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Calorie Restriction Effects on Silencing and Recombination at the Yeast rDNA

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Summary

Aging research has developed rapidly over the past decade, identifying individual genes and molecular mechanisms of the aging process through the use of model organisms and high throughput technologies. Calorie Restriction (CR) is the most widely researched environmental manipulation that extends lifespan. Activation of the NAD^+-dependent protein deacetylase Sir2 (Silent Information Regulator 2) has been proposed to mediate the beneficial effects of CR in the budding yeast *S. cerevisiae*, as well as other organisms. Here we show that in contrast to previous reports, Sir2 is not stimulated by CR to strengthen silencing of multiple reporter genes in the rDNA of *S. cerevisiae*. CR does modestly reduce the frequency of rDNA recombination, although in a *SIR2*-independent manner. CR-mediated repression of rDNA recombination also does not correlate with the silencing of Pol II-transcribed non-coding RNAs derived from the rDNA intergenic spacer, suggesting that additional silencing-independent pathways function in lifespan regulation.

Keywords

Sir2; aging; calorie restriction; silencing; recombination; rDNA; yeast

Introduction

Calorie restriction (CR) extends both average and maximal life span in most organisms tested, suggesting that the fundamental processes of aging are being broadly delayed as opposed to adjusting only specific causes of early death. Although this effect of age delay has been known for over 70 years (McCay & Crowell 1934; McCay *et al.* 1935), the molecular basis for CR's effects on aging has only recently been studied and remains largely unclear. Much of the research in understanding the mechanisms of CR has been performed in model organisms, including the budding yeast *S. cerevisiae*. Reduction of calories by decreasing the level of glucose, the primary carbon source in yeast growth media, results in an increase in both replicative life span (RLS) and chronological life span (CLS) (Jiang *et al.* 2000; Lin *et al.* 2000; Kaeberlein *et al.* 2002; Smith *et al.* 2007). Previous research has shown that deletion of the *SIR2* gene shortens RLS, while *SIR2* over-expression extends RLS (Kaeberlein *et al.* 1999). Sir2 is an NAD^+-dependent histone deacetylase required for heterochromatic gene silencing in yeast (Imai *et al.* 2000; Landry *et al.* 2000b), and is the

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founding member of a large family of proteins called the Sirtuins (Brachmann *et al.* 1995; Frye 1999). Deleting *SIR2* causes genomic instability in the ribosomal DNA (rDNA) array of yeast where homologous recombination is normally suppressed by the enzymatic activity of Sir2 (Gottlieb & Esposito 1989). In the absence of Sir2, increased recombination leads to high levels of extra-chromosomal rDNA circles (ERCs) (Kaeberlein *et al.* 1999), which are causative in the senescence of old mother cells (Sinclair & Guarente 1997).

Early experiments in yeast showed that *SIR2* was required for CR-induced RLS extension (Lin *et al.* 2000), suggesting that CR stimulates Sir2 activity. Supporting this model, transcriptional silencing assays with the repressible *MET15* reporter gene integrated within the rDNA locus indicated that CR strengthened the silencing in a *SIR2*-dependent manner (Lin *et al.* 2002). This type of silencing in the rDNA (rDNA silencing) is highly responsive to increased *SIR2* gene dosage (Fritze *et al.* 1997; Smith *et al.* 1998). One model for the stimulation of Sir2 activity by CR proposes that the cellular NAD^+/NADH ratio is altered by a CR-induced shift from fermentation toward respiration, causing a relative increased abundance of NAD^+ (Lin *et al.* 2002; Lin *et al.* 2004). CR does increase the NAD^+/NADH ratio in yeast cells, but not through an increase in NAD^+. Rather, there is a decrease in the NADH concentration (Lin *et al.* 2004). Sir2 was reported to be inhibited by NADH (Lin *et al.* 2004), so the stimulation in Sir2 activity could actually be a relief of inhibition. A different model suggests that CR induces expression of the nicotinamidase protein Pnc1 (Anderson *et al.* 2003), which converts nicotinamide (NAM) to nicotinic acid as part of the NAD^+ salvage pathway (Ghislain *et al.* 2002; Anderson *et al.* 2003; Gallo *et al.* 2004). NAM is a strong non-competitive inhibitor of Sirtuins that is also a byproduct of the NAD^+-dependent deacetylase reaction (Landry *et al.* 2000a; Tanny & Moazed 2001; Bitterman *et al.* 2002). Therefore, increased Pnc1 would prevent the accumulation of excess NAM, thus promoting Sir2 activity (Anderson *et al.* 2003; Gallo *et al.* 2004). While different, these two models are not necessarily mutually exclusive.

SIR2 homologues have also been suggested to function in regulating lifespan and mediating the CR response in *C. elegans* and *D. melanogaster* (Tissenbaum & Guarente 2001; Rogina & Helfand 2004). While the role of specific Sirtuins in modulating lifespan appears to be conserved from yeast to metazoans, the role of Sir2 in mediating the extension of lifespan caused by CR in yeast has been controversial. Experiments from some labs have challenged the requirement of *SIR2* in mediating the effect of CR in yeast RLS extension (Jiang *et al.* 2002; Kaeberlein *et al.* 2004), as well as in worms (Kaeberlein *et al.* 2006b; Lee *et al.* 2006; Hansen *et al.* 2007; Schulz *et al.* 2007). Additional studies have shown that in CLS, the presence or absence of *SIR2* has little effect on CLS during “moderate” CR (Fabrizio *et al.* 2005; Smith *et al.* 2007), and *sir2Δ* mutants actually have a very long lifespan during “extreme” CR (Fabrizio *et al.* 2005). Thus, it appears that in yeast CLS and RLS, there exists a *SIR2*-independent/ERC-independent aging pathway that is sensitive to calorie restriction by glucose limitation. To further shed light on the mechanism of CR modulation of lifespan, we have investigated the ability of CR to increase Sir2 activity in yeast using rDNA silencing and recombination as indicators.

