2 MATERIALS AND METHODS

2.1 Chemicals, Buffers, Solutions, Media

If not noted otherwise, chemicals were purchased from Sigma or Fisher. Chemicals used for cell culture media were purchased from Becton Dickinson/Difco.

Solutions were either autoclaved for at least 20 min at 121 °C or filter sterilized.

YEPD	20 g Bacto Peptone, 10 g Yeast extract, 950 ml H_20, (20 g agar for plates); add 50 ml 40 % dextrose after autoclaving
SD	7 g yeast nitrogen base without amino acids, 950 ml H_20, (20 g agar for plates); add 50 ml 40 % dextrose, 2 g drop out amino acid powder after autoclaving (for the composition of the dropout powder, please refer to Guthrie and Fink, 1991)
SOB	20 g tryptone, 5 g yeast extract, 0.5 g NaCl, 10 ml 250 mM KCl, ad 1l H_20
LB	10 g Peptone, 5 g NaCl, 5 g Yeast Extract, H_2O ad 1 l
TBS	20 mM Tris-HCl pH 7.6, 200 mM NaCl
FA lysis buffer	50 mM HEPES-KOH pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 0.5 % SDS, 0.1 % sodium deoxycholate, 1 mM PMSF
TE	10 mM Tris-HCl pH 8, 1 mM EDTA
TBE	90 mM Tris-borate, pH 8, 1 mM EDTA
TAE	40 mM Tris-HCl pH 8, 1 mM EDTA
Elution buffer	50 mM Tris-HCl pH 7.5, 10 mM EDTA, and 1 % SDS
TB Buffer	10 mM Pipes, 55 mM $MnCl_2$, 15 mM $CaCl_2$, 250 mM KCl, pH 6.7
Gel loading buffer (10X)	50 mM Tris-HCl pH 8, 50 % sucrose, 4 M urea, 0.05 % bromophenolblue

Polyacrylamide gel solution (6 %)	10.5 ml H_2O, 5.5 ml acrylamide solution (29:1) (BioRad), 4 ml TBS, 200 µl APS, 12 µl TEMED (BioRad)

Solutions for yeast transformations:

Lithium acetate mix	0.1 ml of 1 M lithium acetate pH 7, 0.1 ml 10X TE, 0.8 ml water
PEG mix:	5 ml of 1 M lithium acetate, 5 ml 10X TE, 20 g polyethylenglycol, H_20 ad 50 ml
Salmon Sperm DNA	10 mg/ml

2.2 Laboratory Devices

Clinical centrifuge	International Equipment Co.
Table-top centrifuges	Eppendorf
Sorvall centrifuge RC-5B	DuPont
Agarose-gel electrophoreses chambers	Owl Scientific
Polyacrylamide-gel electrophoreses chamber	Aladin
Electronic precision Scale	Sartorius
Microscope Eclipse E800	Nikon
Monochrome CCD camera	Princeton Instruments
Water Baths	VWR
Heat blocks	Laboratory Supplies Co.
Phosphor imager	BioRad
Phosphor imager cassette	Fuji
Spectral photometer	Beckman
Thermocycler	MJ Research
Vortex	VWR
Mini bead beater	Biospec

Digital Sonifier 450	Branson
Nutator	VWR
DCS 420 grayscale digital camera	Kodak
Gel dryer	BioRad
Gel Documentation Imaging system	Alpha Innotech Co.
Shaker incubators (30 and 37 °C)	New Brunswick Scientific
Incubators (30 and 37 °C)	VWR

2.3 Handling of DNA

2.3.1 Preparation of Plasmid DNA

Plasmid DNA was extracted from overnight LB cultures containing Ampicillin. The Qiagen Miniprep Kit was used according to the manufacture's instruction. The procedure is based on the alkaline lysis method followed by adsorption of DNA onto a silica-gel membrane in the presence of high salt (Vogelstein and Gillespie, 1979; Birnboim and Doly, 1979).

