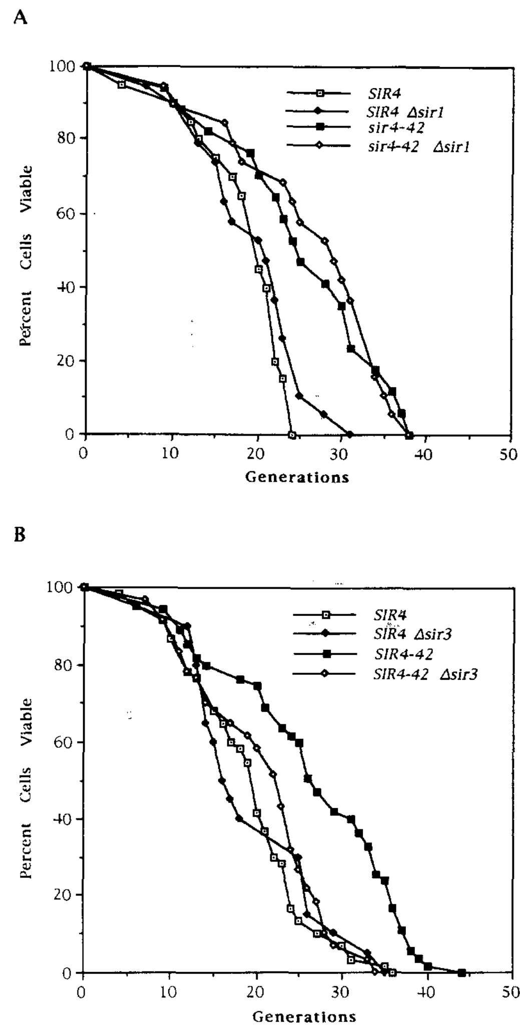

Figure 5. The Increased Longevity Conferred by *sir4-42* Requires *SIR3* but Not *SIR1*

Mortality curves are shown for BKy1-14c (*SIR4*), *sir4-42*, and their isogenic *Δsir1* and *Δsir3* derivatives. The sample sizes were as follows.

(A) BKy1-14c (*SIR4*), 20 cells; BKy102 (*Δsir1*), 19 cells; *sir4-42*, 18 cells; and Bky105 (*sir4-42 Δsir1*), 19 cells.

(B) BKy1-14c (*SIR4*), 60 cells; BKy103 (*Δsir3*), 20 cells; *sir4-42*, 19 cells; and BKy106 (*sir4-42 Δsir3*), 30 cells.

disruptions did not exert any effect on the *sir4-42* mutant or its *SIR4* parent (Figure 5A). In contrast, the *sir3* disruption abolished the extension in life span conferred by *sir4-42* (Figure 5B). This shortening of life span in the *sir4-42* strain was specific, because disruption of *SIR3* did not greatly alter the life span of the *SIR4* parent (Figure 5B). The number of cells examined in this experiment was not sufficiently large to reveal any small reduction in the life span of the *Δsir3* strain. Similarly, we found that deleting *SIR2* abolished the extention in life span conferred by *sir4-42* (data not shown). Thus, the gain of function caused by *sir4-42* is probably an activity of the entire SIR complex, and not *SIR4* alone.

Effects of the *sir4-42* Mutation on Telomeres

Because the *sir4-42* mutation results in a loss of activity at *HM* loci, we considered the possibility that it redirects the SIR complex to another chromosomal location, resulting in the observed extension in life span. One obvious possible location was telomeres, because loss-of-function mutations in *SIR2*, *SIR3*, or *SIR4* relieve silencing at telomeres and also result in shorter telomeres (Aparicio et al., 1991; Palladino et al., 1993). In mammalian cells, telomeres have been shown to shorten with age (Harley et al., 1990), and

