Modulation of Life-span by Histone Deacetylase Genes in *Saccharomyces cerevisiae*

Sangkyu Kim, Alberto Benguria,* Chi-Yung Lai,* and S. Michal Jazwinski †

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

Submitted April 1, 1999; Accepted July 30, 1999
Monitoring Editor: Pam Silver

The yeast *Saccharomyces cerevisiae* has a limited life-span, which is measured by the number of divisions that individual cells complete. Among the many changes that occur as yeasts age are alterations in chromatin-dependent transcriptional silencing. We have genetically manipulated histone deacetylases to modify chromatin, and we have examined the effect on yeast longevity. Deletion of the histone deacetylase gene *RPD3* extended life-span. Its effects on chromatin functional state were evidenced by enhanced silencing at the three known heterochromatic regions of the genome, the silent mating type (*HM*), subtelomeric, and rDNA loci, which occurred even in the absence of *SIR3*. Similarly, the effect of the *rpd3Δ* on life-span did not depend on an intact Sir silencing complex. In fact, deletion of *SIR3* itself had little effect on life-span, although it markedly accelerated the increase in cell generation time that is observed during yeast aging. Deletion of *HDA1*, another histone deacetylase gene, did not result in life-span extension, unless it was combined with deletion of *SIR3*. The *hda1Δ sir3Δ* resulted in an increase in silencing, but only at the rDNA locus. Deletion of *RPD3* suppressed the loss of silencing in rDNA in a *sir2* mutant; however, the silencing did not reach the level found in the *rpd3Δ* single mutant, and *RPD3* deletion did not overcome the life-span shortening seen in the *sir2* mutant. Deletion of both *RPD3* and *HDA1* caused a decrease in life-span, which resulted from a substantial increase in initial mortality of the population. The expression of both of these genes declines with age, providing one possible explanation for the increase in mortality during the life-span. Our results are consistent with the loss of rDNA silencing leading to aging in yeast. The functions of *RPD3* and *HDA1* do not overlap entirely. *RPD3* exerts its effect on chromatin at additional sites in the genome, raising the possibility that events at loci other than rDNA play a role in the aging process.

**INTRODUCTION**

Individual cells of the budding yeast *Saccharomyces cerevisiae* undergo a finite number of cell divisions (Mortimer and Johnston, 1959; Muller et al., 1980). Thus, the life-span of this unicellular eukaryote can be defined as the total number of times the cell divides or the number of daughter cells it produces before dying. Yeast aging is accompanied by many morphological and physiological changes (reviewed in Jazwinski, 1996), including increased cell generation time (Egilmez and Jazwinski, 1989) and sterility (Muller, 1985; Smeal et al., 1996). One of the hallmarks of mammalian cellular senescence is the gradual loss of telomere DNA sequences, as cultures exhaust population doublings. This telomere attrition has been proposed to play a causal role in cellular senescence (Harley et al., 1990; Harley, 1991; Allsopp et al., 1992). In fact, inactivation of telomerase activity in human cells hastened cellular senescence and was accompanied by shortened telomeres (Feng et al. 1995). In contrast, constitutive activation of telomerase maintained telomere length and postponed cellular senescence (Bodnar et al., 1998). In yeast, aging cells do not suffer telomere shortening (D’mello and Jazwinski, 1991); however, another age-related event is associated with telomeres. This is the loss of transcriptional silencing at at least one telomere (Kim et al., 1996). Loss of silencing also has been described at the silent mating type (*HM*) loci of old yeast (Smeal et al., 1996).

Transcriptional silencing is a manifestation of chromosomal position effect, in which a euchromatic gene translocated to a heterochromatic region is expressed in a portion of a cell population, resulting in a mosaic or variegated phenotype (Spofford, 1976; Henikoff, 1990). In the yeast genome, the *HM* loci, telomeres, and the rDNA locus are known to exhibit transcriptional silencing (Klar et al., 1981; Nasmyth et al., 1981; Gottschling et al., 1990; Bryk et al., 1997; Smith and Boeke, 1997; Smith et al., 1998). Efficient transcriptional silencing at the *HM* loci and telomeres requires a number of...
genes. These include SIR1, SIR2, SIR3, SIR4, and RAP1 and genes encoding histones H3 and H4 (Laurenson and Rine, 1992; Loo and Rine, 1995). Increased copy numbers of SIR3, but not of SIR2 or SIR4, resulted in spreading of silenced telomeric domains (Renaut et al., 1993; Hecht et al., 1996; Strahl-Bolsinger et al., 1997). This observation implies that the SIR3 gene product may be a limiting, major structural component of the silencing machinery. SIR2, which is known to suppress meiotic and mitotic recombination involving rDNA repeats (Gottlieb and Esposito, 1989), enhanced rDNA silencing in a dosage-dependent manner (Bryk et al., 1997; Fritz et al., 1997; Smith and Boeke, 1997; Smith et al., 1998). To the contrary, SIR4 inhibited rDNA silencing (Smith and Boeke, 1997; Smith et al., 1998).