2.3.2 Gel Electrophoresis

After addition of sample loading buffer (1/10 the volume), samples were loaded onto an agarose gel of the appropriate percentage, containing 0.25 μg/ml ethidium bromide. Gels were run horizontally in TAE buffer at 120 to 150 V. If fragment of interest had less than 500 bp in length, ethidium bromide was also added to the running buffer. DNA bands were visualized in an imaging system using ultraviolet light.

2.4 Enzymatic Reactions with DNA

2.4.1 Restriction Digest

Restriction digests were performed as recommended by the manufacturer (NEB or Boehringer Ingelheim) using the supplied buffer. Usually, digests were performed using 1 to 2 units of enzyme for 1 microgram of DNA. Reactions were incubated for a minimum of 2 hours at 37 °C.

2.4.2 Ligations

Gel electrophoreses was performed as described in 2.5.2. The gel was wiped dry and the bands of interest were excised with a clean razor blade under ultraviolet light. Samples were melted at 70 °C for 5 minutes and the fragments were combined in a fresh tube to a total volume of 9 microliters. Usually, the ratio between insert and vector was estimated to be 3:1. The gel slices were then incubated at 42 °C for 5 more minutes and then placed on ice for 5 minutes. 11 microliters of a mixture of 8 microliters water, 2 microliters ligation buffer, and 1 microliter T4 ligase (both from NEB) were pipetted to the gel, mixed immediately and incubated over night at room temperature. Transformation into bacteria was performed as described in section 2.8.5

2.5 Construction of Plasmids

pDM271 contains a *Eag*I-*Hin*dIII fragment corresponding to the coding region of *GFP* in a pRS304 backbone. To create *SIR2-GFP* alleles, a portion of the *SIR2* open reading frame was PCR-amplified from plasmids that contained either wild type *SIR2* or *sir2-H364Y*. The PCR products were digested with *Sac*I (cuts uniquely in *SIR2*) and *Eag*I (introduced by PCR) and fused in frame to the N-terminus of *GFP* in plasmid pDM271 to create plasmids pJT18 pSIR2-GFP and pJT19 p*sir2*-H364Y-GFP.

pDM272 (*SIR3-GFP-URA3*) was constructed as follows. A PCR generated *Asp718-EagI* fragment containing the C-terminal 150 amino acids of Sir3 and *Eag*1-*Hin*dIII fragment containing GFP(S65T, V167A) was subcloned into the *Asp*718 and *Hin*dIII digested Yplac111, a yeast *LEU2* marked integrating vector, to generate pDM367. A *Sac*I-*Hin*dIII fragment containing the *SIR3-GFP* fragment from pDM267 was then subcloned into pMR52, a derivative of pRS306 containing the *ACT1* terminator, to generate pDM272.

pDM274 was constructed by subcloning a PCR generated GFP fragment into the *Eco*RI site of pDM158 to replace 6HIS-3HA with GFP. pDM158 contains the entire *SIR4* open reading frame fused to 6HIS-3HA.

pSIR2-LEU2, pSIR2-H364Y-LEU2, pSIR2-G262A-LEU2 were generated as follows (Tanny et al., 1999; Tanny and Moazed, 2001): pGAL-GST was constructed by ligation of an *Xba*I-*Bam*HI fragment containing the GAL1 promoter driving GST from pRD56 (a gift of R. Deshaies) into *Xba*I-*Bam*HI-digested pRS315. The entire SIR2 open reading frame was PCR amplified as an *Eco*RI fragment and ligated into the *Eco*RI site of pRD56 in frame with the C terminus of GST to generate pDM114a. A *Xba*I-*Xho*I fragment containing GAL1-GST-SIR2 was then ligated into *Xba*I-*Xho*I-digested pRS315 to generate pGAL1-GST-SIR2. The mutations were generated by overlap PCR as follows: SIR2 fragments were generated using the overlapping primers JT7 (GTGCTATGGCTCTTTTGCTAC) and JT8 (GCCATAGCACTGCACCAGTTTATC) for H364Y and JT34 (ACTGCTGCAGGTGTTTC) and JT35 (TGCAGCAGTCAGGAC CAA) for G262A; and the outside primers JT9c (GGCGAATTCGGAAGTGCAATA TCTGTC) and JT10 (CGCGAATTCATCACGATTAATCAGGACTTG). Separate PCR reactions were performed with primer pairs JT7/JT10, JT34/JT10 and JT8/JT9c, JT35/JT9c (30 cycles of 94°C for 30 sec, 55 °C for 30 sec, and 72 °C for 2 min). Portions of the resulting PCR products were then mixed and reamplified with primers JT9c and JT10. The final PCR products containing the appropriate point mutation was digested with *Stu*I and *Bgl*II and used

