Identification of High-Copy Disruptors of Telomeric Silencing in *Saccharomyces cerevisiae*

Miriam S. Singer,* Alon Kahana,*,† Alexander J. Wolf,† Lia L. Meisinger,* Suzanne E. Peterson,† Colin Goggin,* Maureen Mahowald* and Daniel E. Gottschling†

**Department of Molecular Genetics and Cell Biology, The University of Chicago, Chicago, Illinois 60637 and †Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, Washington 98109*

Manuscript received April 20, 1998
Accepted for publication July 14, 1998

ABSTRACT

The ends of chromosomes in *Saccharomyces cerevisiae* initiate a repressive chromatin structure that spreads internally and inhibits the transcription of nearby genes, a phenomenon termed telomeric silencing. To investigate the molecular basis of this process, we carried out a genetic screen to identify genes whose overexpression disrupts telomeric silencing. We thus isolated 10 *DOT* genes (disruptor of telomeric silencing). Among these were genes encoding chromatin component Sir4p, DNA helicase Dna2p, ribosomal protein L32, and two proteins of unknown function, Asf1p and Ifh1p. The collection also included genes that had not previously been identified: *DOT1, DOT4, DOT5, DOT6,* and *TLC1,* which encodes the RNA template component of telomerase. With the exception of *TLC1,* all these genes, particularly *DOT1* and *DOT4,* also reduced silencing at other repressed loci (*HM* loci and rDNA) when overexpressed. Moreover, deletion of the latter two genes weakened silencing as well, suggesting that *DOT1* and *DOT4* normally play important roles in gene repression. *DOT1* deletion also affected telomere tract length. The function of Dot1p is not known. The sequence of Dot4p suggests that it is a ubiquitin-processing protease. Taken together, the *DOT* genes include both components and regulators of silent chromatin.

THE natural ends of linear eukaryotic chromosomes are made up of specialized DNA sequences and additional factors that are associated with them. The resulting macromolecular structures, called telomeres, are important in maintaining the integrity of the genome. Whereas broken chromosome ends, which lack telomeres, are commonly substrates for DNA joining, recombination, and degradation, telomeres are poor substrates for such reactions; hence, telomeres serve as protective "caps" for the DNA ends (Zakian 1996; Pryde and Louis 1997; van Steensel *et al.* 1998).

Telomeres not only define the physical nature of the DNA termini, but they also affect the nearby sequences that make up the distal regions of the chromosomes. In a phenomenon that is likely related to their role as protectors of the DNA ends, telomeres render these telomere-proximal domains inert, or inaccessible, relative to other regions of the genome. This protection has been observed physically, as a decreased accessibility of telomere-proximal DNA to the activity of DNA modifying enzymes expressed *in vivo* (Gottschling 1992). It has also been observed genetically because telomeres in *Saccharomyces cerevisiae,* as well as Drosophila and Trypanosoma, repress, or silence, the expression of nearby genes (Levis *et al.* 1985; Gottschling *et al.* 1990; Zomerdijk *et al.* 1991; Horn and Cross 1995).

The phenomenon of telomere-mediated gene silencing has been used to analyze the molecular basis of the telomere's effects on nearby DNA; the understanding that has emerged from this work is that a repressive chromatin structure initiates from the telomere and extends inward along the chromosome, rendering the enveloped DNA inaccessible to factors such as those of the transcriptional machinery (Renauld *et al.* 1993). Structural components of silent telomeric chromatin include the telomere sequence DNA-binding protein Rap1p, nucleosomal core histones H3 and H4, and nonhistone chromatin components Sir2p, Sir3p, and Sir4p (Aparicio *et al.* 1991; Kyrion *et al.* 1993; Thompson *et al.* 1994). Current models of telomeric silencing suggest that the Sir proteins are recruited to the telomeres through their interactions with Rap1p and each other, and then "polymerize" along the unique, telomere-adjacent sequences by binding the N-terminal tails of histones H3 and H4 of the associated nucleosomes (reviewed in Grunstein 1997).