Results

Effects of CR on rDNA silencing of a MET15 reporter gene

The exquisite sensitivity of rDNA silencing to Sir2 levels implies that it should be an excellent tool for monitoring the effect of CR on Sir2 activity. We first analyzed the effect of lowering the glucose concentration on the silencing of a *Ty1-MET15* reporter gene integrated into the rDNA non-transcribed spacer (NTS2) (Smith & Boeke 1997). Ty1 is a retrotransposon that carried *MET15* into the rDNA of this strain (see schematic in Fig. 1). *MET15* encodes an O-acetyl homoserine-O-acetyl serine sulfhydrylase required for sulfur

amino acid synthesis. In the presence of Pb^{2+} ions, *met15Δ* strains produce a characteristic dark brown pigment, and *MET15*$^{+}$ strains do not produce the pigment (Cost & Boeke 1996). Silencing of *MET15* in the rDNA results in an intermediate tan colony color (Smith & Boeke 1997). Eliminating silencing by deletion of *SIR2* changes the colony color to white, whereas strengthening silencing by deletion of *SIR4* results in a darker shade of tan (Smith & Boeke 1997). As expected (Smith & Boeke 1997), deletion of *SIR4* caused a darker tan color than WT, indicating that the plates were sensitive to improved silencing of *MET15* on both 2% and 0.5% glucose (Fig. 1). Surprisingly, reducing the glucose concentration from 2% to 0.5% (the typical CR condition) had no measurable effect on the colony color when *MET15* was positioned in the rDNA (Fig. 1). The typical CR condition of 0.5% glucose, therefore, does not appear to have any effect on silencing of *MET15*. This is in contrast to an earlier report that detected a darker colony color with this reporter at 0.5% glucose (Lin *et al.* 2002).

In some yeast replicative lifespan studies, it was reported that glucose concentrations lower than 0.5% were needed to reliably detect CR-mediated extension of lifespan (Jiang *et al.* 2000; Kaeberlein *et al.* 2004). In the case of *MET15*, reducing the glucose to 0.2% clearly resulted in a darker colony color for the WT JS325 strain, suggestive of stronger silencing (Fig. 1). However, the darker color was not dependent on *SIR2*, and also developed when *MET15* was located at a non-rDNA locus that is not subject to *SIR2*-dependent silencing (Fig. 1). This result suggested that the *MET15* reporter gene was potentially transcriptionally repressed by very low glucose concentrations, but the repression was not specific to the rDNA locus. Therefore, the *MET15* reporter system may not be reliable for measuring silencing under CR conditions.

Effects of CR on the rDNA silencing reporter gene mURA3

The development of a dark brown colony color at a 0.2% glucose concentration for all strains in Fig. 1 could also indicate an indirect metabolic effect on pigment formation, rather than a transcriptional effect. To test this possibility, we utilized a different rDNA silencing reporter gene called *Ty1-mURA3* that is also highly sensitive to changes in Sir2 dosage or activity (Smith *et al.* 1998; Sauve *et al.* 2005). The *mURA3* reporter is a modified *URA3* gene in which the promoter has been replaced with a *TRP1* promoter (Smith & Boeke 1997). Silencing in these strains can be easily measured by spotting 5-fold serial dilutions of cells onto SC-ura plates. Improved silencing of the *Ty1-mURA3* reporter results in diminished growth on SC-ura. Strains with *Ty1-mURA3* integrated within an rDNA gene at NTS1 or NTS2 were compared to a control strain with *Ty1-mURA3* integrated at a non-rDNA chromosomal position, and to congenic strains where *SIR2* was deleted (Fig. 2). The strains were all spotted onto SC and SC-ura plates containing 2%, 0.5%, 0.2%, or 0.05% glucose. It should be noted that each of the reduced glucose concentrations tested have previously shown some benefit to RLS (Jiang *et al.* 2000; Kaeberlein *et al.* 2002), as well as to CLS (Smith *et al.* 2007). Slight decreases in growth were observed for the NTS2 and NTS1 positions on SC-ura plates when the glucose concentration was lowered (Fig. 2). However, this difference was accounted for by a progressive reduction in colony growth on the SC control plates (Fig. 2), suggesting that silencing was not actually being strengthened. Similar results were observed with the *Ty1-mURA3* reporter using a different strain background (BY384) related to BY4741/42 (data not shown), indicating that this was not a strain specificity issue. This result also suggests that the effect of CR on *MET15* that caused a darker colony color in Fig. 1 was most likely not transcriptional.

We next tested whether CR induces the spreading of rDNA silencing through the use of a *mURA3-HIS3* cassette integrated 50bp left (50L) of the rDNA tandem array, within unique chromosome XII sequence (Buck *et al.* 2002). Unlike genes integrated within the tandem array, the *RDN1(50L)::mURA3-HIS3* cassette is not subject to high recombination

frequencies (Buck *et al.* 2002), allowing the reliable use of 5-FOA to detect improved silencing of *mURA3* as in indicator of spreading (McClure *et al.* 2008). 5-FOA is a uracil analog that kills cells expressing the Ura3 protein (Boeke *et al.* 1984). The induction of spreading by *SIR2* overexpression strengthens *mURA3* silencing such that growth is permitted on FOA (McClure *et al.* 2008). As expected, introduction of a *2μ SIR2/LEU2* plasmid (pSB766) resulted in strong growth on FOA with this reporter, serving as a positive control for the spreading phenotype (Fig. 3A, *2μ SIR2* rows). However, reducing the glucose concentration had no effect on either the SC-leu-ura, or SC-leu FOA plates (Fig. 3A, vector rows), indicating that CR did not induce the spreading of rDNA silencing. The polyphenolic compound, resveratrol, has been reported as a small molecule activator of Sir2 and other Sirtuins (Howitz *et al.* 2003). We therefore tested whether the addition of resveratrol would strengthen rDNA silencing in combination with CR. Using the *RDN1(50L)::mURA3-HIS3* reporter cassette again, 100 μM resveratrol had no effect on rDNA silencing regardless of the glucose concentration. (Supplemental Fig. 1A).

In yeast, there is a limiting pool of Sir2 shared between the rDNA, telomeres and silent mating loci (Buck & Shore 1995; Smith *et al.* 1998), raising the possibility that the Sir2 protein pool is normally fully activated, making any potential positive effect by CR on silencing undetectable. Therefore, we hypothesized that increased *SIR2* dosage would promote the enhancement of silencing by CR. However, as shown in Fig. 3A, the addition of a *2μ SIR2* plasmid strengthened *mURA3* silencing at the 50L position so much that no growth was observed on the SC-leu-ura plates. Thus, although there were no differences in silencing with reduced glucose concentrations, the assay was already at the detection limit even at the 2% glucose level. Therefore, we also measured the effect of a *2μ SIR2* plasmid on silencing of a *LEU2* reporter integrated within the tandem array. This marker is not silenced as well as *mURA3* (Smith & Boeke 1997), allowing for added strengthening of repression by CR. *SIR2* overexpression improved silencing of *LEU2* as expected, but reducing the glucose concentration did not improve the silencing any further (Fig. 3B). If anything, there could be slight depression. Together, these results show that in our assays we can easily detect Sir2-mediated improvements in rDNA silencing, but never CR-mediated improvements.