Transcriptional silencing is also affected by modification of the core histones by acetylation or deacetylation. Specific histone domains required for efficient silencing are localized of the core histones by acetylation or deacetylation. Specific chromatin functional state and aging, we deleted association with a number of corepressors (reviewed by et al. HDA1 yeast.

yeast. dependent transcriptional silencing at the rDNA locus in the core histones is reversibly catalyzed by histone acetylation or deacetylation. Histones H4 and H3 are homologous to yeast HDAC1 and IME2, which are required for efficient silencing are localized. HDAC2 is also known to affect transcription of various other genes, including repression of HO, TRK2, STE6, PHO5, SPO13, and IME2 (reviewed by Grunstein, 1997; Struhl, 1998). Mammalian histone deacetylase genes HDAC1 and HDAC2, both of which are homologous to yeast RP3D (Taunton et al., 1996; Yang et al., 1996), also mediate transcriptional repression in association with a number of corepressors (reviewed by Grunstein, 1997; Struhl, 1998).

The attenuated silencing observed in old yeast cells suggests a possible connection between chromatin-dependent transcriptional silencing and aging. As an approach to gaining more insight into a possible connection between chromatin functional state and aging, we deleted RP3D, HDA1, SIR2, and SIR3 either singly or in combination, and examined the life-span of the mutants. Chromatin changes were monitored by examination of transcriptional state. Our data show a correlation between life-span and chromatin-dependent transcriptional silencing at the rDNA locus in yeast.

MATERIALS AND METHODS

Yeast Strains and Plasmids

Yeast strains used in this study are listed in Table 1. The diploid YPK4.7 was constructed by “self mating” of a haploid derivative of YPH501 (Sikorski and Hieter, 1989), which was performed by inducing the HO gene (Herskowitz and Jensen, 1991). Haploid segregants of YPK4.7 show no significant differences in mean life-span (Kirchman and Jazwinski, unpublished results).

Genes of interest were disrupted by the “y transformation” procedure (Sikorski and Hieter, 1989). The rp3d deletants were created by replacing 63% of the coding region from the HindIII site (+467) to the EcoRI site (+1292) with pRS306 (the appropriate derivative, pMV130, was provided by Richard Gaber, Northwestern University) (Vidal and Gaber, 1991), pRS403, or pRS404. The pRS series of plasmids has been described (Sikorski and Hieter, 1989). Life-spans were not dependent on the selectable marker.) In strain YAB11, sir2 has an insertion of a PCR-amplified fragment containing UR3 at the BglII site in the coding region at +1023 from the first nucleotide of the translation initiation codon. The hda1 deletants were constructed by replacing 70% of the coding region spanning positions +137 and +2627 with pRS403 or pRS405. To delete SIR3, an ~3-kb Sac–HindIII fragment isolated from plasmid pAR78 (provided by Scott C. Holmes, Princeton University) was used to replace most of the coding region with LEU12. All the deletions/disruptions were confirmed by Southern blot analysis.

Media and Genetic Methods

Yeast media were prepared as described (Rose et al., 1990). The synthetic medium containing 5mM 5-fluoroorotic acid (Gottschling et al., 1990) was prepared as described. Standard genetic methods were used for mating, sporulation, and tetrad analysis (Rose et al., 1990). Transcriptional silencing of TRP1 at the hmr locus or UR3 inserted 2.1 kb from the right telomere of chromosome V was determined quantitatively by plating serial dilutions of cells, as described previously (Gottschling et al., 1990; Sussel et al., 1991). In the presence of 73 mM 5-fluoroorotic acid is converted to a toxic compound. Student’s t test was used to assess the significance of differences in silencing, except when the colony-forming units were very low. In that case, the Poisson 95% central confidence intervals were compared.

Life-span Determination

Life-spans of yeast cells were determined as described elsewhere (Kim et al., 1998). Briefly, cells were grown in liquid YPG medium (1% yeast extract, 2% peptone, 3% glycerol) to suppress growth of petite yeasts. Exponentially growing cells were spotted on standard YPD plates (1% yeast extract, 2% peptone, 2% glucose, 2% agar) at low density. An appropriate number of individual cells were randomly picked under a microscope and aligned in isolated areas with a micromanipulator. After incubation of the plates at 30°C, virgin cells (new buds) were separated from their mother cells and left at the original spot, and the mother cells were discarded. The life-spans of these virgin cells were determined by recording the total number of daughter cells produced and removed. Mother cells were scored dead when budding ceased completely and they lost refractility. The nonparametric Wilcoxon signed rank test was performed to assess significance of differences in life-span.

Northern Blot Analysis and Quantitation of mRNA Levels

Age-synchronized cell populations of X2180-1A were prepared by rate-zonal sedimentation in sucrose density gradients (Egilmez et al., 1993). To detect age-dependent changes in mRNA levels, Northern blot analysis was performed as described (Sun et al., 1994). RT-PCR Analysis of mRNA Levels