to replace a *Stu*I-*Bgl*II fragment in pGAL/GST-Sir2 to generate pGAL-GST-H364Y and pGAL-GST-G262A. A *Bgl*II–*Stu*I fragment encoding the C-terminus of Sir2 and containing the H364Y and G262A mutations, respectively, was then subcloned into the *Bgl*II–*Stu*I sites of pSIR2-LEU2, which contains the entire SIR2 genomic region in the pRS315 backbone, to generate pH364Y-LEU2 and pG262A-LEU2. All PCR-generated regions were entirely sequenced at the Harvard Medical School Biopolymers Facility to ensure the absence of PCR-introduced errors.

pDM311 (*HIS3-mURA3::rDNA*) was constructed by ligation of a 1 kb *Eco*RI-*Xho*I fragment containing a portion of the rDNA NTS, and an *Eco*RI-*Eag*I fragment containing the *mURA* gene (Smith and Boeke, 1997), into pRS303 digested with *Xho*I and *Eag*I.

pJSS60-2 (*mURA3::rDNA25S/LEU2*) was a gift from M. Grunstein. pDM554 (*sir4*Δ*::LEU2*) was a gift from D. Gottschling. pJ107.1 (*sir3*Δ*::LEU2*) was a gift from A. Kahana.

Plasmids pRS305, and pRS304 are integrating vectors; pRS315, and pRS313 contain *CEN/ARS* elements. All are described in Guthrie and Fink (1991).

2.6 Bacteria

Plasmids were propagated in the DH5α. strain (Vieira and Messing, 1982): F^- , *end*A1, *hsd*R17, (rK^-, mK^+), *sup*E44, *thi*-1, *rec*A1, *gyr*A96, *rel*A1, Δ(*arg*F-*lac*ZYA)U169, Φ80d*lac*ZΔM15, λ-

2.6.1 Storage and Reactivation

Bacterial strains were stored at –80 °C in 50 % glycerol. For reactivation, material scraped off the top of the vial was patched onto LB/ampicillin plates and incubated over night at 37 °C.

2.6.2 Plate Cultures

Plates (containing ampilcillin where appropriate) were obtained from the media facility of the Harvard Medical School. Cells were incubated over night at 37 °C.

2.6.3 Liquid Cultures

Cells were cultivated in LB media containing ampicillin. Cells were grown at 37 °C.

2.6.4 Preparation of competent bacteria

DH5α cells were grown form a fresh plate in 200 ml SOB at room temperature over night. After in incubation on ice for 10 min, cells were spun down at 5000 rpm for 10 min in a GSA rotor at 4 °C. Cells were resuspended in 80 ml ice cold TB, and incubated on ice for 10 min, before they were spun down again as previously. Cells were then resuspend in 20 ml TB (with 7 % DMSO added) and incubated in an ice bath for 10 min. Cells were aliquoted into 1.5 ml Eppendorf tubes, frozen in liquid nitrogen and stored at -80 °C.

2.6.5 Transformations

100 microliters competent *Escherichia coli* cells were thawed from -80 °C on ice. They were then dispensed into 15 ml thin wall tubes (Falcon). 4.5 microliters of the melted ligation reaction were added to the cells and mixed immediately. After incubate on ice for 30 min, cells were heat-shocked at 42 °C for exactly 45 seconds and then incubated on ice for 2 min. 800 microliters LB were added and the vials were shaken at 37 °C for 1 hour. Then, 200 microliters were plated on a LB plate containing ampicillin. The rest of the cell suspension was spun down and all of the media was discarded save 200 microliters, which was resuspended and also plated.