Evaluating the utility of TPE as an indicator of Sir2 activity

The effect of *SIR2* overexpression on telomere position effect (TPE) has been less clear than with rDNA silencing, with one study reporting no effect (Renauld *et al.* 1993), and at least two reporting improved silencing (Cockell *et al.* 2000; Kaeberlein *et al.* 2005a). To evaluate the utility of TPE as an indicator of Sir2 activity levels, we transformed high copy *LEU2/SIR2* (pSIR2μ) or *LEU2/SIR3* (pLP304) plasmids into a reporter strain that contains a telomeric *URA3* gene. *SIR3* overexpression was already known to strengthen TPE (Renauld *et al.* 1993), and was used as a positive control in the assay. Improved TPE was indicated by less colony growth on SC plates lacking uracil (SC-leu-ura) and better growth on SC containing 5-FOA). As predicted, the *SIR3* control plasmid strengthened TPE (Fig. 3C). However, the *SIR2* plasmid actually weakened TPE, as indicated by less growth on FOA compared to the empty vector control (Fig. 3C). As a result of this finding, we concluded that TPE is not a consistent indicator of Sir2 activity. Similarly, Alan Morgan and coworkers have also determined that CR does not strengthen TPE (personal communication).

CR represses rDNA recombination independently of SIR2

The above results with rDNA silencing do not support a model for Sir2 activation by CR growth conditions. However, Sir2 is also involved in repressing recombination within the rDNA (Gottlieb & Esposito 1989), leaving open the possibility that stimulation of Sir2 activity by CR could improve the repression of rDNA recombination without strengthening

rDNA silencing. Such a model is supported by the previous identification of genes that differentially affect rDNA silencing and recombination when mutated, including *SET1*, *FOB1*, and *HHO1* (Bryk *et al.* 2002; Huang & Moazed 2003; Li *et al.* 2008). To evaluate the effect of CR on rDNA recombination, WT, *cac1Δ*, and *sir2Δ* strains containing the *mURA3-HIS3* cassette within the 35S transcribed region of the rDNA were used to measure the frequency of *HIS3* marker loss on various glucose concentrations by a replica-plating assay for ½ sectored colonies on SC-his media. The frequency of rDNA recombination in a WT strain is quite low, so the *cac1Δ* mutation was chosen to increase the recombination frequency (Smith *et al.* 1999), thus making it easier to detect any decrease in the frequency caused by CR. As shown in Fig. 4A, the *cac1Δ* and *sir2Δ* mutations both increased the frequency of recombination compared to WT when grown on 2% glucose. The typical CR condition (0.5% glucose) modestly reduced the recombination frequency for each strain, including the *sir2Δ* mutant, by ~2-fold (Fig. 4A). Stronger CR (0.05% glucose) further reduced the recombination frequency in both mutants by ~5-fold (Fig. 4A). To confirm these results we also measured recombination frequency with an independent strain set that contained the *Ty1-MET15* reporter within NTS2. In this case, the frequency of ½ sectored colonies (white/brown) grown on Pb^{2+}-containing plates was calculated for WT, *sir2Δ*, and *sir2Δ fob1Δ* strains (Fig. 4B). Again, the recombination frequency was greatly elevated on 2% glucose by the *sir2Δ* mutation and repressed by ~2-fold on 0.5% glucose. The 0.05% glucose concentration blocked colony growth on Pb^{2+} plates, so this recombination data from this condition was not obtained. Similar to the silencing results, resveratrol did not suppress rDNA recombination using this assay (Supplemental Fig. 1B). Deletion of *FOB1* was already known to partially block the hyper recombination caused by a *sir2Δ* mutation (Kaeberlein *et al.* 1999), which we also observed in the *sir2Δ fob1Δ* double mutant. These results support a model in which CR suppresses rDNA recombination without affecting the transcriptional silencing of Pol II-transcribed reporter genes, although the suppression does not depend on *SIR2*.

Effects of CR on endogenous transcripts produced from the rDNA NTS

One of the current models for Sir2-mediated suppression of rDNA recombination involves the silencing of a bidirectional Pol II promoter within NTS1 called E-pro (Kobayashi & Ganley 2005). Deletion of *SIR2* results in the transcription of E-pro, and the transcription process results in the displacement of cohesin from the NTS (Kobayashi *et al*. 2004; Kobayashi & Ganley 2005). The loss of cohesin stimulates unequal crossing over between the sister chromatids and a subsequent change in rDNA copy number. It was therefore possible that CR suppressed rDNA recombination in our assays by improving the silencing of transcription derived from E-pro or other Pol II-derived transcripts recently discovered from NTS1 and NTS2 (Li *et al.* 2006). To test this possibility, we utilized Northern blotting to measure the steady state level of each NTS transcript from WT and *sir2Δ* strains grown in 2%, 0.5%, 0.2%, and 0.05% glucose. These RNAs are transcribed from both the top and bottom strands of NTS1 and NTS2 (Fig. 5A). As shown in Fig. 5B for the NTS1-top RNA species, deletion of *SIR2* greatly increases the expression due to a loss of silencing. Similar results were observed for the other three transcripts (data not shown). Although silencing of these transcripts depends on Sir2, CR did not strengthen their repression. Instead, for each of the four RNAs, CR conditions either had no effect or may have slightly elevated their expression (Fig. 5C). CR did not further increase the expression in the *sir2Δ* background compared to 2% glucose (Fig. 5C). This data strongly suggests that CR does not suppress rDNA recombination by improving the silencing of non-coding transcripts from the NTS.