Telomeric silencing in *S. cerevisiae* is inherited in a semistable manner (Gottschling *et al.* 1990); *i.e.,* the repressed transcriptional state is generally present through multiple generations of a growing clonal population, but it is occasionally reversed in a stochastic manner. However, the resulting transcriptionally competent

Corresponding author: Daniel E. Gottschling, Division of Basic Sciences, Mail-Stop A3-025, Fred Hutchinson Cancer Research Center, 1100 Fairview Ave. North, P.O. Box 19024, Seattle, WA 98109-1024. E-mail: dgottsch@fhcrc.org

state is itself only heritable in the same limited way. When a color marker gene such as *ADE2* is located near a telomere (Gottschling *et al.* 1990), this switching of transcriptional states results in red-and-white sectored colonies. The switching between expression levels can be explained in part as the effect of shifts in a competition between silencing components and transcriptional-activating factors for assembly onto telomere-proximal DNA (Aparicio and Gottschling 1994).

The fact that under normal circumstances the preexisting transcriptional state is most often inherited despite this competition indicates the existence of some mechanism to favor the status quo through the successive cell cycles. In particular, assembly (or reassembly) of the silent chromatin must occur during or shortly after each round of DNA replication. Consistent with this idea, a number of unrelated mutations or drug treatments that lengthen S phase, and presumably affect the kinetics and coordination of molecular events in S phase, are able to suppress defects in silencing (Axelrod and Rine 1991; Laman *et al.* 1995). Furthermore, silencing is sensitive to mutations in subunits of chromatin assembly factor I, an activity that has been found *in vitro* to facilitate assembly of newly replicated DNA into nucleosomes (Enomoto *et al.* 1997; Kaufman *et al.* 1997). Hence, there appears to be an intimate coordination between silent chromatin assembly and DNA replication.

Telomeric silencing is mechanistically similar to silencing of the nontelomeric, cryptic mating type loci *HML* and *HMR* (Laurenson and Rine 1992; Loo and Rine 1995). In fact, all the factors described above as components of the telomeric silencing apparatus are required at the *HM* loci, although additional factors are also needed at *HML* and *HMR*, some of which appear to be involved in the recruitment of the Sir proteins to these sites (Loo and Rine 1995; Triolo and Sternglanz 1996; Fox *et al.* 1997). The ribosomal RNA gene locus (rDNA/*RDN1*) is another region of the *S. cerevisiae* genome that can silence genes. However, silencing at this locus is qualitatively different than at telomeres or *HM* loci. For example, of the known silencing components, only the *SIR2* gene product is required for rDNA silencing (Bryk *et al.* 1997; Fritze *et al.* 1997; Smith and Boeke 1997).

Given that various silent loci use common factors (such as certain Sir proteins), the pattern of silencing achieved in the cell must reflect the equilibrium reached in the competition between these loci for factor binding (see Lloyd *et al.* 1997). For example, the distance that silent chromatin spreads internally along the chromosome from a telomere is directly related to the amount of Sir3p in the cell (Renauld *et al.* 1993; Hecht *et al.* 1996), indicating that this component of silencing in *S. cerevisiae* is normally in limited supply (also see Maillet *et al.* 1996). If a nontelomeric silencing locus developed a relative advantage in Sir3p binding, it would follow that the spread of telomeric silencing would decrease below its normal levels. Such a competitive interaction between sites would constitute a level of cellular regulation. Supporting this notion is the finding that certain genetic and physiological changes, such as aging, cause a shift in the relative abundance of silencing components between different loci (Buck and Shore 1995; Kennedy *et al.* 1997). Thus, to understand how silent telomeric chromatin is established or maintained, it is necessary to identify the silencing factors and to appreciate how they compete at a given locus with transcription activation components, how the assembly of silent chromatin is coordinated with DNA replication, and how limited silencing factors are distributed between different silent loci.