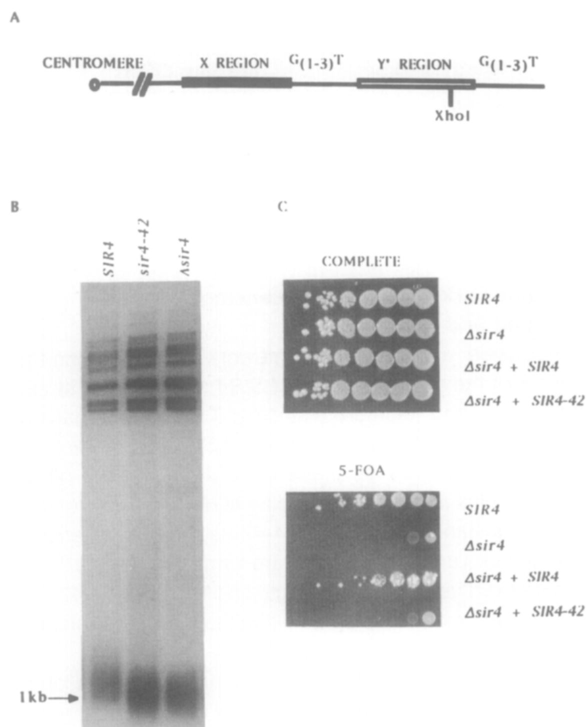

Figure 6. Telomere Length Shortens and Telomere Silencing Is Eliminated in the *sir4-42* Mutant

(A) The structure of most yeast telomeres. The variable G(1–3)T region at the chromosmome end is flanked by the conserved Y′ region, which contains a XhoI site (Walmsley et al., 1984). The average length from the XhoI site to the terminus of the chromomsome is approximately 1.2 kb.

(B) XhoI-digested DNA is probed with a labeled 600 bp fragment that hybridizes to Y′ DNA that is telomere-distal to the XhoI site. The broad smear just above a 1 kb size marker consists of yeast telomeres.

(C) Viability of strains having *URA3* at a chromosome VIIL telomeric locus on complete synthetic media with or without 5-FOA. Strains used were UCC1001 (*SIR4*) or BKy109 (*Δsir4*) containing the *ARS–CEN* vector pRS314, or BKy109 (*Δsir4*) with *SIR4* or *sir4-42* carried on pRS314. Cells were pregrown in synthetic liquid media and then plated at 10-fold serial dilutions from right to left on either complete media or media containing 5-FOA. Silencing of *URA3* gives rise to a Ura$^-$ phenotype and growth on 5-FOA.

this shortening has been proposed as a causative agent of aging (Allsopp et al., 1992; Olovnikov, 1973). If telomere shortening imposed a limit to life span in yeast, then excessive recruitment of the SIR complex might counter aging by lengthening telomeres.

Therefore, we determined the length of telomeres and the degree of silencing at telomeres in *Δsir4* and *sir4-42* mutants. The 1.2 kb band in the *SIR4* parent represents the G1–3T repeats and Y′ region distal to the XhoI sites at most yeast chromosomes (Figure 6A) (Chan and Tye, 1983; Walmsley et al., 1984). As previously reported, deletion of *SIR4* in BKy1-14c resulted in a shortening of telomeres of approximately 50–100 bases (Palladino et al., 1993). Surprisingly, the length of telomeres in the *sir4-42* mutant was indistinguishable from their length in the *Δsir4* mutant, indicating that the mutant behaved like the deletion with respect to activity at telomeres (Figure 6B). Next,

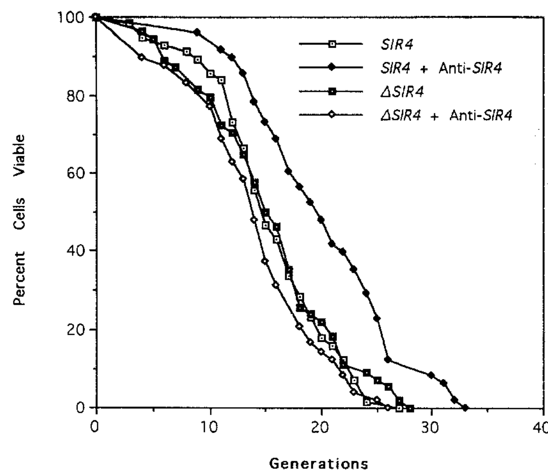

Figure 7. The Dominant Negative Anti-*SIR4* Construct Extends Yeast Life Span

The strain labeled *SIR4* + Anti-*SIR4* is BKy1-14c transformed with the plasmid pJH3A, a 2μ plasmid containing the C-terminal 154 amino acids of the *SIR4* gene (Ivy et al., 1986). The strain labeled *Δsir4* + Anti-*SIR4* is BKy104 transformed with plasmid pJH3A. The control strains (*SIR4* or *Δsir4*) contain a 2μ *LEU2* vector plasmid. Mortality curves for all four strains are from two experiments. The mean life span of the strain labeled *SIR4* + Anti-*SIR4* was shown to be statistically different from that of the *SIR4* strain (see Experimental Procedures). The sample sizes were as follows: *SIR4*, 56 cells; *SIR4* + anti-*SIR4*, 48 cells; *Δsir4*, 54 cells; and *Δsir4* + anti-*SIR4*, 48 cells.