To examine age-dependent expression of RP3D, RT-PCR analysis was performed because of the low levels of RP3D mRNA. Total RNA was isolated from cells of different ages using glass beads and hot acidic phenol (Ausubel et al., 1993). To detect age-dependent changes in mRNA levels, Northern hybridization with DNA probes and quantitation of mRNA levels were performed as described (Sun et al., 1994).
Table 1. Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>YPK4.7</td>
<td>MATα/α, MATα/α, ura3-52/ura3-52, lys2-801m/lys2-801m, ade2-101m/ade2-101m, trp1-Δ63/trp1-Δ63, his3-Δ200/his3-Δ200, leu2-Δ1/leu2-Δ1</td>
<td>Kirchman et al., 1999</td>
</tr>
<tr>
<td>YSK631</td>
<td>YPK4.7 with +/rd3Δ::URA3 +/hda1Δ::HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>YSK668</td>
<td>YPK4.7 with +/rd3Δ::URA3 +/sr3Δ::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>YSK661</td>
<td>MATα, rd3Δ::URA3, haploid segregant of YSK631</td>
<td>This study</td>
</tr>
<tr>
<td>YSK662</td>
<td>MATα, rd3Δ::URA3, hda1Δ::HIS3, haploid segregant of YSK631</td>
<td>This study</td>
</tr>
<tr>
<td>YSK663</td>
<td>MATα, haploid segregant of YSK631</td>
<td>This study</td>
</tr>
<tr>
<td>YSK664</td>
<td>MATα, hda1Δ::HIS3, haploid segregant of YSK631</td>
<td>This study</td>
</tr>
<tr>
<td>YSK710</td>
<td>MATα, rd3Δ::URA3, sr3Δ::LEU2, haploid segregant of YSK668</td>
<td>This study</td>
</tr>
<tr>
<td>YSK711</td>
<td>MATα, sr3Δ::LEU2, haploid segregant of YSK668</td>
<td>This study</td>
</tr>
<tr>
<td>YSK712</td>
<td>MATα, haploid segregant of YSK668</td>
<td>This study</td>
</tr>
<tr>
<td>YSK713</td>
<td>MATα, rd3Δ::URA3, haploid segregant of YSK668</td>
<td>This study</td>
</tr>
<tr>
<td>YSK770</td>
<td>YSK663 with sir3Δ::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>YSK771</td>
<td>YSK664 with sir3Δ::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>DY2126</td>
<td>MATα, ade2-1 can1-1 his3-10 leu2-1 trp1-ura3-3 hmr::TRP1</td>
<td>Yablon and Stillman, 1996</td>
</tr>
<tr>
<td>YSK694</td>
<td>DY2126 with hda1Δ::HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>YSK696</td>
<td>DY2126 with rd3Δ::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>YSK726</td>
<td>DY2126 with sir3Δ::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>YSK730</td>
<td>DY2126 with hda1Δ::HIS3 sir3Δ::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>YSK728</td>
<td>DY2126 with rd3Δ::URA3 sir3Δ::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>YDS21U</td>
<td>MATα, ade2-1 can1-100, his3-11, 15 leu2-3, 112 trp1-1 URA3; TelVR (2.1 kb from Tel)</td>
<td>Rundlett et al., 1996</td>
</tr>
<tr>
<td>YSK831</td>
<td>YDS21U with hda1Δ::HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>YSK830</td>
<td>YDS21U with rd3Δ::TRP1</td>
<td>This study</td>
</tr>
<tr>
<td>YSK829</td>
<td>YDS21U with sir3Δ::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>YSK833</td>
<td>YDS21U with hda1Δ::HIS3 sir3Δ::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>YSK832</td>
<td>YDS21U with rd3Δ::TRP1</td>
<td>This study</td>
</tr>
<tr>
<td>M1</td>
<td>MATα, his3Δ::200, leu2Δ, ura3-167, trp1Δ-Δ63, met15Δ, RDN1::Tel1-MET15</td>
<td>Smith and Boeke, 1997</td>
</tr>
<tr>
<td>M9</td>
<td>Same as M1 except that Tel1-MET15 is inserted in a non-cDNA locus</td>
<td>Smith and Boeke, 1997</td>
</tr>
<tr>
<td>JS218</td>
<td>M1 with sir3Δ::HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>YSK757</td>
<td>M1 with sir3Δ::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>YSK753</td>
<td>M1 with hda1Δ::pRS405 (LEU2)</td>
<td>This study</td>
</tr>
<tr>
<td>YSK781</td>
<td>M1 with sir3Δ::LEU2 hda1Δ::pRS405 (HIS3)</td>
<td>This study</td>
</tr>
<tr>
<td>YSK755</td>
<td>M1 with rd3Δ::pRS404 (TRP1)</td>
<td>This study</td>
</tr>
<tr>
<td>YSK779</td>
<td>JS218 with rd3Δ::pRS306 (URA3)</td>
<td>This study</td>
</tr>
<tr>
<td>YCY711</td>
<td>M9 with rd3Δ::pRS404 (TRP1)</td>
<td>This study</td>
</tr>
<tr>
<td>YSK783</td>
<td>M1 with sir3Δ::LEU2 rd3Δ::pRS406 (URA3)</td>
<td>This study</td>
</tr>
<tr>
<td>YPK9</td>
<td>MATα, ura3-52 lys2-801m/lys2-801m, ade2-101m/ade2-101m, trp1-Δ63/trp1-Δ63, his3-Δ200/leu2-Δ1</td>
<td>From YPK4.7 (Kirchman et al., 1999)</td>
</tr>
<tr>
<td>YAB11</td>
<td>YPK9 with sir2::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>YAB12</td>
<td>YPK9 with rd3Δ::pRS306 (URA3)</td>
<td>This study</td>
</tr>
<tr>
<td>YAB13</td>
<td>YAB11 with rd3Δ::pRS403 (HIS3)</td>
<td>This study</td>
</tr>
<tr>
<td>X2180-1A</td>
<td>MATα, SUC2, mal1-1, mel1-1, gal2-1, CUP1</td>
<td>The Berkeley Yeast</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Genetic Stock Center</td>
</tr>
</tbody>
</table>

did not result in RNA degradation. At the same time, to ensure that DNA present in the RNA preparations was completely digested by DNase I, 0.1 µg of DNase I-treated RNA was subjected to PCR analysis with the same primers used for RT-PCR (see below for the PCR reaction conditions). The DNase I-treated RNA was precipitated with 2.5 volumes of 100% ethanol and resuspended in 9 µl of diethylpyrocarbonate-treated water.