In a number of genetic systems, increased dosage or inappropriate expression of gene products in mutant or wild-type forms have been used in the analysis of complex biological assembly processes (reviewed in Herskowitz 1987). We have adopted these approaches to investigate telomeric silencing in *S. cerevisiae*. Based on the assumption that telomeric silencing is the result of a multimeric complex of factors that is assembled in a coordinated fashion, and that the assembly process might be easily disrupted by a stoichiometric imbalance of its components, we screened for gene products whose increased dosage disrupted telomeric silencing. Here we describe the genes identified in this screen.

MATERIALS AND METHODS

Yeast strains and media: *S. cerevisiae* strains used in this study are shown in Table 1. Strain UCC3511 was constructed in several steps. The *SIR2* gene was disrupted in YPH250 by transformation with pJR531 (gift of J. Rine), followed by selection for His^+ cells, thus producing UCC2666. *HMR*a was then disrupted by transformation with pVZ+HMRa::URA3 digested with *Bam*HI and *Sal*I, followed by selection for Ura^+ transformants, to produce UCC2670. UCC2670 was transformed with pJH423 (gift of R. Esposito), a *SIR2*-containing plasmid (YEp13, *LEU2*), and crossed with YPH102 (Sikorski and Hieter 1989), producing a diploid strain that was then sporulated to give UCC2675. Finally, UCC2675 was crossed with YPH499 and sporulated to give UCC3511.

UCC3532 was made by transforming YPH499 (Sikorski and Hieter 1989) with pHR10-6 (Singer and Gottschling 1994). UCC4566 was created by transforming UCC3532 with pAK4 (Huang *et al.* 1997) that was digested with *Sal*I and *Not*I and selecting for Ura^+ transformants. *PPR1* was disrupted in UCC4566 using pΔPPR1::LYS2 (Renauld *et al.* 1993) to create UCC4567.

UCC3500 was made by transforming UCC111 (Aparicio and Gottschling 1994) with pHR10-6 (Singer and Gottschling 1994). UCC3503 was made by successively transforming YPH102 (Sikorski and Hieter 1989) with pVII-L URA3-TEL (Gottschling *et al.* 1990) and pHR10-6 (Singer and Gottschling 1994). UCC3500 and UCC3503 were then crossed to create the diploid UCC3519. Finally, *DOT1* was disrupted by transforming UCC3519 with pVZ28::LEU2, which was digested with *Sph*I and *Xba*I, and selecting for Leu^+ transformants, thus creating UCC4551. UCC4551 was then