Discussion

Understanding the molecular mechanism of CR could provide a great benefit in combating age-related degeneration and disease. The highly researched *SIR2* pathway has been proposed to mediate the lifespan effects of CR in yeast, worms, flies and even rodents (Lin *et al.* 2000; Tissenbaum & Guarente 2001; Lin *et al.* 2002; Anderson *et al.* 2003; Rogina & Helfand 2004; Chen *et al.* 2005; Bordone *et al.* 2007; Boily *et al.* 2008). Sir2 and other Sirtuins have clearly been implicated in longevity control of yeast and the other model organisms, but the data presented here challenge the initial interpretation that Sir2 activity at the rDNA locus is increased by CR in yeast. By tracking commonly used rDNA reporter genes, as well as endogenous non-coding RNAs from the "non-transcribed spacer", we were unable to detect improved Sir2-dependent silencing as a result of CR growth conditions. These findings are consistent with previous RLS and CLS studies showing lifespan extension by CR even in the absence of *SIR2* (Kaeberlein *et al.* 2004; Fabrizio *et al.* 2005; Smith *et al.* 2007). It has been proposed that the Sir2 homolog, Hst2, can partially substitute for Sir2 in suppressing recombination to extend lifespan in response to CR (Lamming *et al.* 2005), although a separate study showed that CR of varying glucose concentrations extends RLS even in a *fob1Δ sir2Δ hst1Δ hst2Δ* quadruple mutant (Kaeberlein *et al.* 2006a). While we have not tested the effects of the other sirtuins on recombination in this study, it is clear that CR can suppress recombination independently of Sir2 and in the absence of enhanced Pol II silencing within the rDNA. If CR does stimulate Sir2 in yeast cells, then a novel mechanism would have to be invoked in which the pool of Sir2 utilized for rDNA silencing is not subject to the activation. This seems unlikely because activation of Sir2 *in vivo* by the addition of isonicotinamide generally strengthens *HM*, telomeric, and rDNA silencing (Sauve *et al.* 2005). Alternatively, one or more of the other Sirtuins could become engaged at the rDNA without the need to modulate Sir2 activity.

At least three previously published experiments have used silencing as a read-out of Sir2 activation. The first study used a *MET15* reporter to show that CR strengthened rDNA silencing to produce a darker colony color (Lin *et al.* 2002). We did not observe a darker colony color with "moderate" CR of 0.5% glucose, but found that "extreme" CR conditions below 0.5% glucose result in a dark colony color that does not depend on *SIR2* or location in the rDNA (Fig. 1). CR either causes metabolic effects that affect colony color development with this system that are variable according to differences in media formulation, or causes a general transcriptional repression of the *MET15* promoter regardless of its genomic location. The other two studies measured telomeric silencing of *ADE2* or *URA3* reporters to show that neither resveratrol nor CR improved Sir2 activity in TPE (Kaeberlein *et al.* 2005a; Kaeberlein *et al.* 2005b). Overexpression of *SIR2* strengthened repression of the telomeric *ADE2* reporter, but did not extend RLS in the PSY316 strain background (Kaeberlein *et al.* 2005a). Our *SIR2* overexpression data in Fig. 3C using a strain background related to BY4741/42 and S288C did not demonstrate improved TPE, which may suggest that the sensitivity of TPE to Sir2 activity is specific to particular reporter genes, strain backgrounds, or even specific levels of *SIR2* overexpression (Cockell *et al.* 2000). In contrast, *SIR2* overexpression has universally resulted in stronger silencing at the rDNA regardless of the strain background, reporter gene, or level of *SIR2* overexpression (Fritze *et al.* 1997; Smith *et al.* 1998; Smith *et al.* 2007). We propose that rDNA silencing is better suited than TPE in detecting improved Sir2 activity. Despite using rDNA silencing as the reporter assay, we were also unable to detect any effect of resveratrol, even in combination with CR growth conditions.

One of the potential limitations of using reporter genes such as *mURA3* or *LEU2* as a readout for Sir2 stimulation is that small differences might be difficult to detect due to the qualitative nature of the 5-fold dilution spot assays. However, previous studies have shown

that even one extra copy of *SIR2* produces an easily visible enhancement of rDNA silencing using the 5-fold spot assays (Smith *et al.* 1998; Smith *et al.* 2007). Using 2-fold serial dilutions have not resulted in any improved resolution because colony size also contributes to the readout (data not shown). Additionally, quantitative measures of silencing under CR conditions have similarly failed to demonstrate Sir2 activation (personal communication A. Morgan). Finally, in addition to the spot assays for measuring silencing, northern blotting for NTS-derived non-coding RNAs showed that CR does not improve Sir2-mediated repression of these transcripts.

If CR does not stimulate Sir2 for silencing or recombination suppression in yeast cells, then what alternative pathways could be involved in the suppression of recombination by CR and in the extension of lifespan? Inhibition of the TOR signaling pathway has been shown to extend both RLS and CLS in yeast (Kaeberlein *et al.* 2005b; Powers *et al.* 2006; Medvedik *et al.* 2007), and to extend the lifespan of *Drosophila* and *C. elegans* (Vellai *et al.* 2003; Kapahi *et al.* 2004). When nutrients are high, TOR activity is high, and when nutrients are reduced, TOR activity becomes decreased. Accordingly, genetic epistasis experiments in yeast have indicated that TOR acts downstream of CR in the extension of lifespan (Kaeberlein *et al.* 2005b; Powers *et al.* 2006; Medvedik *et al.* 2007). As with CR, there is some debate whether *SIR2* and *HST2* are required for the TOR inhibition-mediated extension of yeast RLS (Kaeberlein *et al.* 2005b; Medvedik *et al.* 2007). At least for CLS, it has been reported that the effect of reducing TOR signaling is through increased respiration and upregulation of mitochondrial gene expression (Bonawitz *et al.* 2007). Alternatively, the effects could be mediated by a decrease in translation capacity (Hansen *et al.* 2007; Steffen *et al.* 2008). Interestingly, deletion of TOR pathway genes or the addition of low concentrations of rapamycin both cause a reduction in the frequency of rDNA recombination ((Medvedik *et al.* 2007); A. Morgan, personal communication), suggesting that even if Sir2 is not mediating the effect, the control of rDNA circles could still be involved in the extension of RLS in a WT strain background. Of particular interest is the recent finding that positioning of the rDNA array near the inner nuclear membrane (INM) is required for maintaining the stability of the rDNA repeats, but not for rDNA silencing of reporter genes (Mekhail *et al.* 2008). CR could potentially enhance this association with the INM. In conclusion, CR does not appear to activate Sir2 at the rDNA or telomeres in *Saccharomyces cerevisiae*. Additional effort will be needed to uncover the molecular details of CR-mediated replicative and chronological longevity.