2.7 Yeast

2.7.1 Storage and Reactivation

For storage, yeast strains were grown to saturation in the appropriate media. Cells were mixed with an equal volume of a 50 % glycerol solution. They were stored in cryotubes at -80 °C. For reactivation, material scraped off the top of the vial was patched onto the appropriate media plate and incubated at 30 °C.

2.7.2 Plate Cultures

Yeast cells were streaked onto agarose media plate in a diluting fashion, so that single colonies could be picked for experiments. Cells were plated on YEPD plates for routine use, or minimal synthetic media lacking either uracil, tryptophan, histidine, or leucin, according to the strain's auxotrophy. For selection of the kanamycin marker (Longtine et al., 1998), cells were streaked onto YEPD plates containing G418. In this case, cells had to be replica plated after three days of growth at 30 °C to obtain single colonies.

2.7.3 Liquid Cultures

Yeast cells were usually grown in a volume of 5 ml at 30 °C in YEPD or appropriate minimal synthetic media lacking either uracil, tryptophan, histidine, or leucin, according to the strain's auxotrophy. Overnight cultures were usually diluted 1:25 and grown to log phase (OD_{600} between 0.5 and 1) before harvest.

2.7.4 Transformations

Strains were grown overnight in 5 ml of the appropriate media. The overnight culture was then diluted 1:25 into 5 ml of fresh media. After 5 hours, cell density was usually between OD of 0.6 and 1, or at 10^7 cells per ml. 5 microliters miniprep DNA and

5 microliters of 10 mg/ml sonicated salmon sperm DNA were pipetted into an 1.5 ml eppendorf tube. Cultures were spun down in a clinical centrifuge for 5 minutes at setting 5. The supernatent was discarded. Yeast cells were washed in 1 ml lithium acetate mix, and resuspended in 500 microliters. 50 microliters of the cells were added to the DNA/salmon sperm mixture, together with 0.7 ml of PEG mix. Cells were incubated at 30 °C for 30 minutes. The tubes were then transferred to a 42 °C heatblock for 15 minutes. Cells were spun down in a tabletop centrifuge at top speed for 15 seconds and the supernatants were aspirated off. Cells were resuspended in 0.2 ml TE and plated onto appropriate selective media plates.

2.7.5 Strains

Yeast strains used in this study are listed in Table 1. Transformations of all yeast strains were performed using the lithium chloride method (section 2.9.4, and Guthrie and Fink, 1991).

To create the strains used in figures 3.1, 3.2, 3.3, 3.7A, and 3.9, the plasmids pSIR2-LEU2, pSIR2-H364Y-LEU2, pSIR2-G262A-LEU2 and pRS315 (all of which contain a CEN/ARS element) were transformed into strain JRY3433.

The strains used in figure 3.5 were generated as follows: To create *SIR2-GFP* strains, plasmids pJT18 and pJT19 were integrated at the *SIR2* locus in strain JRY3433 after digestion with *Bgl*II. This yielded strains DMY2163 (*SIR2-GFP*) and DMY2164 (*sir2-H364Y-GFP*). To create the *SIR3-GFP* strains, plasmid pDM272 was integrated at the *SIR3* locus in strain JRY3433 after digestion with *Xho*I. This yielded strain DMY1943. This strain was then transformed with plasmids pSIR2-LEU2 or pSIR2-H364Y-LEU2, generating strains DMY2165 and DMY2166, respectively. To create the *GFP-SIR4* strains, plasmid pDM274 was integrated at the *SIR4* locus in strain JRY3433 after digestion with *Sph*I. This yielded

strain DMY1944, which was then transformed with plasmids pSIR2-LEU2 or pSIR2-H364Y-LEU2, to generate strains DMY2167 and DMY2168, respectively.