we determined the degree of silencing of *URA3* positioned at telomere VIIL in strain UCC1001 and derivatives in which *SIR4* was deleted and *sir4-42* (or *SIR4*) was reintroduced. Deleting *SIR4* greatly relieved silencing, as expected, resulting in sensitivity to 5-fluoro-orotic acid (5-FOA) (Figure 6C). Introduction of *SIR4* but not *sir4-42* restored silencing to the *Δsir4* strain. There are two important conclusions from these findings. First, the *sir4-42* mutant behaves like the *SIR4* deletion at telomeres; i.e., it exhibits a loss-of-function phenotype. Second, since *sir4-42* extends life span and *Δsir4* does not, the lengthened life span is probably unrelated to telomere length or telomere silencing.

Expression of the Carboxyl Terminus of SIR4 Also Extends Life Span

Our results indicate that the *sir4-42* mutation abolishes activity at the *HM* loci and at telomeres and endows the SIR complex with the ability to extend life span. This extension in life span could be because the *sir4-42* complex is more available to function at yet another chromosomal location that is crucial in the determination of life span. By this logic, a different means of preventing recruitment of the SIR complex to the *HM* loci and to telomeres should also extend life span.

Since the *sir4-42* mutation removes the carboxyl terminus of the protein, it seemed possible that this fragment of SIR4 localized the complex to *HM* loci and telomeres. Thus, overexpression of a carboxy-terminal fragment of SIR4 might compete with the wild-type protein for recruitment to *HM* loci and telomeres. Indeed, a construct ex-

Table 3. Phenotypes of *SIR4* Alleles

Allele	Amino Acids	Mating	Stress Response	Life Span Effects
SIR4	1–1358	+	Sensitive	–
sir4-42	1–1237	–	Resistant	Increase
sir4Δ	–	–	Resistant	Decrease
SIR4 + Anti-*SIR4*	1–1358 and 1205–1358	–	Resistant	Increase

pressing only the carboxyl 154 residues of SIR4 has been shown to behave as an anti-*SIR4* dominant negative mutant with respect to silencing at *HM* loci (Ivy et al., 1986; Marshall et al., 1987). We introduced this construct into BKy1-14c and confirmed that it functioned as a dominant negative inhibitor of mating. The transformant was also stress resistant, as expected. Strikingly, the construct extended the life span of BKy1-14c by about 30% (Figure 7). To verify that this extension in life span was due to the wild-type SIR complex, we expressed the construct in the BKy1-14c *Δsir4* strain and observed no extension (Figure 7).

Discussion

In S. cerevisiae, the number of cell divisions that mother cells undergo is relatively fixed and is termed their life span (Mortimer and Johnston, 1959). We have exploited a correlation between stress resistance and life span within a set of yeast strains to isolate mutations that increase the life span of mother cells. Mutations were isolated by an increased tolerance to stress, and some extended the life span by 20%–55%. This screen resulted in mutations in four genes, termed *UTH1–UTH4*. One of these genes, *UTH2*, is identical to *SIR4*, known to be required for silencing regions of yeast chromosomes (Rine and Herskowitz, 1987).

A correlation between stress tolerance and life span may be more general than what we have observed here. In Drosophila melanogaster, selective breeding of flies at an advanced age results in strains with an elongated life span (Luckinbill et al., 1984; Rose and Charlesworth, 1981). These flies are also more resistant to stress treatments such as dessication and starvation (Arking et al., 1991). In C. elegans, first stage larvae can enter a dormant state during which they are termed dauer larvae and can remain viable for prolonged periods in the absence of nutrients. While this pathway is normally activated by starvation, it can be induced inappropriately when *daf-2* (for abnormal dauer formation) temperature-sensitive mutants are shifted to the restrictive temperature (Vowels and Thomas, 1992). Such induction of dauer gene expression does not enable postlarval worms to enter the dormant state. However, adults display a life span that is elongated 2-fold compared with the uninduced control (Kenyon et al., 1993). These findings indicate that a pathway that functions to promote stress resistance in starved larvae can extend the life span of adults.