Experimental Procedures

Yeast strains and media conditions

All strains used for rDNA silencing were derived from the GRF167/JB740 background commonly used for Ty1 transposition and rDNA silencing assays (Smith & Boeke 1997), and listed in Table 1. Synthetic Complete (SC) and Yeast Extract/Peptone/Dextrose (YPD) media were formulated as previously described (Burke *et al.* 2000). Strains were transformed with plasmids where indicated, including the empty *2μ LEU2* plasmid (pRS425), *2μ LEU2 SIR2* plasmids (pSIR2μ or pSB766), and *2μ LEU2 SIR3* plasmid (pLP304). In Fig. 3B, an empty *2μ HIS3* plasmid (pRS423) and *2μ HIS3 SIR2* plasmid (pJSS71-13) were used because *LEU2* was the silencing reporter.

Silencing and recombination assays

For the *MET15* reporter strains, cells were patched onto YPD plates and allowed to grow overnight at 30°C. Cells were then streaked onto YPD plates containing ½ the normal concentration of Bacto peptone and Bacto yeast extract, and lead nitrate at 0.1% lead nitrate. Using ½ concentrated YPD (0.5xYPD) improved color development compared to typical

YPD (data not shown). Glucose concentrations ranged from 2% to 0.05%. The lead plates were then incubated at 30°C for 5 days before photographs were taken. For spot test silencing assays, cells were patched onto the appropriate SC plates containing the indicated glucose concentration and/or missing the indicated amino acids or uracil. After overnight growth at 30°C, the cells were serially diluted in 5-fold increments in 96-well plates and then 5 μl spotted onto the indicated plates. For recombination assays, cells were pre-grown overnight in SC media containing either 2% or 0.5% glucose. JS306, JS400, and JS576 were diluted in water and plated onto YPD containing 2% or 0.5% glucose, and allowed to grow into colonies for 2 days (average of ~200 colonies per plate). The colonies were then replica plated onto SC and SC-his to identify ½ sectored colonies where only half the colony grew on SC-his. The number of half-sectored colonies was divided by the total number of His^+ or sectored colonies, and multiplied by 1000 to give the recombination frequency per 1000 cells. For DSY380, DSY394, and DSY390, the culture dilutions were plated directly onto the 0.1% lead nitrate plates (2% or 0.5% glucose) and allowed to grow for 5 days before the frequency of half sectored (brown/white) colonies was calculated. Resveratrol (Sigma) was added to the media were indicated at a concentration of 100 μM. The recombination assays were performed in triplicate starting from three independent colonies for each strain.

Northern blotting

Yeast strains were grown to saturation in YPD containing 2% glucose and supplemented with tryptophan (0.4 mM) and adenine (40 μg/mL) at 30°C. Cultures were then diluted to a final density of 2.5×10^6 cells/ml in 50 ml of fresh media with 2%, 0.5%, 0.2% or 0.05% glucose. The diluted cultures were grown at 30°C to $2\text{-}3\times10^7$ cells/ml. Total RNA was isolated as described previously (Bryk *et al.* 1997). Northern analyses were performed as described in (Swanson *et al.* 1991). Strand-specific ^{32}P-labeled RNA probes used to detect NTS transcripts are described in (Li *et al.* 2006). An *ACT1* (+564 to +1200) probe was made by PCR amplification of yeast genomic DNA, purified from an agarose gel and labeled with [α-^{32}P]-dATP by random priming (Ausubel *et al.* 2000). Northern blots were quantified on a GE Healthcare Storm 860 PhosphorImager by using ImageQuant software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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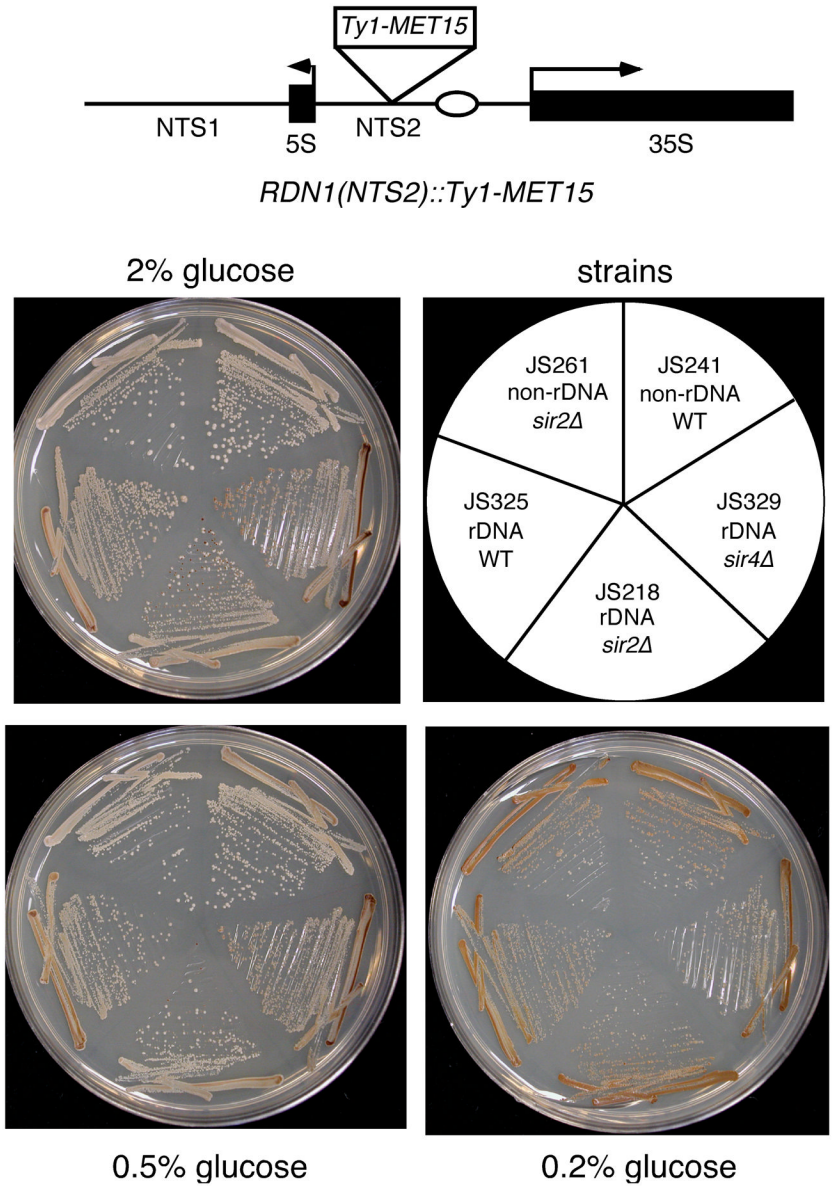

Fig. 1.