To synthesize first-strand cDNA, 1 µl (0.5 µg) of oligo d(T)12-18 (Life Technologies-BRL) was added to the tubes containing 9 µl of the DNase I-treated RNA. After 10 min incubation at 70°C, the tubes were chilled in an ice slurry. After brief centrifugation, the following ingredients were added to each tube containing the RNA and oligo d(T)12-18: 5 µl of 5× first-strand buffer (Life Technologies-BRL), 250 mM Tris·HCl, pH 8.3, 375 mM KCl, 15 mM MgCl2, 2 µl of 0.1 M dithiothreitol, 1 µl of 10 mM each dATP, dGTP, and dTTP mix, 1 µl of 0.1 mM dCTP, and 1 µl (−10 µCi) of [α-32P]dCTP (3000 Ci/ mmol). After incubation of the reaction mixture for 2 min at 42°C, 1 µl (200 U) of Superscript II RNase H-Reverse Transcriptase (Life Technologies-BRL) was added to each tube. The first-strand cDNA synthesis was performed for 50 min at 42°C. The reaction was stopped by incubating the tubes for 15 min at 70°C.

To quantitate the amount of cDNA synthesized in each tube, 3 µl of the cDNA synthesis reaction mixture were heat treated with the same volume of sequencing stop buffer (90% deionized formamide, 20 mM EDTA, pH 8.0, 0.05% bromophenol blue, 0.05% xylene cyanol). The tubes were heated for 3 min at 90°C, and the samples were loaded onto a 6% polyacrylamide, 7 M urea sequencing gel, alongside 32P-labeled size marker DNAs. The amount of cDNA synthesized in each sample was determined by phosphorimaging.

After normalization of the amount of cDNA present in each tube, 1 µl of the cDNA sample was dialysed 1:10 in water to obtain a 0.1× cDNA sample in addition to the 1× cDNA sample. For each cDNA sample, 1.25 µl of 0.1×, 2.5 µl of 0.1×, 5 µl of 0.1×, 1 µl of 1×, and 2 µl of 1× cDNA sample were added to five separate, fresh tubes.
After deionized water was added to each tube to bring the volume up to 37 μl, the following ingredients were added: 5 μl of 10X PCR buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl, 25 mM MgCl₂), 1 μl of 10 mM each dATP, dTTP, dGTP, and dCTP mix, 1 μl of each RPD3 primer at 50 mM concentration, 1 μl (10 μCi) of [α-32P] dCTP (3000 Ci/mmol), and 1 μl (5 U) of Taq polymerase (Promega, Madison, WI). The primers were 5’- (+470 from the first nucleotide of the ATG start codon) GGTGTCGCTCTATGGAAAGGA-3’ and 3’-GGATCCCTACGGCTTCTAAA CCC (1305)-5’. PCR amplification using this primer pair generates a 836-bp product specific to RPD3. After 5-min incubation at 94°C, the PCR amplification continued for 30 cycles, each cycle consisting of 1.5 min at 94°C, 1.5 min at 54°C, and 2.5 min at 72°C. The PCR products were separated on a 6% nondenaturing polyacrylamide gel. After the gel was dried, quantitation of DNA bands amplified from the RPD3 cDNA was performed by phosphorimaging, using the PhosphorImager and ImageQuant Software (Molecular Dynamics, Sunnyvale, CA).

Quantitation of Extrachromosomal rDNA Circles

Yeast cells were grown to an OD₆₀₀ of 0.8, and DNA was extracted from harvested cells using the Easy DNA kit (Invitrogen, San Diego, CA). RNA-free DNA (20 μg) was electrophoresed for 20 h at 1 V/cm in an 0.6% agarose gel containing 40 mM Tris-acetate, 1 mM EDTA, pH 8.0, in the absence of ethidium bromide. The separated DNA was transferred to a Nytran membrane (Schleicher and Schuell, Keene, NH). rDNA was detected by hybridization with a 32P-labeled DNA probe and quantitated by phosphorimaging. Normalization for DNA loading was performed with an actin (ACT1) DNA probe. The identity of rDNA circles was further confirmed by two-dimensional gel electrophoresis in the presence of chloroquine, as described (Sinclair and Guarente, 1997).