A Framework for Relating Silencing to Aging and Stress

We consider a speculative framework for interpreting the effects of the various alleles of *SIR4* on life span, stress resistance, and telomere silencing. Table 3 summarizes the effects of three mutant alleles of *SIR4*: *Δsir4*, *sir4-42*, and the anti-*sir4* in the presence of the wild-type gene. These alleles all alleviate silencing at *HM* loci and promote stress resistance. *Δsir4* and *sir4-42* also alleviate silencing at telomeres and reduce telomere length. However, these alleles exert very different effects on life span. *sir4-42* and the anti-*sir4* extend life span, while the *Δsir4* shortens life span.

To explain these findings, we first propose that a locus that is repressed by the SIR complex can promote resistance to stress when repression is eliminated (Figure 8). In principle, this locus could be linked to *HML*, *HMR*, or a telomere, or it could reside at some other location. Linkage to *HM* loci is not likely, however, because deletion of *SIR1*, which weakens repression at the *HM* loci, does not promote stress resistance. For simplicity, we show the case of a telomere-linked, stress-resistant locus under SIR control.

We further suggest that life span is controlled by a different locus (termed *AGE*, Figure 8) that is unlinked to *HM* loci or telomeres, but is regulated by the SIRs. The repression of the *AGE* locus by *SIR4* is essential to longevity, according to this view, and aging may result from a breakdown in the silencing of that locus. Thus, *Δsir4* exhibits a shortened life span. It is, of course, possible that silencing at more than one chromosomal region governs aging. In any case, the *AGE* locus is shown as unlinked to telomeres or *HM* loci, because the *sir4-42* mutation and *Δsir4* exhibit similar effects at *HM* loci and at telomeres, but have opposite effects on life span. Further, the extension of life span by *sir4-42* is semidominant in a strain also containing *SIR4*, indicating that it is a gain-of-function mutation with regard to life span. The function gained in the mutant must relate to the normal silencing activity of the SIR complex, because the ability of *sir4-42* to promote longevity requires the integrity of *SIR3* and *SIR2*.

Why is the *sir4-42* mutant more able to repress a hypothetical *AGE* locus? We suggest that the mutation prevents recruitment of the SIR complex to *HML*, *HMR*, and telomeres, rendering the complex more available for any other site of action in the cell. The carboxyl 121 residues that are missing in the *sir4-42* mutant may be important in the recruitment of the SIR complex to these chromosomal sites. Consistent with the view that the carboxyl terminus

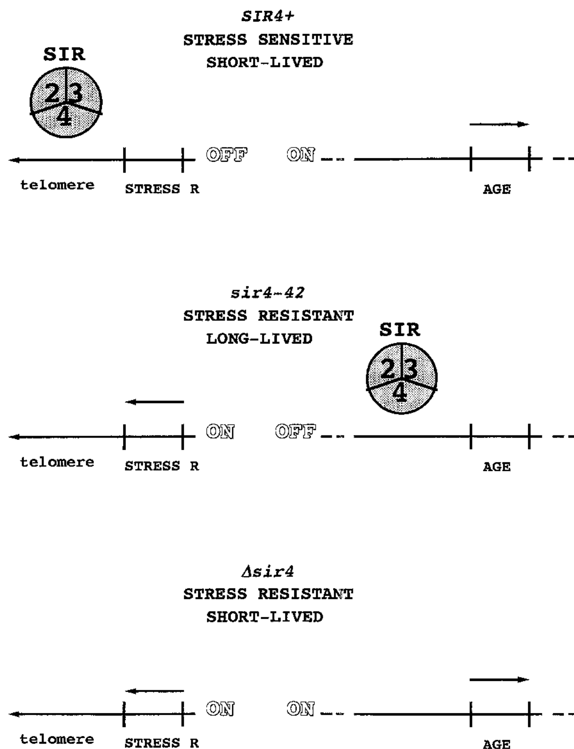

Figure 8. A Model for the Effects of Silencing on Yeast Life Span

A locus that confers stress resistance is labeled STRESS R and is shown located near a telomere. A nontelomeric locus that causes aging when expressed in old cells is labeled AGE. The arrows indicate transcription. Silencing by SIR2, SIR3, and SIR4 is indicated. In a *SIR4* strain, the majority of the SIR complex is localized at telomeres, at the expense of the *AGE* locus. The *sir4-42* mutation prevents recruitment of the SIR complex to telomeres (and *HM* loci), resulting in increased occupancy at *AGE* and a concomitant slowing of the aging process.