TABLE 1

Yeast strains

Strain	Genotype	Source
JSS125 (S3)	*MAT*α *his3-*Δ*200 leu2*Δ*1 ura3-167 RDN1::URA3*	Smith and Boeke (1997)
JSS128 (S6)	*MAT*α *his3-*Δ*200 leu2*Δ*1 ura3-167 RDN1::URA3*	Smith and Boeke (1997)
UCC3503	*MAT*α *ura3-52 lys2-801 ade2-101 leu2-*Δ*1 his3-*Δ*200 adh4::URA3-TEL-VIIL DIA5-1*	This study
UCC3504	*MAT*α *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 ura3-52 ppr1::LYS2 adh4::URA3-TEL-VIIL DIA5-1*	This study
UCC3505	*MAT***a** *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*63 ura3-52 ppr1::HIS3 adh4::URA3-TEL-VIIL DIA5-1*	Singer and Gottschling (1994)
UCC3511	*MAT*α *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*63 ura3-52 hmr::URA3*	This study
UCC3515	*MAT*α *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*63 ura3-52 hml::URA3*	Singer and Gottschling (1994)
UCC3611	*MAT*α *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 ura3-52 ppr1::LYS2 adh4::URA3-TEL-VIIL DIA5-1 asf1::HIS3*	This study
UCC3612	*MAT*α *lys2-801 trp1-*Δ*63 hml::URA3 ade2-101 his3-*Δ*200 leu2-*Δ*1 ura3-52 asf1::HIS3*	This study
UCC3615	*MAT*α *ura3-52 lys2-801 ade2-101 leu2-*Δ*1 his3-*Δ*200 trp1-*Δ*63 hmr::URA3 ppr1::LYS2 asf1::HIS3*	This study
UCC3617	*MAT***a** *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*63 ura3-52 ppr1::LYS2 adh4::URA3 DIA5-1 asf1::HIS3*	This study
UCC4554	*MAT***a** *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*63 ura3-52 adh4::URA3-TEL-VIIL DIA5-1 dot1::LEU2*	This study
UCC4555	*MAT***a** *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*63 ura3-52 adh4::URA3-TEL-VIIL DIA5-1 dot1::LEU2*	This study
UCC4560	*MAT***a** *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 ura3-52 ppr1::HIS3 adh4::URA3-TEL-VIIL DIA5-1 dot1::LEU2*	This study
UCC4561	*MAT*α *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 ura3-52 ppr1::HIS3 adh4::URA3-TEL-VIIL DIA5-1 dot1::LEU2*	This study
UCC4562	*MAT***a** *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*63 ura3-52 adh4::URA3-TEL-VIIL DIA5-1*	This study
UCC4563	*MAT*α *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 ura3-52 ppr1::HIS adh4::URA3-TEL-VIIL DIA5-1*	This study
UCC4564	*MAT*α *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*63 ura3-52 ppr1::LYS2 hmr::URA3*	This study
UCC4565	*MAT*α *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*63 ura3-52 ppr1::LYS2 hml::URA3*	This study
UCC4566	*MAT***a** *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*63 ura3-52 adh4::URA3 DIA5-1*	This study
UCC4567	*MAT***a** *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*63 ura3-52 ppr1::LYS2 adh4::URA3 DIA5-1*	This study
UCC4571	*MAT***a** *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*63 ura3-52 ppr1::LYS2 adh4::URA3 DIA5-1 dot1::HIS3*	This study
UCC4574	*MAT*α *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*63 ura3-52 hml::URA3 dot1::HIS3*	This study
UCC4576	*MAT***a** *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*63 ura3-52 ppr1::LYS2 adh4::URA3 DIA5-1 dot4::HIS3*	This study
UCC4579	*MAT*α *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*63 ura3-52 ppr1::LYS2 hmr::URA3 dot4::HIS3*	This study
UCC4580	*MAT***a** *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*63 ura3-52 ppr1::HIS adh4::URA3 dot4::HIS3*	This study
UCC4583	*MAT*α *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 ura3-52 ppr1::LYS2 adh4::URA3-TEL-VIIL DIA5-1 dot4::HIS3*	This study
UCC4586	*MAT*α *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*63 ura3-52 hmr::URA3 dot1::HIS3*	This study
UCC4591	*MAT*α *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 ura3-52 adh4::URA3-TEL-VIIL DIA5-1 dot4::HIS3*	This study
UCC4594	*MAT*α *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 ura3-52 ppr1::LYS2 adh4::URA3-TEL-VIIL DIA5-1 dot4::HIS3*	This study
UCC4595	*MAT*α *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 ura3-52 ppr1::LYS2 adh4::URA3-TEL-VIIL DIA5-1*	This study

(*continued*)

TABLE 1

(Continued)