CR effects on silencing of a *MET15* reporter integrated into the NTS2 region of an rDNA repeat. The indicated strains were streaked for single colonies onto 0.5xYPD plates containing 0.1% lead nitrate and either 2%, 0.5%, or 0.2% glucose. Darker colony color indicates better rDNA silencing of *MET15*. Colonies were grown for 5 days.

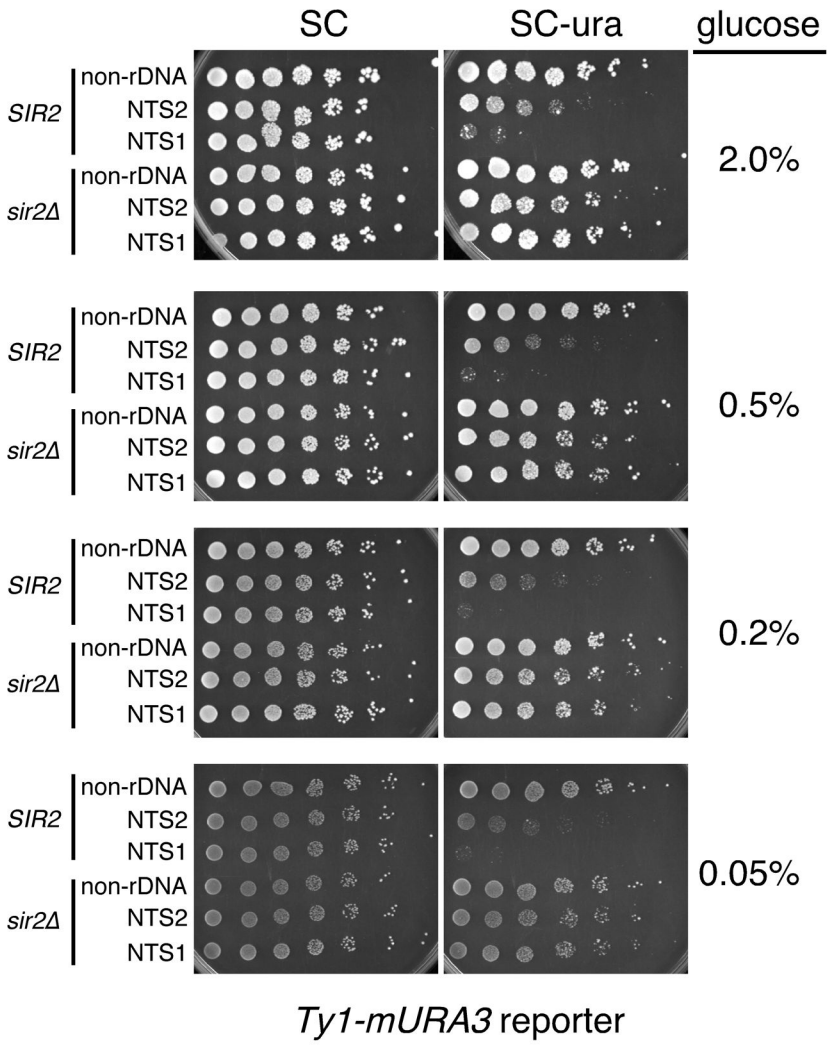

Fig. 2.

CR effects on silencing of a *Ty1-mURA3* marker within the tandem array. Strains with *Ty1-mURA3* integrated within the tandem array at NTS2 (JS125) and NTS1 (JS128), or at a non-rDNA genomic location (JS122) were spotted as 5-fold serial dilutions onto SC and SC-ura plates containing 2%, 0.5%, 0.2%, or 0.05% glucose. The *sir2Δ* control strains were JS151 (non-rDNA), JS155 (NTS2), and JS163 (NTS1). Plates were incubated for 3 days prior to photography.