of SIR4 helps localize the SIRs to *HM* loci and telomeres, overexpression of the carboxyl 163 residues of SIR4 is known to exert a dominant negative effect on repression at *HM* loci (Ivy et al., 1986; Marshall et al., 1987). We expressed this SIR4 fragment and found that in addition to blocking repression at *HML* and *HMR*, it promoted longevity in a *SIR4* strain but not in a *Δsir4* strain. This finding in consistent with the above recruitment model and suggests that the *sir4-42* mutation does not function by creating some novel activity of the SIR complex. Determining the identity of new sites of repression by the SIR complex will be an important goal for future studies.

Aging and Chromosome Silencing

Our findings lead to the suggestion that a breakdown in silencing by the SIR complex may be causally related to aging in S. cerevisiae. Is there any indication that a breakdown in chromosomal silencing is related to aging in any other system? X chromosome inactivation is perhaps the most extensive example of heterochromatic gene inactivation in mammals (Lyon, 1961). By employing strains with X-autosomal translocations, it was shown that X-linked markers, normally silenced in embryos and young adults, became reactivated as a function of age in the mouse (Wareham et al., 1987). It is intriguing that the inactivated X chromosome displays a hypoacetylation of histone H4 (Jeppesen and Turner, 1993), as do the silenced *HM* loci (Braunstein et al., 1993). In the latter case, hypoacetylation is abolished by mutations in *SIR2*, *SIR3*, or *SIR4*.

Further, in cultured human diploid fibroblasts, CpG methylation, which correlates very well with gene inactivation, is lost as the cells progress toward senescence (Wilson and Jones, 1983). The rate of loss is greater in fibroblasts derived from shorter-lived mammals, such as mice, than in cells from humans. While these examples only demonstrate correlations between aging and a loss of silencing, they are consistent with the possibility that a loss of silencing may be a primary cause of aging in a wide range of biological systems.

Aging and Telomeres

An interesting current hypothesis proposes that a progressive loss of telomeres causes senescence in cultured animal cells and aging in the intact animal (Allsopp et al., 1992; Olovnikov, 1973). This view is based on several observations on cell cultures and intact animals. In cell cultures, there is a good correlation between the length of telomeres and the replicative capacity of primary human fibroblasts taken from a range of different individuals (Allsopp et al., 1992; Harley et al., 1990). Cells from individuals with the premature aging syndrome Huntington's–Gilford progeria display the shortest telomeres and weakest replicative capacity. In rare instances, cells emerge from crisis and become immortalized in culture. In such cells, the telomeres lengthen, and telomerase activity increases (Allsopp et al., 1992). In humans, telomeres from several human organs, such as lymphocytes or peripheral blood leukocytes, have been shown to shorten with age (Schwartz et al., 1993; Vaziri et al., 1993).

The identification of *SIR4* as a gene that affects life span in yeast appears to relate telomeres and aging in that organism. However, our findings show that telomeres in the *sir4-42* strain, just as in the *Δsir4* null mutant, are shorter than in the wild type. This suggests that telomere length is not causally related to aging. However, this conclusion must be qualified, because we have not examined the effect of the *sir4-42* mutation on the state of telomeres specifically in old cells. It is theoretically possible that the mutation counters telomere shortening selectively in old cells. Owing to the technical difficulties in obtaining a large population of old cells, we have not yet analyzed the effects of aging on telomere length in wild-type or mutant senescent cells. D'Mello and Jazwinski have followed cells out to 80% of their life spans and found that telomeres have not shortened (D'Mello and Jazwinski, 1991). We are currently developing methods to obtain the large numbers of cells at the ends of their life spans that will make these important experiments possible.

Overview

We describe the start of a genetic analysis on aging in S. cerevisiae. More broadly, genetics is likely to be a useful approach, whatever the underlying mechanism of aging. If aging is based on a developmental program, the identification of important genes will be a crucial first step toward an understanding of the process, just as in embryonic development. If, rather, aging is a response to accumulated damage, mutations that extend life span will shed light on which processes are most damage sensitive.