Strain	Genotype	Source
UCC4602	*MAT*α *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 ura3-52 adh4::URA3-TEL-VIIL DIA5-1*	This study
UCC6008	*MAT*α *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 ura3-52 ppr1::LYS2 adh4::URA3-TEL-VIIL DIA5-1 dot1::HIS3*	This study
UCC6541	*MAT***a** *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 trp1-*Δ*63 ura3-52 ppr1::LYS2 adh4::URA3 DIA5-1 sir4::HIS3*	This study
UCC6542	*MAT*α *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 ura3-52 ppr1::LYS2 adh4::URA3-TEL-VIIL DIA5-1 sir4::HIS3*	This study
UCC6550	*MAT*α *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 ura3-52 ppr1::LYS2 adh4::URA3-TEL-VIIL DIA5-1 dot5::HIS3*	This study
UCC6552	*MAT*α *ade2-101 his3-*Δ*200 leu2-*Δ*1 lys2-801 ura3-52 ppr1::LYS2 adh4::URA3-TEL-VIIL DIA5-1 dot6::HIS3*	This study
UCC6555	*MAT*α *ade2*Δ*::hisG his3-*Δ*200 leu2-*Δ*0 lys2*Δ*0 met15*Δ*0 trp1*Δ*63 ura3*Δ*0 adh4::URA3-TEL-VIIL ppr1::LYS2*	This study
UCC6562	*MAT*α *ade2*Δ*::hisG his3-*Δ*200 leu2-*Δ*0 lys2*Δ*0 met15*Δ*0 trp1*Δ*63 ura3*Δ*0 adh4::URA3-TEL-VIIL ppr1::LYS2 asf1::HIS3*	This study
UCC6605	*MAT*α *his3-*Δ*200 leu2-*Δ*1 ura3-167 RDN1::URA3 dot1::HIS3*	This study
UCC6606	*MAT*α *his3-*Δ*200 leu2-*Δ*1 ura3-167 RDN1::URA3 dot4::HIS3*	This study
UCC6607	*MAT*α *his3-*Δ*200 leu2-*Δ*1 ura3-167 RDN1::URA3 dot5::HIS3*	This study
UCC6608	*MAT*α *his3-*Δ*200 leu2-*Δ*1 ura3-167 RDN1::URA3 dot6::HIS3*	This study
UCC6609	*MAT*α *his3-*Δ*200 leu2-*Δ*1 ura3-167 RDN1::URA3 asf1::HIS3*	This study
UCC6616	*MAT*α *his3-*Δ*200 leu2-*Δ*1 ura3-167 RDN1::URA3 sir4::HIS3*	This study

DIA5-1 refers to a direct integration of *ADE2* adjacent to the right telomere of chromosome *V* (Singer and Gottschling (1994).

sporulated, yielding the haploid segregants UCC4554, UCC4555, UCC4560, UCC4561, UCC4562, and UCC4563.

UCC3503 was transformed with pΔPPR1::LYS2 (Renauld *et al.* 1993) to create UCC3504. Strains UCC3503 and UCC3505 (Singer and Gottschling 1994) were crossed to each other, and the resulting diploid was sporulated to yield UCC3537. UCC3537 and UCC3504 were crossed to create UCC3542. *DOT4* was then disrupted by transforming UCC3542 with plasmid pdot4::HIS3(−), which was digested with *Sph*I and *Bam*HI, and selecting for His^+ transformants, thus creating two independent transformants, UCC4572 and UCC4573. UCC4572 was sporulated to yield the haploid segregant UCC4602. UCC4573 was sporulated to yield the haploid segregants UCC4591, UCC4594, and UCC4595.

Strains UCC4586 and UCC4574 were made by transforming UCC3511 and UCC3515, respectively, with pdot1::HIS3(+), which was digested with *Sph*I and *Xba*I. Similarly, UCC4577 and UCC4578 were created by transforming UCC3511 and UCC3515, respectively, with pdot4::HIS3(−), which was digested with *Sph*I and *Bam*HI. In all these transformations, His^+ transformants were selected.

UCC4564 and UCC4565 were made by deleting *PPR1* in UCC3511 and UCC3515, respectively, using plasmid pΔPPR1::LYS2 (Renauld *et al.* 1993). Strains UCC4579 and UCC4580 were then made by transforming UCC4564 and UCC4565, respectively, with pdot4::HIS3(−), which was digested with *Sph*I and *Bam*HI, and selecting for His^+ transformants.