Strains used in figures 3.6 and 3.7B were generated as follows: DMY754 was generated by transformation of strain BJ5459 with pDM271 after digestion with *Xho*I. pSIR2-H364Y-TRP1 was used to transform BJ5459 to yield strain DMY1371. To create strains DMY1923 and DMY1925, strains DMY754 and DMY1371 were transformed with plasmid pDM554 digested with *Bgl*II and *Pvu*I.

rDNA silencing strains in figure 3.8A (NTS), are based on strains DMY477 and DMY479. Strain DMY2121 was made by transformation with pJ107.1 after digestion with *Bam*HI and *Hin*dIII. To generate strains DMY2171 and DMY 2123, transformations with the PCR products generated (30 cycles of 94 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 2 min) with primers GH54 and GH55 (CATTCAAACCATT TTTCCCTCATCGGCACATTAAAGCTGGCGGATCCCCGGGTTAATTAA and TTTGGCACTTTTAAATTATTAAATTGCCTTCTACTTAGAGGAATTCGAGC TCGTTTAAAC) as well as GH75 and GH76 (ATGCCAAATGACAATAAGAC ACCCAATAGGTCCAGCACTCCGGATCCCCGGGTTAATTAA and GCATAATT TTGGCATCTGCCATATGCTCGGGCTCGACCACGAATTCGAGCTCGTTTAAAC) were performed according to Longtine et al. (1998). To establish equal auxotrophy of all strains, transformation with pRS313 and pRS305 cut with *Afl*II were performed where needed.

rDNA silencing strains figure 3.8B (25S), were made by transforming plasmid pJSS60-2 cut with either *Bst*XI or *Mlu*I for integration into *LEU2* or *rDNA/25S*, respectively. In addition, plasmid pRS304 cut with *Mfe*I and pRS303 cut with *Nhe*I was used to generate strains with equal auxotrophy.

rDNA silencing strains (Figure 3.10) were made by transforming plasmid pDM311 cut with either Sma I or Nhe I for integration into RDN1 and HIS3, respectively. Construction of sir2-H364Y and sir2-G262A has been previously described (Tanny and Moazed, 2001).

Correct integration was confirmed by PCR.

Strain	**Genotype**	**Source/Reference**
W303-1a	*MAT**a** ade2-1 can1-100 his3-11 leu2-3,112 trp1 ura3-1*	R. Rothstein
JRY3433	W303-1a with *sir2Δ::HIS3*	J. Rine
JRY3289	W303-1a with *sir3Δ::TRP1*	"
JRY3411	W303-1a with *sir4Δ::HIS3*	"
	(Figures 3.1, 3.2, 3.3, 3.7A, 3.9)	
DMY1864	JRY3433 with pSIR2-LEU2	Tanny, 1999
DMY1865	JRY3433 with pRS315	"
DMY1866	JRY3433 with pSIR2-H364Y-LEU2	"
DMY1867	JRY3433 with pSIR2-G262A-LEU2	"
	(Figure 3.5)	
DMY2163	JRY3433 with SIR2-GFP::URA3	this work
DMY2164	JRY3433 with SIR2-H364Y-GFP::URA3	"
DMY1943	JRY3433 with SIR3-GFP::URA3	"
DMY1944	JRY3433 with GFP-SIR4::URA3	"
DMY2165	DMY1942 with pSIR2-LEU2	"
DMY2166	DMY1942 with pSIR2-H364Y-LEU2	"
DMY2167	DMY1944 with pSIR2-LEU2	"
DMY2168	DMY1944 with pSIR2-H364Y-LEU2	"
	(Figures 3.6, 3.7B)	
BJ5459	*MAT**a** ura3-52 trp1 lys2-801 leu2 Δ1 pep4 Δ::HIS prb1Δ1.6R can1 GAL*	E. Jones
DMY754	BJ5459 with *SIR3-GFP::URA3*	this work
DMY1923	DMY754 with *sir4 Δ::LEU2*	"
DMY1925	DM13714 with *sir4 Δ::LEU2*	"
DMY1371	BJ5459 with *SIR2-H364Y::TRP1*	"
	(Figure 3.8A)	
DMY477	*MATα his3Δ200 leu2Δ1 ura3-167 (not rDNA)::Ty1-mURA3*	M. Grunstein
DMY479	*MATα his3Δ200 leu2Δ1 ura3-167 RDN1::Ty1-mURA3*	M. Grunstein
DMY2127	DMY479 with *pRS313(CEN/ARS HIS3) leu2::LEU2*	this work
DMY2125	DMY477 with *pRS313(CEN/ARS HIS3) leu2::LEU2*	"
DMY2171	DMY479 with *pRS313(CEN/ARS HIS3) leu2::LEU2 sir2Δ::kan^R*	"
DMY2121	DMY479 with *pRS313(CEN/ARS HIS3) sir3Δ:: LEU*	"
DMY2123	DMY479 with *pRS313(CEN/ARS HIS3) leu2::LEU2sir4Δ:: kan^R*	"
	(Figure 3.8B)	
DMY2177	W303-1a with *trp1::TRP1 his3::HIS3 mURA3:: LEU2*	this work
DMY2111	W303-1a with *trp1::TRP1 his3::HIS3 mURA3:: 25S/LEU2*	"
DMY2183	JRY3433 with *trp1::TRP1 mURA3:: 25S/LEU2*	"
DMY2187	JRY3289 with *his3::HIS3 mURA3:: 25S/LEU2*	"
DMY2191	JRY3411with *trp1::TRP1 mURA3:: 25S/LEU2*	"