Experimental Procedures

Strains, Plasmids, and Media

Yeast strains (see Table 1) were grown using standard media and conditions (Sherman et al., 1979). The *STE4* disruption was constructed using a *ste4::URA3* plasmid as described (Whiteway et al., 1989). Disruption of *STE12* was constructed using plasmid pNC163 as described (Company et al., 1988). The plasmid pKC2 containing the *MATa* locus was kindly provided by G. Fink. The region from 153 bp 5′ to *SIR4* through the entire open reading frame was deleted and replaced with the *URA3* gene by use of the plasmid pAR59, provided by J. Broach (Marshall et al., 1987). *sir4Δ* was confirmed by Southern blot analysis. *sir1Δ* was generated by use of plasmid pJI23.2, which removes the C-terminal 335 amino acids from the 648 amino acid protein (Ivy et al., 1986). *sir3Δ* was constructed with plasmid pDM42, which deletes 123 amino acids at the carboxyl terminus of SIR3 (Mahoney and Broach, 1989). pJH3A was originally described by Ivy et al. (1986).

Integration plasmids were generated by subcloning the entire library insert containing *SIR4* from pBK40 into pRS305 or pRS306 by a NotI–SalI double digest (Sikorski and Hieter, 1989). Integration was directed to the *URA3* locus by a StuI digest. Integration was directed to the *LEU2* locus by an XcmI digest. All integration events were verified by Southern analysis.

To generate BKy5, strain BK1-14c was transformed with a GAL-HO plasmid and plated on galactose medium to induce switching of mating type (Herskowitz and Jensen, 1991). Colonies were tested by mating to CKy20 or CKy21 to determine their mating type. A *MATa* colony was picked, and the GAL-HO plasmid was segregated by using 5-FOA (Boeke et al., 1987). This strain, BKy5, was mated to BKy1-14c, and zygotes were isolated by micromanipulation to generate BKy6. To verify that BKy6 is a diploid, the strain was shown to be sporulation competent. The *SIR4/sir4-42* heterozygote (BKy17) was generated by mating the *sir4-42* mutant containing pBK40 to BKy1-14c and subsequently losing the plasmid with 5-FOA. BKy17 was sporulated, and a *MATa sir4-42* segregant (BKy21) was chosen to generate the homozygous *sir4-42* diploid (BKy28). BKy21 carrying pBK40 was mated to the *sir4-42* mutant also carrying pBK40, and diploids were isolated.

Life Span Analysis

To determine the life span of a strain, cells were taken from logarithmically growing liquid cultures and plated at low density on complete medium. The plates were incubated at 30°C for approximately 3 hr. At this time, daughter cells were isolated as buds that had emerged from mother cells and were moved with a Zeiss Micromanipulator to uninhabited regions of the plate. The life spans of these cells were determined by noting and removing all subsequent daughters they generated. The plates were incubated at 30°C during working hours and shifted to 4°C overnight. Life spans generated by this incubation schedule do not differ significantly from those generated by incubating cells continuously at 30°C (data not shown). On very rare occasions, an apparently young cell was observed to lyse immediately after micromanipulation and was excluded from the data set.

Mutagenesis

Growing BKy1-14c cells were mutagenized with ethyl methanesulfonate (approximately 60% of cells killed) and allowed to form colonies on rich media (yeast extract–peptone–dextrose, or YEPD). These colonies were then replica-plated to sporulation plates and allowed to starve for 7–9 days. At this point, the cells were returned to YEPD and incubated for 2 days. While the majority of colonies were unable to grow appreciably, a small percentage were able to resume growth. These colonies were isolated and their starvation-resistant phenotype confirmed. There were 39 mutants recovered that reproducibly tested starvation-resistant. Life spans of all 39 mutants were determined for a minimum of 20 cells of each mutant. Eight mutants demonstrated significantly higher mean life spans as determined by the Wilcoxen signed rank test. These eight mutants were used for subsequent analysis.

Cloning of *UTH2*

A genomic library, CT3, supplied by R. Young (Thompson et al., 1993), was transformed into the *uth2-42* mutant by standard methods. Transformed colonies were tested for their ability to complement the mating defect in the *uth2-42* mutant. Plates containing library-transformed colonies were replica-plated onto permissive plates containing a lawn of strain CKy21. Cells were incubated at room temperature for 1 day to allow mating and then replica-plated to plates selective for diploid growth. Colonies were picked that clearly grew on the selective plates. Plasmids were recovered from these colonies by standard methods and retransformed into *uth2-42* mutant cells. One plasmid was isolated that restored mating efficiency in the *uth2-42* mutant. This plasmid, pBK40, also conferred heat shock sensitivity and starvation sensitivity to *uth2-42*, making it a good candidate for the *UTH2* gene.