RESULTS

Deletion of RPD3 Results in Life-span Extension

We first examined the effect of genes encoding histone deacetylases on yeast replicative life-span. RPD3 and HDA1 were deleted from a diploid strain (YPK4.7). After sporulation and tetrad dissection, germinated rpd3Δ, hda1Δ, and rpd3Δ hda1Δ segregants were examined for their life-spans (Figure 1A). The average life-span of the rpd3Δ segregant was extended by 41% compared with the wild-type control. The hda1Δ segregant showed little change compared with the wild-type control. The rpd3Δ hda1Δ double mutant had a significantly shorter mean life-span because of high initial mortality.

Most of the rpd3Δ hda1Δ mother cells that had stopped cell division either early or late were attached to a large bud. The budding pattern was frequently random during the life-span, after the initial drop in survival. The increase in frequency of random budding is an age-related phenotype (Jazwinski et al., 1998). Consistent with the shorter mean life-span associated with high initial mortality, the germinated rpd3Δ hda1Δ segregants formed much smaller colonies, compared with the other segregants (Figure 1B). These results suggest that RPD3 and HDA1 share some essential function, but their effects on yeast aging are different. Similar results were obtained with the same segregants from other tetrads. Deletion of HOS1 or HOS2, both of which share sequence homology with RPD3 and HDA1 (Rundlett et al., 1996), showed no effect on life-span (our unpublished results).

Deletion of SIR3 from the hda1Δ Strain Results in Life-span Extension

To determine whether the life-span extension shown by the rpd3Δ segregants is mediated by the Sir silencing complex, Sir3Δ was deleted from a diploid heterozygous for rpd3Δ, and meiotic segregants were examined (Figure 2A). The rpd3Δ sir3Δ segregant showed as much life-span extension as the rpd3Δ alone, whereas the sir3Δ segregant was virtually the same as the wild-type control in life-span. This result indicates that life-span extension by RPD3 deletion does not require the intact Sir silencing complex needed for efficient silencing at telomerers and at HM loci.

Sir3Δ was also deleted from the hda1Δ strain, and the life-span of the hda1Δ sir3Δ strain was analyzed (Figure 2B). Interestingly, the average life-span of the double mutant was extended by as much as 38%, whereas either single mutant
showed little change as observed before. While measuring the life-spans, we noticed differences among different strains in the length of time taken for mother cells to generate consecutive buds (generation time) (Figure 2C). The mother cells of the sir3Δ strain had shorter generation times at early ages than the wild-type control. With age, however, they exhibited an exponential increase in generation time, compared with the control. The generation time of the hda1Δ strain remained close to that of the wild-type control throughout the life-span. The generation time of the hda1Δ sir3Δ double mutant was initially as short as that of the sir3Δ single mutant but did not increase with age at the same rapid rate. Therefore, the synthetic life-span extension phenotype of the hda1Δ sir3Δ strain is the result of generation of more daughter cells for a prolonged time, compared with either single mutant or the control. The exponential increase in generation time in the sir3Δ strain represents an acceleration of an aging phenotype (Egilmez and Jazwinski, 1989). To our knowledge, this is the second example in which an age-related phenotype has been separated from longevity. In the other case, an earlier than usual increase in cell size was obtained when life-span was extended by other means (Chen et al., 1990).

Transcriptional Silencing in the rpd3Δ and hda1Δ sir3Δ Strains

We next wanted to determine whether the average life-spans of the deletion mutants for RPD3, HDA1, or SIR3 correlate with chromatin changes. For this purpose, transcriptional silencing of each mutant was examined at HMR, a subtelomeric site, and at RDN1. At the silent mating-type locus, silencing was significantly increased in the rpd3Δ strain but not in the hda1Δ strain (Table 2). At the subtelomeric locus, both the rpd3Δ and hda1Δ strains showed increased silencing, with the effect of rpd3Δ being greater than that of hda1Δ (Table 3), as observed previously (Rundlett et al., 1996). At both loci examined, silencing was abolished by deletion of SIR3, as expected.

For rDNA silencing, we used the color assay of colonies arising from cells containing MET15 integrated in the RDN1 locus (Cost and Boeke, 1996; Smith and Boeke, 1997). As shown previously, rDNA silencing was dependent on SIR2, as judged by the lighter background colony colors in the

<table>
<thead>
<tr>
<th>Strain (all hmr::TRP1)</th>
<th>No. of cultures</th>
<th>Frequency of Trp⁺ colonies (mean ± SD)</th>
<th>Relative frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>DY2126 (control)</td>
<td>8</td>
<td>5.19 ± 2.74 × 10⁻⁶</td>
<td>1.00</td>
</tr>
<tr>
<td>YSK694 (hda1Δ)</td>
<td>8</td>
<td>6.12 ± 3.97 × 10⁻⁶</td>
<td>1.18</td>
</tr>
<tr>
<td>YSK696 (rpd3Δ)</td>
<td>11</td>
<td>2.08 ± 1.40 × 10⁻⁶</td>
<td>0.40</td>
</tr>
<tr>
<td>YSK726 (sir3Δ)</td>
<td>9</td>
<td>1.03 ± 0.08</td>
<td>1.98 × 10⁶</td>
</tr>
<tr>
<td>YSK730 (hda1Δ sir3Δ)</td>
<td>9</td>
<td>0.98 ± 0.08</td>
<td>1.89 × 10⁶</td>
</tr>
<tr>
<td>YSK728 (rpd3Δ sir3Δ)</td>
<td>12</td>
<td>0.92 ± 0.08**</td>
<td>1.77 × 10⁶</td>
</tr>
</tbody>
</table>

*P < 0.05 compared with the control.