To make UCC4571, UCC4567 was transformed with pdot1::HIS3(+), which was digested with *Sph*I and *Xba*I. To make UCC4576, UCC4567 was transformed with pdot4::HIS3(−), which was digested with *Sph*I and *Bam*HI. To make UCC3617, UCC4567 was transformed with pasf1::HIS3 digested with *Not*I and *Sal*I. To make UCC6541, UCC4567 was transformed with pRS4.2 (Kimmerly and Rine 1987), which was digested with *Pvu*II. In all cases, His^+ transformants were selected.

UCC6008, UCC4583, UCC3611, and UCC6542 were made by fragment-mediated transformation of UCC3504 using DNA from plasmids pdot1::HIS3(+), pdot4::HIS3(−), pasf1::HIS3, and pRS4.2, respectively. The plasmids were digested as described above. UCC6550 was created by transforming UCC3504 with plasmid pBlu49::HIS3#1 digested with *Xho*I and *Eco*RI. UCC6552 was created by transforming UCC3504 with plasmid pBlu23::HIS3#1 digested with *Xho*I and *Not*I. In all cases, His^+ transformants were selected.

UCC6555 was made by transforming BY4705 (Brachmann *et al.* 1998) with pVII-L URA3-TEL (Gottschling *et al.* 1990), creating UCC1091, which was subsequently transformed with pΔPPR1::LYS2 (Renauld *et al.* 1993). To make UCC6562, UCC6555 was transformed with pasf1::HIS3 digested with *Not*I and *Sal*I, and His^+ transformants were selected.

UCC3615 and UCC3612 were made by deleting *ASF1* in UCC4564 and UCC3515, respectively, using pasf1::HIS3 as described above.

UCC6605, UCC6606, UCC6607, UCC6608, and UCC6609 were made by fragment-mediated transformation of JS125 (Smith and Boeke 1997) using DNA from plasmids pdot1::HIS3(+), pdot4::HIS3(−), pBlu49::HIS3#1, pBlu23::HIS3#1, and pasf1::HIS3, respectively. The plasmids were digested as described above. To make UCC6616, JS128 (Smith and Boeke 1997) was transformed with pRS4.2 (Kimmerly and Rine 1987) digested with *Pvu*II.

TLC1 disruptions were made in UCC3503, UCC3504, UCC3511, UCC4564, UCC3515, UCC4565, and JS125 by fragment-mediated transformation using DNA from pSD166 cut with *Not*I and *Sal*I. His^+ colonies were simultaneously streaked onto fresh plates with media lacking histidine, and were subjected to colony PCR to check for proper integration. Colonies

from the restreak were then used in serial dilution assays and in overnight cultures for isolation of genomic DNA before telomeric shortening could cause senescence.

S. cerevisiae cultures were grown at 30° and liquid cultures were agitated at ~200 rpm. YEPD (rich) growth medium contains 10 g yeast extract, 20 g Bacto-peptone, and 20 g glucose/liter. The synthetic (HC) media has been previously described (Adams *et al.* 1998). For silencing assays on galactose-containing media, colonies were pregrown on 3% galactose medium for 4 days and then resuspended in water. Tenfold serial dilutions were then plated onto 3% galactose medium lacking or containing uracil, and the cells were again incubated for 4 days before the colonies were counted. Transformations were carried out according to a standard lithium acetate procedure (Ausubel *et al.* 1995). All 6-azauracil (6-AU)-containing media were made from a 2 g/liter filter-sterilized stock added after the media had been autoclaved and cooled to ~60°.