TABLE 1 Yeast strains used in this study

Strain	Genotype	Source/Reference
	(Figure 3.10)	
DMY1928	*MATα ade2 his3 leu2 trp1 ura3 lys2 dam::LYS2 hht2,hhf2::HIS3 hht1,hhf1::LEU2 pRM200(CEN/ARS HHT2 HHF2-TRP1)*	M. Grunstein
DMY2129	DMY1928 with *sir2Δ::kan^R*	this work
DMY1493	*MATα ade2 his3 leu2 trp1 ura3 lys2 dam::LYS2 hht2,hhf2::HIS3 hht1,hhf1::LEU2 pRM430(CEN/ARS hht2Δ4-30 HHF2-TRP1)*	M. Grunstein
DMY1503	*MATα ade2 his3 leu2 trp1 ura3 lys2 dam::LYS2 hht2,hhf2::HIS3 hht1,hhf1::LEU2 pRM(CEN/ARS HHT2 hhf2K16Q-TRP1)*	M. Grunstein
DMY2137	DMY1928 with *mURA3::rDNA*	this work
DMY2139	DMY1928 with *mURA3::HIS3*	"
DMY2141	DMY2129 with *mURA3::rDNA*	"
DMY2143	DMY2129 with *mURA3::HIS3*	"
DMY2145	DMY1493 with *mURA3::rDNA*	"
DMY2147	DMY1493 with *mURA3::HIS3*	"
DMY2149	DMY1503 with *mURA3::rDNA*	"
DMY2151	DMY1503 with *mURA3::HIS3*	"

TABLE 1 (continued) Yeast strains used in this study

2.8 Microscopy

Cells expressing GFP fusion proteins were grown overnight, diluted 1:20 and grown to log phase in synthetic medium containing 22 mM sodium citrate (Straight et al., 1996), to reduce the background signal. Cells were visualized on a Nikon microscope and images were captured using a monochrome CCD camera. The images were then processed using the Metamorph software and the Canvas program.

2.9 Silencing Assays

rDNA silencing assays were performed as described by Smith and Boeke (1997). A URA3 gene under control of the TRP1 promoter (mURA3) is introduced into the mRNA array. Cells are then plated on YEPD plates, to insure that equal numbers of cells have been plated, and on media lacking uracil. If the marker gene is being silenced (and the gene is not transcribed), growth is poor on media lacking uracil. If the marker is not silenced, cells grow well on such media (see Figure 2.1 for schematic).

Overnight cultures were diluted 1:25 and grown to log-phase at 30 ºC. Three microliters of 10-fold serial dilutions of each culture in water were spotted on YEPD and SD-URA plates. Plates were incubated at 30 ºC for two to three days and photographed using a digital camera.