**P < 0.01 compared with the sir3Δ strain.

Figure 2. Effects of SIR3 deletion on life-span and senescence. (A) Survival curves of wild-type (YSK712), rpd3Δ (YSK713), sir3Δ (YSK711), and rpd3Δ sir3Δ (YSK710) segregants of YSK668. Mean life-span (and the number of cells analyzed) was 19.1 (40) for the wild-type, 27.9 (40) for the rpd3Δ, 17.8 (40) for the sir3Δ, and 28.4 (40) for the rpd3Δ sir3Δ strains. The rpd3Δ and rpd3Δ sir3Δ strains differed from wild-type in life-span (P < 0.00001). (B) Survival curves of wild-type (YSK663), hda1Δ (YSK664), sir3Δ (YSK770), and hda1Δ sir3Δ (YSK771) strains. Mean life-span (and the number of cells analyzed) was 18.6 (50) for the wild-type, 18.3 (50) for the hda1Δ, 19.0 (50) for the sir3Δ, and 25.6 (50) for the hda1Δ sir3Δ strains. The life-span of the hda1Δ sir3Δ strain was significantly longer than that of the control, the sir3Δ, or the hda1Δ strains (P = 0.000002, 0.000028, and 0.000002, respectively). (C) Change in generation time of the hda1Δ, sir3Δ, or hda1Δ sir3Δ mother cells relative to the wild-type control during the life-spans shown in B. From the day life-span determination was started, the total number of buds generated by the wild-type mother cells was divided by the total number of buds generated by the hda1Δ, sir3Δ, or hda1Δ sir3Δ mother cells. The resulting number corresponds to an estimate of the average generation time of each mutant strain relative to that of the wild type. The relative generation times were calculated until day 10. By this time, all of the strains completed >96% of their life-spans.

Table 2. Effect of deletion of RPD3, HDA1, and SIR3 on HMR silencing

Vol. 10, October 1999 3129
sir2Δ strain, compared with the intermediate brown colors of the control colonies (Figure 3, compare A, B, and C). In addition, the more frequent appearance of dark brown colonies or colony sectors in the sir2Δ strain, which results from mitotic recombination leading to complete loss of MET15, indicates that SIR2 is also required for suppression of mitotic recombination involving rDNA repeats. In contrast, colonies of the rpd3Δ or the rpd3Δ sir3Δ strains showed uniformly intensified brown colors, indicating that both rDNA silencing and recombinational suppression were increased by deletion of RPD3 (Figure 3, G and H). Colony colors of the hda1Δ or the sir3Δ strains were not substantially different from those of the control, although colony colors of the sir3Δ strain appeared to be slightly intensified (Figure 3, D and E), as reported previously (Smith et al., 1998). Interestingly, colonies of the hda1Δ sir3Δ double mutant developed in uniformly intense brown colors as the rpd3Δ mutant (Figure 3F). This indicates that deletion of both HDA1 and SIR3 increased rDNA silencing and recombinational suppression to an extent similar to the increase by RPD3 deletion. Deletion of RPD3 had little effect on transcription of MET15 located outside the RDN1 locus (Figure 3J).

Deletion of RPD3 Partially Suppresses the Silencing Defect in the sir2Δ and sir3Δ Strains

We noticed that the polar budding of mother cells occurred less frequently in the rpd3Δ sir3Δ double mutant than in the sir3Δ single mutant. In contrast, the hda1Δ sir3Δ double mutant maintained a polar budding pattern. This suggests suppression of the HM silencing defect of the sir3Δ by deletion of RPD3, but not by deletion of HDA1. In fact, the sir3Δ rpd3Δ strain showed a slight but significant increase in HMR silencing compared with the sir3Δ strain (Table 2). This small increase in silencing in a sir3Δ rpd3Δ strain was observed at the subtelomeric locus as well (Table 3). Deletion of RPD3 was also able to partially suppress the increased mitotic recombination in the sir2Δ strain at the RDN1 locus, as indicated by less frequent appearance of dark brown-colored colonies and sectors, and to enhance rDNA silencing. The uniformly brown colony colors of the rpd3Δ sir2Δ double mutant were nearly, but not quite, as intense as those of the rpd3Δ single mutant (Figure 3I). This indicates that deletion of RPD3 can overcome the loss of silencing and increased mitotic recombination in rDNA caused by deletion of SIR2 (Figure 3, compare B and C). The sir2Δ, however, prevents the rpd3Δ from maximally enhancing silencing of rDNA (Figure 3, compare G and I). We also determined the life-span of the sir2 mutant, in which rDNA silencing is severely abated (Bryk et al., 1997; Fritze et al., 1997; Smith and Boeke, 1997; Smith et al., 1998). The mean life-span of the sir2 mutant was significantly reduced, compared with the wild-type control (Figure 4A). The deletion of RPD3 did not suppress this decline in life-span in the sir2Δ strain (Figure 4A), despite its enhancement of rDNA silencing.