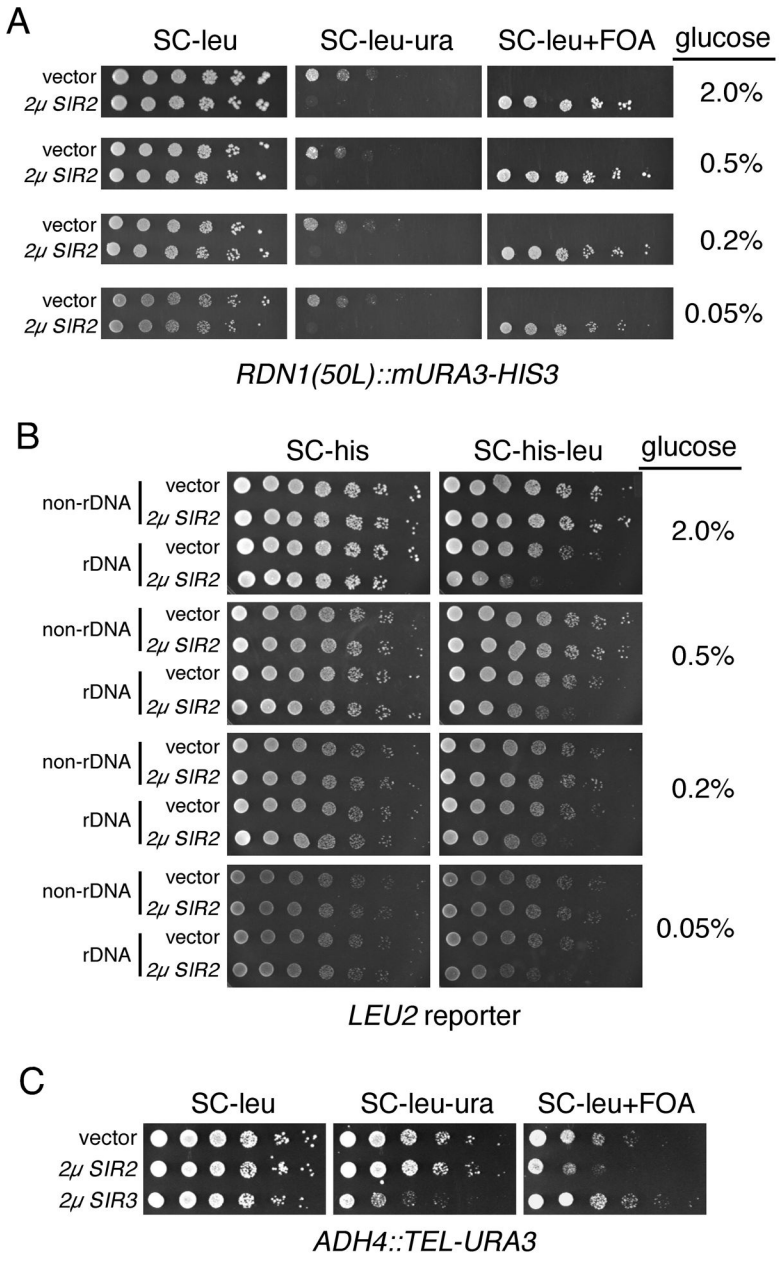

Fig. 3.

SIR2 overexpression strengthens rDNA silencing, but CR does not. A) YSB348 was transformed with either an empty *2μ LEU2* vector (pRS425) or a *SIR2 2μ LEU2* plasmid (pSB766). YSB348 contains a *mURA3-HIS3* silencing reporter cassette integrated within unique chromosome XII sequence just 50 bp to the left (centromere proximal) of the tandem array (Buck *et al.* 2002). All plates lacked leucine to select for the plasmids, and contained the indicated glucose concentration. B) JS210 and JS215 were transformed with either an empty *2μ HIS3* vector (pRS423) or a *SIR2 2μ HIS3* plasmid (pJSS71-13). JS215 contains *LEU2* integrated within the rDNA, and JS210 contains *LEU2* outside the rDNA as a control (Smith & Boeke 1997). The SC plates lacked histidine to select for the plasmids. Silencing of *LEU2* is indicated by poor growth on SC-his-leu. C) Telomere silencing assay showing the effects of overexpressing *SIR2* (strain JS717) or *SIR3* (strain JS718) compared to an

empty vector (strain JS716). These strains contain a *URA3* marker integrated at the *ADH4* gene, and flanked by a telomere. The plates lacked leucine to select for the *LEU2*-containing plasmids.

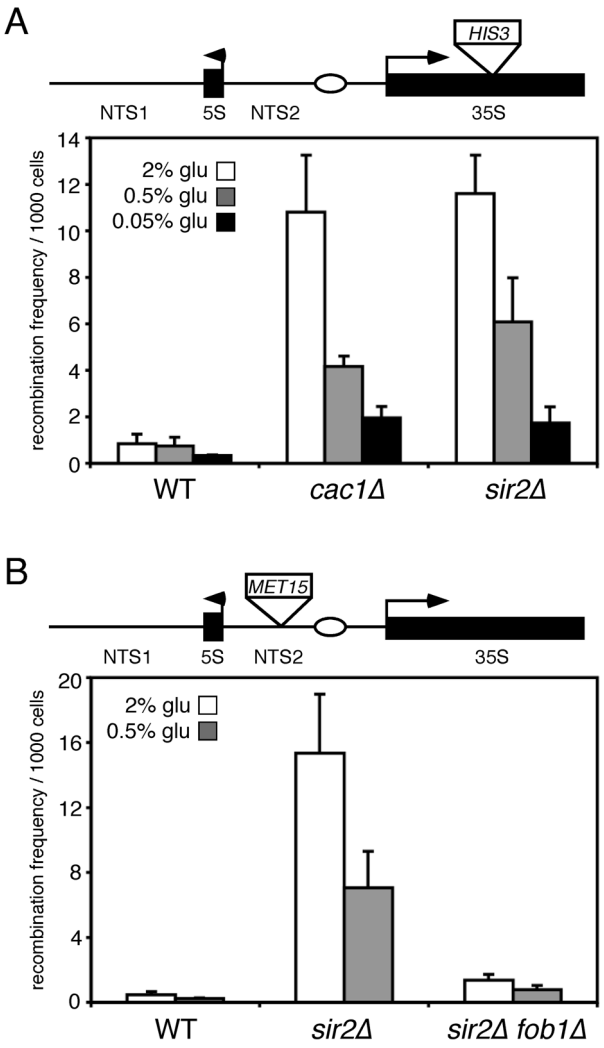

Fig. 4.

CR suppresses rDNA recombination independently of *SIR2*. A) Recombinational loss of *HIS3* from the rDNA was measured by calculating the frequency of half-sectored His^+/His^- colonies per 1000 cells. Strains tested were JS306 (WT), JS400 (*cac1Δ*), and JS576 (*sir2Δ*). Strains were grown in SC media containing either 2%, 0.5%, or 0.05% glucose. The schematic diagram indicates the position of *HIS3* (as part of the *mURA3-HIS3* cassette) in the 35S transcribed region of an rDNA repeat. The number of colonies for each replicate ranged from ~2000 to 6000. B) Recombinational loss of *MET15* from the rDNA was measured by calculating the frequency of half-sectored brown/white colonies per 1000 cells. Strains tested were DSY380 (WT), DSY394 (*sir2Δ*), and DSY390 (*sir2Δ fob1Δ*). Lead plates contained 2% or 0.5% glucose. The schematic diagram indicates the position of *MET15* (as part of the *Ty1-MET15* cassette) in the NTS2 region of an rDNA repeat. The number of colonies for each replicate ranged from ~4000 to 12000. Error bars in both panels represent the standard deviation between biological replicates.