Transformation of the pTRP library into UCC3505: UCC3505 cells were pregrown in rich (2% glucose) medium and transformed with the pTRP library DNA (Singer and Gottschling 1994). Transformants were plated onto synthetic medium containing 2% glucose and lacking tryptophan to select for cells that had been transformed with the *TRP1*-bearing plasmids. After 6 days of growth, the colonies were replica plated onto synthetic medium containing 3% galactose (to induce strong transcription from the *GAL1* promoter of the pTRP vector) and lacking tryptophan (HC-trp 3% galactose). After 3 days of growth on HC-trp (3% galactose) medium, the colonies were replica plated onto synthetic medium containing 3% galactose and lacking tryptophan and uracil (to select for strains in which there had been a derepression of the telomeric *URA3* gene). The Ura^+ colonies were then restreaked onto the same medium and the color of individual colonies was inspected. White Ura^+ colonies were then checked for their phenotypes on medium containing 2% glucose. Only those colonies that were Ura^- and red/white sectoring when grown on glucose medium were retained. Plasmid DNA was isolated from each of these transformants (Hoffman and Winston 1987) and reintroduced into UCC3505 to confirm that the galactose-dependent loss of silencing was indeed plasmid linked.

6-AU assay: pTRP plasmid-bearing strains to be tested for 6-AU sensitivity were pregrown on solid HC-trp + 3% galactose medium for 4 days at 30°, and then serial dilutions were plated onto HC-trp-ura + 3% galactose medium containing 6-AU at concentrations ranging from 1 to 5 μg/ml. Strains to be tested that were not carrying plasmids were pregrown on YEPD (rich) medium for 3 days at 30°, and then serial dilutions were plated on HC-ura plates that contained 10, 20, or 30 μg/ml 6-AU.

Plasmid constructions: pVZ+HMRa::URA3 was constructed in multiple steps. pFATRS303 was constructed by cloning an *Xba*I fragment from pFAT10 (Runge and Zakian 1989) into the *Aat*II site of pRS303 (Sikorski and Hieter 1989). pHMRa-lacZ (a gift from M. Hochstrasser) was digested with *Bgl*II and religated to remove the *lacZ* gene and produce pHMRa. A *Pst*I-*Eco*RI fragment (4 kbp) from pHMRa was cloned into pFATRS303 that was digested with *Sma*I to give pFATRS303-HMRa2. A *Sal*I-*Bam*HI fragment (4 kbp) containing *HMRa* was then ligated to pVZ1 (Henikoff and Eghtedarzadeh 1987) that was digested with *Sal*I and *Bam*HI. Finally, a *Bam*HI fragment (1.1 kbp) containing *URA3* from pM20 (a gift from R. Schiestl) was cloned into the *Bgl*II site of pVZ1+HMRa to produce pVZ+HMRa::URA3.

pVZ28 was made by partially digesting pTRP28 with *Xho*I and ligating the 1.9-kb fragment containing *DOT1* into the *Sal*I site of pVZ1. pVZ28::LEU2 was made by blunt-end ligation of a *Bam*HI fragment from YDp-L (Berben *et al.* 1991) into pVZ28 digested with *Afl*II and *Xho*I. pdot1::HIS3(+) was made by blunt-end ligation of a *Bam*HI fragment from YDp-H (Berben *et al.* 1991) into pVZ28 digested with *Afl*II and *Xho*I such that the *HIS3* and *DOT1* genes had the same transcriptional orientation.

pVZDOT4 was made by blunt-end ligation of an *Xho*I fragment containing *DOT4* from pTRP50 into pVZ1(-H3) (the *Hin*dIII site was destroyed by digestion with *Hin*dIII, followed by T4 polymerase treatment and blunt-end ligation) digested with *Pst*I/*Hin*cII. pdot4::HIS3(−) was made by blunt-end ligation of a *Bam*HI fragment from YDp-H (Berben *et al.* 1991) into pVZDOT4 that was digested with *Hin*dIII and *Nco*I such that the *HIS3* and *DOT4* genes had the same transcriptional orientation.