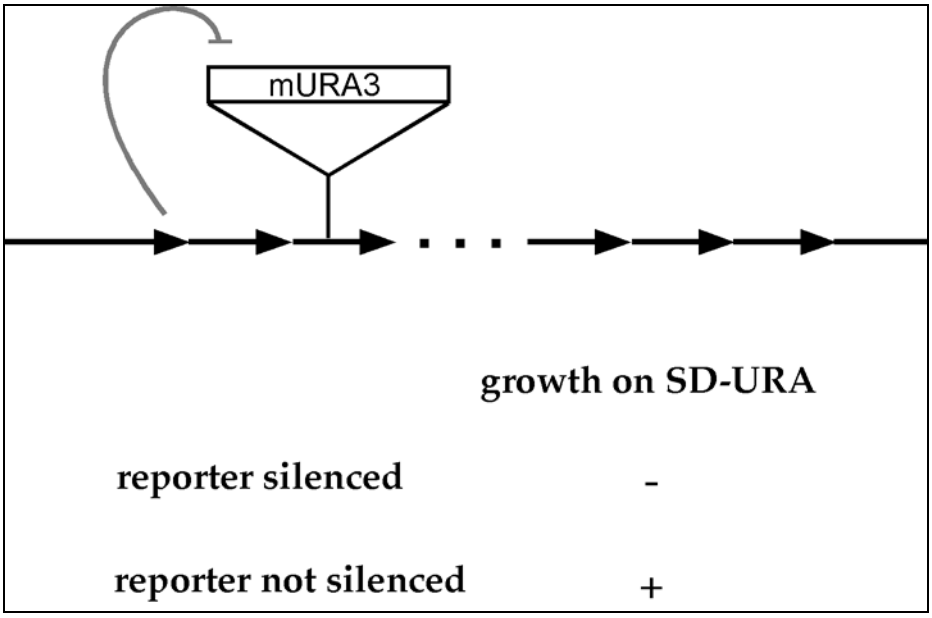

FIGURE 2.1 The principle of the silencing growth assay. A mURA3 reporter gene is inserted into the rDNA array. Growth of cells with a silenced or non-silenced reporter on media lacking uracil is indicated.

2.10 Chromation Immunoprecipitation (ChIP)

2.10.1 Principle

The aim of the Chromatin immunoprecipitation (ChIP) assay is to determine whether a particular protein is bound to a specific DNA sequence. It is also possible to assess the amount of protein bound when appropriate controls are being used.

In a ChIP assay, live cells are cross-linked with formaldehyde. This way, all protein-DNA as well as protein-protein interactions are being fixed. After cell lysis, the chromatin is sheared to produce soluble chromatin fragments. These can then be used in immunoprecipitations, using antibodies against a specific protein associated with DNA. The DNA that was part of the eluate is then freed by reversal of the cross-links. Using polymerase chain reaction (PCR) with primers specific for a certain DNA fragment, the amount of protein

that was originally bound can be assessed. The more protein was bound to the DNA, the more DNA serves as a template in the PCR and the stronger the signal.

The assay used in this study was performed as described by Kuras and Struhl (1999), and Strahl-Bolsinger et al. (1997).

2.10.2 Preparation of soluble chromatin

400 ml of yeast cells were grown to an OD_{660} of 1.5 and cross-linked with 1 % formaldehyde for 20 min at room temperature. Glycine was added to a final concentration of 125 mM and incubation was continued for 5 min. After washing twice with ice cold TBS, cells were resuspended in FA lysis buffer. Lysis was performed using zirconia/silica beads (Biospec) in a mini-bead beater with 4 pulses of 90 sec followed by 90 sec on ice. The lysate was sonicated three times at 4 ºC for 20 sec using a Sonifier equipped with a microtip to generate a mean DNA size of 0.1-1 kb, as checked on an agarose mini-gel. The lysate was cleared by centrifugation and frozen in liquid nitrogen for storage at –80 °C.