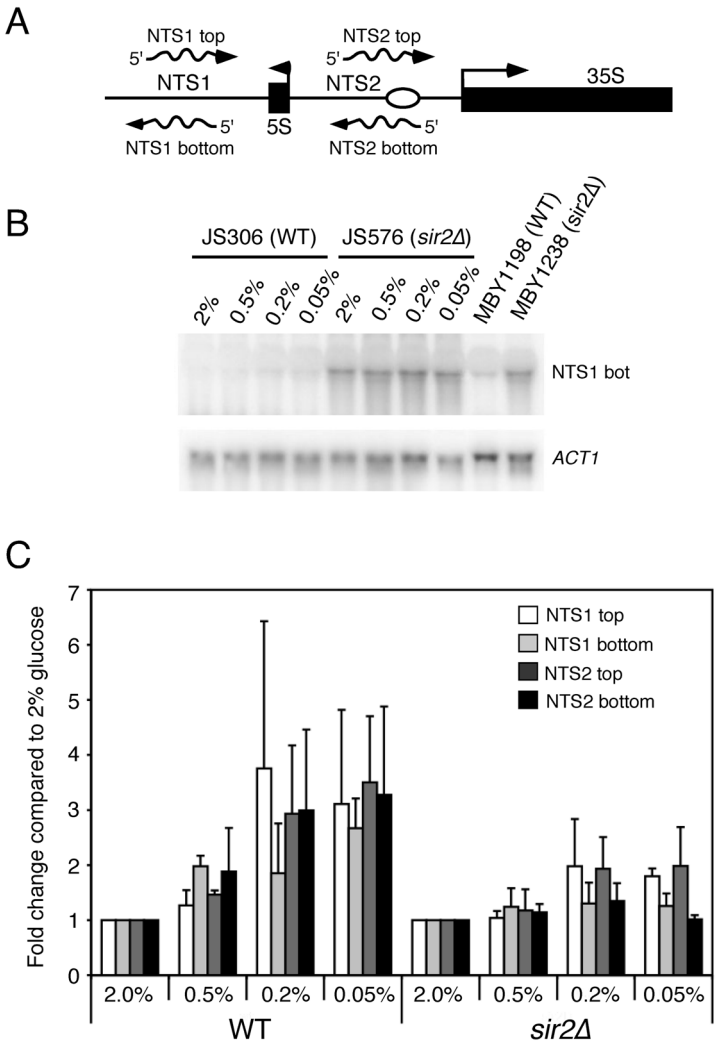

Fig. 5.

Analyzing CR effects on the silencing of non-coding RNAs derived from the NTS1 and NTS2 regions of the rDNA. A) Schematic diagram depicting the Pol II-driven expression of non-coding RNAs from both strands of NTS1 and NTS2. B) Representative Northern blot showing silencing of the NTS1-bottom strand RNA by Sir2. Note the large increase in expression in the *sir2Δ* mutants JS576 and MBY1238. Expression level of *ACT1* was used as a loading control. C) Quantitation of CR effects on the expression of the non-coding RNAs. Since the *sir2Δ* mutant JS576) caused a large increase in expression for each RNA, we normalized the expression level at 2% glucose to 1.0 for both WT and *sir2Δ*. Changes in expression relative to 2% glucose are shown for both WT (JS306) and *sir2Δ* (JS576). Standard deviations from 3 independent experiments are shown.

Table 1

Yeast strains used in the study.

Strain	Genotype
DSY380	*MAT***a** *his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1(18S)::mURA3-HIS3 RDN1(NTS2)::Ty1-MET15*
DSY390	DSY390 *sir2Δ::kanMX4*
DSY394	DSY380 *sir2Δ::kanMX4 fob1Δ::kanMX4*
JS122[a]	*MATα his3Δ200 leu2Δ1 ura3-167 ????::Ty1-mURA3*[b]
JS125[a]	*MATα his3Δ200 leu2Δ1 ura3-167 RDN1(NTS2)::Ty1-mURA3*
JS128[a]	*MATα his3Δ200 leu2Δ1 ura3-167 RDN1(NTS1)::Ty1-mURA3*
JS151[a]	JS122 *sir2Δ::HIS3*
JS155[a]	JS125 *sir2Δ::HIS3*
JS163[a]	JS128 *sir2Δ::HIS3*
JS210[a]	*MATα his3Δ200 trp1 Δ63 ura3-167 leu2Δ1::pJSS60-2*[c]
JS215[a]	*MATα his3Δ200 trp1 Δ63 ura3-167 leu2Δ1 RDN1(35S)::pJSS60-2*[c]
JS218[a]	*MATα his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1(NTS2)::Ty1-MET15 sir2Δ::HIS3*
JS241[a]	*MATα his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 ????::Ty1-MET15*[b]
JS261	JS241 *sir2Δ::HIS3*
JS306[d]	*MAR***a** *his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1(18S)::mURA3-HIS3 RDN1(NTS2)::Ty1-MET15*
JS325[d]	*MATα his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1(NTS2)::Ty1-MET15*
JS329[d]	*MATα his3Δ200 leu2Δ1 met15Δ0 trp1Δ63 ura3-167 RDN1(NTS2)::Ty1-MET15 sir4Δ::HIS3*
JS400[d]	JS306 *cac1Δ::kanMX4*
JS576[d]	JS306 *sir2Δ::kanMX4*
JS716	*MAT***a** *his3Δ200 lys2Δ202 trp1Δ63 leu2Δ1::TRP1 ura3-52 ADH4::URA3-TEL* +*p*RS425 (2μ *LEU2* empty)
JS717	*MAT***a** *his3Δ200 lys2Δ202 trp1Δ63 leu2Δ1::TRP1 ura3-52 ADH4::URA3-TEL* +*p*SIR2μ (2μ *LEU2 SIR2*)
JS718	*MAT***a** *his3Δ200 lys2Δ202 trp1Δ63 leu2Δ1::TRP1 ura3-52 ADH4::URA3-TEL* +pLP304 (2μ *LEU2 SIR3*)
MBY1198[e]	*MAT*α, his*3Δ200, ade2Δ::his*G, *leu2Δ0, ura2Δ0, met15Δ0, trp1Δ63, Ty1his3AI*-236r, Ty1ade2AI-515
MBY1238[e]	MBY1198 *sir2Δ*
YSB348[f]	*MATα his3Δ200 leu2Δ1 ura3-167 RDN1(50L)::mURA3-HIS3*

[a] Strains described in reference (Smith & Boeke 1997).

[b] *Ty1-mURA3* is integrated at a non-rDNA location in the genome.

[c] Plasmid pJSS60-2, containing a *LEU2* marker, was integrated into the genome at the indicated locus.

[d] Strains described in reference (Smith *et al.* 1999).

[e] Strains described in reference (Li *et al*. 2006).

[f] Strain described in reference (Buck *et al*. 2002).