It has been shown recently that extrachromosomal rDNA circles (ERCs) accumulate in old yeast cells, presumably through recombination at RDN1 and amplification, and that induction of ERCs can cause yeast aging (Sinclair and Guarente, 1997). Deletion of SIR2 increases recombination at RDN1 (Gottlieb and Esposito, 1989; Smith and Boeke, 1997). We compared the relative amounts of ERCs present in wild-type and sir2 mutant cells and found that their amount in sir2 mutants did not exceed that present in the wild-type control (Figure 4B). This suggests that ERC production is not the cause of the curtailed life-span in the sir2 mutant.

Changes in RPD3, HDA1, and SIR3 mRNA Levels with Age

To obtain more insight into how RPD3, HDA1, and SIR3 affect life-span, we determined their expression patterns as a function of age. The mRNA levels of HDA1 and SIR3, normalized to the level of TLC1 RNA, which remains relatively constant with age, dropped sharply from generation 2 to 5 and after this remained low in older cells (Figure 5). For RPD3, RT-PCR analysis was performed because we encountered difficulty detecting its mRNA on Northern blots. The results indicate that the amount of RPD3 mRNA also decreases with age (Figure 6). A substantial decrease in SIR1 mRNA levels was also observed in older cells (our unpublished results). These patterns of decrease in gene expression were always reproducible in at least three determinations in each case. It has been shown previously that a decrease in transcript levels is not characteristic of the majority of genes during the yeast life-span (Eglintez et al., 1989).

DISCUSSION

Histone Deacetylase Genes Play a Role in Determining Yeast Life-span

Deletion of RPD3 or HDA1 (in the presence of a sir3Δ) results in a substantial increase in yeast life-span. This suggests that the acetylation profile of the core histones, which determines the degree of accessibility of the DNA in chromatin, is a determinant of yeast longevity. Indeed, the deletion of these deacetylase genes causes changes in chromatin, as evidenced by alterations in transcriptional silencing at three known heterochromatic loci in yeast.

It would be a mistake, however, to interpret the data solely in terms of the changes in gene activity at HM, telomeres, and rDNA, which were assayed to confirm that the deletion of the deacetylases resulted in predictable functional consequences. It is known that RPD3 impinges on the
Figure 3. Effect of various deletions on rDNA silencing. Cells from (A) M9 (Ty1-MET15 inserted in a non-rDNA locus), (B) M1 (RDNI1:Ty1-MET15), (C) JS218 (M1 with sir2:: HIS3), (D) YSK757 (M1 with sir3Δ:: LEU2), (E) YSK753 (M1 with hda1Δ:: LEU2), (F) YSK781 (M1 with sir3Δ:: LEU2, hda1Δ:: HIS3), (G) YSK755 (M1 with rpd3Δ:: TRP1), (H) YSK783 (M1 with sir3Δ:: LEU2, rpd3Δ:: URA3), (I) YSK779 (M1 with rpd3Δ:: URA3, sir2:: HIS3), and (J) YCYL11 (M9 with rpd3Δ:: TRP1) were streaked on modified YPD agar medium containing Pb2⁺. The plates were incubated for 1 wk at 30°C. MET15⁺ cells grown on Pb²⁺ medium form white colonies (A), but met15 mutant cells develop dark brown colonies on the same medium. In the control M1 (B), the Ty1-MET15 is located upstream of the 5S rDNA in the RDNI locus (Smith and Boeke, 1997).
expression of at least several yeast genes outside these heterochromatic loci, and it is likely that HDA1 similarly affects several genes. Apart from these local, gene-specific effects, these deacetylases may exert more global effects on larger chromatin domains. They may also be more generally involved in chromatin remodeling. Nevertheless, it is interesting to explore the potential role of chromatin changes at HM, telomeres, and rDNA in yeast aging that our results support.

**Increased Heterochromatic Silencing by rpd3Δ and Partial Suppression of Silencing Defect by rpd3Δ**

Hyperacetylation of the core histones is expected to “loosen” chromatin assembly, resulting in decreased silencing (Wolfe, 1996; Grunstein, 1997). In fact, silent heterochromatic regions of metazoan genomes are generally hypoacetylated compared with those of euchromatic regions (reviewed by Grunstein, 1997). Mutation in RPD3, however, increases silencing despite its hyperacetylation effect on all of the N-terminal lysine residues of histones H3 and H4 examined (Sussel et al., 1995; Rundlett et al., 1996; Vannier et al., 1996). It has been speculated that hyperacetylation might result in increased transcription of SIR3 or other genes involved in silencing, hence enhanced silencing (Rundlett et al., 1996; Grunstein, 1997). Our data, however, indicate that this may not be the case; deletion of RPD3 from the sir3Δ or sir2Δ strains resulted in partial yet significant restoration of silencing. (After this article was submitted, Smith et al. [1999] reported that an rpd3 mutation enhances silencing of rDNA and at the HM locus in a sir3 mutant, in agreement with our findings.) Moreover, it is not SIR4 whose transcription could be induced by RPD3 deletion, because increase in SIR4 dosage results in reduced rDNA silencing (Smith et al., 1999).
Histone Deacetylases and Yeast Aging

The mechanisms of aging in which they are involved may differ. RPD3 may function in both rDNA and telomeric silencing, whereas HDA1 may impinge on rDNA silencing alone.