2.10.3 Immunoprecipitation

Immunoprecipitations were carried out in 500 microliters of lysate with either 1 microliter anti-Sir2 antibody, 0.5 microliter anti-Sir3 antibody (Tanny et al., 1999), 1 microliter anti-Sir4 antibody, or 5 microliters of anti-acetyl-H4 (Upstate Biotechnology) overnight at 4 ºC. 20 microliters protein-A Sepharose CL-4B beads (Amersham Pharmacia) were added and incubation was continued for 90 min. Using a nutator, beads were washed for 5 min at room temperature in the following buffers: twice in FA lysis buffer/0.1 % SDS, twice with FA lysis buffer/0.1 % SDS/500 mM NaCl, once in 10 mM Tris-HCl pH 8, 0.25 M LiCl, 1 mM EDTA 0.5 % NP-40, 0.5 % sodium deoxycholate and once in TE. Elution was performed by incubating the beads in 250 microliters elution buffer at 65 ºC for 10 min. Samples were treated with 800 µg/ml Pronase (Boehringer Mannheim) and incubated for 1 h

at 42 ºC followed by reversal of cross-links at 65 ºC for 5 h. Samples were Phenol/Chloroform extracted and ethanol precipitated by addition of 20 microliters 4 M LiCl and 1 ml ethanol, followed by an overnight incubation at -20 °C.

2.10.4 Polymerase chain reaction (PCR)

PCRs were carried out in a volume of 20 microliters with $1/50^{th}$ of the immunoprecipitated material and $1/25{,}000^{th}$ of input material. The reaction was carried out with 1 μM of primers, 0.1 mM of dNTPs, and 0.1 mCi/ml of [α-^{32}P]-dCTP with a specific activity 3000 Ci/mmol (Amersham Pharmacia). The number of cycles was determined in initial experiments using 2.5-fold dilutions to insure linearity of the signal. Cycling parameters were: 1 cycle of 95 ºC for 2 min, 55 ºC for 30 sec, 72 ºC for 1 min, followed by cycles of 95 ºC for 30 sec, 55 ºC for 30 sec, 72 ºC for 1 min, and a final step at 72 ºC for 4 min. Typically, 20 cycles were used for rDNA and 26 cycles for telomeres and *HM* loci.

2.10.5 Separation of bands and quantitation

Samples were run on an 8 % polyacrylamide gel at 200 V for 1 h in a vertical gel electrophoreses chamber using TBE. The gel was dried and exposed to a Fuji Phosphorimager screen and scanned on a BioRad Phosphorimager. Quantitation was performed using QuantityOne software (BioRad). Standard deviation was calculated using Microsoft Excel.

2.12.6 Oligonucleotides

The following primer pairs, purchased from Operon, were used for ChIP experiments:

TEL0.6	CAGGCAGTCCTTTCTATTTC and GCTTGTTAACTCTCCGACAG
TEL1.4	AATGTCTTATCAAGACCGAC and TACAGTCCAGAAATCGCTCC
TEL2.8	CTGATCTGATGTTCTCACGC and TCTGTATGAGTCATCGAAGC
TEL4.7	GGTTCGTGACTACAAAGG and CTACCAACAGATGAGGTC
NTS1/1	CTCCTCCGATATTCCTAC and TGCAAGATGAATAGCCAG
NTS1/2	GCT TCC TAT GCT AAA TCC and AGAAGCAACTAAACGAGG
NTS2/1	GGTAACCCAGTTCCTCAC and CTTTCCTGTTATGGCATGG
NTS2/2	GCATGAAGTACCTCCCAACT and CGCTTCCGCTTCCGCAGTAA
25S/1	GACGTCATAGAGGGTGAGAA and TTGACTTACGTCGCAGTCCT
25S/2	ATTTCACTGGCGCCGAAGCT and TACGGACAAGGGGAATCTGA
18S	TAGAGTGTTCAAAGCAGGCG and CCCAGAACCCAAAGACTTTG
HML-E	GGATGGATCTAGGGTTTTATGCC and TTTGGCCCCCGAAATCG
HML0.6	AGACGGCCAGAAACCTC and TCGCCTACCTTCTTGAAC
CUP1	TTTTCCGCTGAACCGTTCCA and CATTGGCACTCATGACCTTC or GAGAAGCAAATAACTCCTTGTC
ACT1	CCAATTGCTCGAGAGATTTC and CATGATACCTTGGTGTCTTG