A 1.6 kb fragment located entirely within the pBK40 library insert was random primed according to the protocol of the manufacturer (United States Biochemical Corporation) and used to probe a λ clone library (Riles et al., 1993). Only one clone, which contained the *SIR4* gene, showed a distinguishable signal.

Determination of Stress Phenotypes

To determine starvation resistance, haploid cells were grown in rich media to log phase, collected by centrifugation, and resuspended in minimal sporulation media for a period of 7–9 days. After starvation, cells were again collected by centrifugation and plated on YEPD to measure colony-forming units per milliliter. Colonies could be assayed for their ability to withstand starvation by utilizing sporulation plates instead of liquid culture.

Heat shock resistance was determined by collecting logarithmically growing cells and plating them at a known concentration on YEPD plates. The cells were heat shocked at 55°C for periods varying from 5 min to 1 hr. Plates were then incubated at 30°C for 3 days, and the number of colonies was noted.

Saturation density was measured by suspending logarithmically growing cells in YEPD liquid culture at a density of 10^6 cells/ml. Cultures were incubated for a period of 5 days, with the number of cells per milliliter counted in a hemacytometer on a periodic basis. Control experiments indicated that the media was completely saturated after this time period.

Growth on ethanol was measured by directly streaking a strain on either rich media containing ethanol or synthetic media supplemented with necessary nutrients and containing ethanol as the sole carbon source.

DNA Sequencing

Gap repair was utilized to clone both the wild-type *SIR4* allele from BKy1-14c and the *sir4-42* allele from the *SIR4* mutant strain. A SmaI–AatII double digest was performed to remove the coding region of *SIR4* from pBK40. The linear plasmid was gel purified and transformed into either BKy1-14c or the *sir4-42* mutant. Ura^+ colonies were picked, and the plasmids were recovered by standard methods. Restriction digests were conducted to determine whether the gap repair event was successful. To localize the mutation within *SIR4*, digests were conducted with AatII, SmaI, and SphI, all of which have one site in the *SIR4* gene and another within the pBK40 insert either 5′ or 3′ to *SIR4*. These linearized plasmids were transformed into *sir4-42* and transformants were tested for their ability to complement the *sir4-42* associated mating defect. This analysis localized the mutation to the region from amino acid 743 to the 3′ end of the gene.

A SmaI fragment containing this region was gel purified from both the BKy1-14c *SIR4* allele and the mutant allele from *sir4-42* and subcloned into Bluescript (Stratagene). Sequencing primers were made approximately 200 bp apart for this entire region, and it was sequenced

by the single-strand approach (Sequenase version 2; United States Biochemical Corporation). Only one base pair change was discovered, generating a stop at codon 1237.

Determination of Telomere Length and Silencing

Total genomic DNA was isolated from BKy1-14c, 14c *sir4Δ*, and *sir4-42*, digested with XhoI, and separated on a 0.7% agarose gel and transferred to a GeneScreen Plus hybridization transfer membrane (New England Nuclear Research Products). Hybridization and wash conditions were as suggested by the manufacturer of the membrane. A plasmid containing 600 bp located within the conserved Y′ region of yeast telomeres, supplied by V. Zakian, was nick translated (GIBCO BRL) and used as a probe (Chan and Tye, 1983). This probe overlaps the XhoI site and thus hybridizes to fragments both telomere-proximal and telomere-distal to the restriction site. Most yeast telomeres contain the Y′ region (Walmsley et al., 1984).

A strain (UCC1001) with the *URA3* gene located in the telomeric region of the left arm of chromosome VII was provided by D. Gottschling (Renauld et al., 1993). For this experiment, *SIR4* had to be disrupted with *LEU2* (Ivy et al., 1986) to generate BKy109. Either the wild-type *SIR4* gene or *sir4-42* was reintroduced into BKy109 on a *TRP1*-marked *ARS-CEN* vector (pRS314) (Sikorski and Hieter, 1989). UCC1001 and BKy109 were transformed with pRS314 as a control. All media used in the experiment lacked trytophan to maintain selection for the plasmids.

Statistical Analysis

Deterimination of the significance of differences in mean life span between two strains was performed using the nonparametric Wilcoxen signed rank test (Systat5 Statistical Software, Systat, Incorporated). Whenever the mean life spans of two strains are said to be statistically significant, the analysis showed a confidence level greater than 99%.

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