One possibility may be that the loss of silencing at HM and telomeres during aging results in age changes, such as sterility (Smeal et al., 1996) and increased generation time (Egilmez and Jazwinski, 1989; Figure 2), that themselves do not affect the life-span. For an effect on life-span to be observed, events at the rDNA locus either alone or in conjunction with the HM and telomere changes may be required. Some of the latter age changes may even have a salutary effect, such as the increase in stress resistance on loss of telomeric silencing (Kennedy et al., 1995).

In evaluating the physiological significance of the enhanced silencing afforded by rpd3Δ or hda1Δ, the magnitude of the actual silencing should be kept in mind. The silencing increases seen in a rpd3Δ sir3Δ strain, although significant, do not approach wild-type levels at HM and telomeres, yet life-span is extended. This focuses attention on rDNA. Life extension is correlated with an increase in rDNA silencing in the rpd3Δ strain (with or without the sir3Δ) and the hda1Δ sir3Δ strain; however, the assay is not easy to quantitate. Furthermore, this assay monitors the activity of an integrated RNA polymerase II-dependent gene and not rRNA transcription. Thus, the physiological significance of the silencing changes is not entirely clear. Nevertheless, the state of rDNA chromatin appears to be important for life-span.

The lack of extension of life-span in the rpd3 single mutant (Figure 3, G and I). Alternatively, the deletion of RPD3 may exert an effect on life-span outside the RDN1 locus. This effect may require concomitant events at RDN1. It is noteworthy that the extension of life-span by deletion of RPD3 is associated with silencing of rDNA well beyond that in the wild type (Figure 3, B and G). Similar silencing is seen in the rpd3Δ sir3Δ and hda1Δ sir3Δ strains (Figure 3, F and H), in which extension of life-span is also observed (Figures 1 and 2).

rDNA Silencing and Generation of ERCs

Recently, accumulation of ERCs has been proposed as a cause of yeast aging (Sinclair and Guarente, 1997). This
The decrease in the old mother cells may be accounted for by a substantial increase in sterility (Muller, 1985; Smeal et al., 1996; Strahl-Bolsinger et al., 1996), because Sir3p is a limiting factor for the silencing effect exerted by the Sir complex (Renauld et al., 1996), because Sir3p is a limiting factor for the silencing processes that are dependent on accessibility to DNA. Plausible candidates, suggested by the studies, are alterations in transcriptional status. In fact, chromatin-dependent silencing with age is the relocalization of the Sir3p and Sir4p products are low. The relatively low level of SIR3 mRNA in old cells provides a plausible explanation for the decrease in telomeric and HM silencing in older cells (Kim et al., 1996; Smeal et al., 1996), because Sir3p is a limiting factor for the silencing effect exerted by the Sir complex (Renaud et al., 1993; Hecht et al., 1996; Strahl-Bolsinger et al., 1997). An alternative but not mutually exclusive explanation for the loss of telomeric silencing with age is the relocalization of the Sir3p and Sir4p to the nucleolus that occurs in old cells (Kennedy et al., 1997). The loss of HM silencing (Smeal et al., 1996), and therefore the increase in sterility (Muller, 1985; Smeal et al., 1996), of old mother cells may be accounted for by a substantial decrease in the SIR1 mRNA level in older cells.

Possible Consequences of Alterations in rDNA Chromatin

The studies described here implicate histone deacetylases and chromatin changes as determinants of yeast longevity. These chromatin changes can impinge on various cellular processes that are dependent on accessibility to DNA. Plausible candidates, suggested by the studies, are alterations in transcriptional status. In fact, chromatin-dependent silencing of rDNA appears to most readily explain the observations. Gene-specific regulatory effects at loci throughout the yeast genome may also be involved, but they are more difficult to assess, as are other effects of chromatin structural changes. If indeed transcription of rDNA is the process critical for life-span, what are the ramifications? A proper supply of rRNA might be necessary to guarantee longevity. In fact, the deletion of SIR2 curtails life-span (Figure 4A) and has been suggested to yield excessive production of rRNA (Smith and Boeke, 1997). In contrast, the rpd3Δ (or deletion of HDA1 and SIR3) extends life-span and may tighten control to prevent undue rDNA transcription.

There are data consistent with the model sketched above. During yeast aging, there is an increase in cellular rRNA content that does not keep up with the increase in cell volume (Motizuki and Tsurugi, 1992; Jazwinski, 1996). Concomitantly, a decline in protein synthesis rate occurs, which may be one of the key reasons for the increase in generation time and ultimately death. These excessive rRNA levels may not be matched by ribosomal protein synthesis, resulting in defective ribosome assembly. The rpd3Δ may mitigate this imbalance to maintain protein synthesis rates longer. This scenario does not take into account, of course, the action of the deacetylases and Sir proteins at other locations, which may further complicate their effects on longevity.

ACKNOWLEDGMENTS

We thank Ashley Schneider in this laboratory for help in construction of strain YAB13. We thank those who provided plasmids and yeast strains (P. A. Kirchman, R. Gaber, M. Grunstein, L. Pillus, D. J. Stillman, S. Holmes, and J. Boeke). We thank B. Villeponteau for stimulating discussion. This work was supported by grants from the National Institute on Aging of National Institutes of Health (United States Public Health Service). A.B. was the recipient of a postdoctoral fellowship from the Ministry of Education and Culture of Spain.

REFERENCES


3134 Molecular Biology of the Cell