ASF1 was cloned as a 1.5-kb PCR product from an amplification from YPH499 (Sikorski and Hieter 1989) genomic DNA. The primers used in the reaction were ASF#1: 5′-CGG GATCCTTGGCGAGAATTTCGATTTTCAGG-3′ and ASF#2: 5′-GACTAGTGTGTTTTATGAACTTTTAGGATGACGTATT G-3′. The PCR reaction used 28 pmol of each primer in a 100-μl reaction, which also included 1× Taq buffer (Promega, Madison, WI), 0.2 mm dNTPs, 2 mm $MgCl_2$, Taq enzyme (Promega), and YPH499 (Sikorski and Hieter 1989) genomic DNA from ~10^7 cells. The PCR product was digested with *Bam*HI and *Spe*I, and ligated to pBluescript II KS− (Stratagene, La Jolla, CA) that was digested with *Bam*HI and *Xba*I, thus constructing pBlueASF1. To construct pasf1::HIS3, a 1.2-kb *HIS3*-containing *Bam*HI fragment from YDp-H (Berben *et al.* 1991) was used to replace a 0.9-kb *Sna*BI-*Nde*I fragment of pBlueASF1 through a blunt-end ligation.

pBlu49 was constructed by ligating the 0.8-kb *Xho*I fragment containing *DOT5* from pTRP49 into the *Sal*I site of pBluescript II KS−. To make pBlu49::HIS3#1, the 1.2-kb *Bam*HI fragment containing *HIS3* from YDP-H (Berben *et al.* 1991) was used to replace the 0.5-kb *Pfl*MI-*Sna*BI internal *DOT5* fragment in pBlu49.

pBlu23 was constructed by ligating the 2.1-kb *Xho*I fragment containing *DOT6* from pTRP23 into the *Sal*I site of pBluescript II KS− (Stratagene). To make pBlu23::HIS3#1, the 1.2-kb *Bam*HI fragment containing *HIS3* from YDP-H (Berben *et al.* 1991) was used to replace the 0.8-kb *Mlu*I-*Afl*II *DOT6* fragment in pBlu23.

pRS313/Y′RsaI was constructed by ligating the 350-bp *Rsa*I Y′ sequence-containing fragment from pY′ARS into the *Sma*I site of pRS313 (Sikorski and Hieter 1989). pY′ARS (made by Jeff Stevenson) was constructed by cloning the 1-kb *Xho*I/*Sph*I fragment from pYP1-L2 (gift from E. Louis) into vector pVZ1, which had been digested with *Sal*I and *Sph*I.

YTCA-1 differs from YTCA-2 (Gottschling *et al.* 1990) only in that the 125-bp *Hae*III-*Mnl*I fragment is present in reversed orientation.

pSD166 was constructed in multiple steps: A 3.9-kb *Eco*RI fragment containing *TLC1* was excised from pAZ1 (Beeler *et al.* 1994) and inserted into the *Eco*RI site of pRS424 (Christianson *et al.* 1992) to create p424/TLC1g. From p424/TLC1g, a 4-kbp *Not*I/*Sal*I fragment was cloned into *Not*I/*Sal*I-digested pRS425 (Christianson *et al.* 1992), creating pSD141. pSD141 was then cut with *Bgl*II and *Nde*I and transformed into UCC3586, and the gap-repaired plasmid was recovered, creating pSD143, a plasmid containing the *tlc1::HIS3* disruption. A *Not*I/*Sal*I fragment from pSD143 was next cloned into *Not*I/*Sal*I-digested pVZ1, creating pSD166.

Analysis of nucleic acids: Methods for DNA preparation and analysis have been described previously (Hoffman and Winston 1987; Gottschling *et al.* 1990). DNA sequencing was carried out using the Taq DyeDeoxy Terminator Cycle Sequencing Kit from Applied Biosystems (Foster City, CA) according to the manufacturer's instructions. To sequence the ends of the *DOT* cDNA clones, *GAL1* and *CYC1* primers

were used (*GAL1*, 5′-CCTCTATACTTTAACGTCAAGGAG; *CYC1*, 5′-GAAAAGGGGCCTGTTTACTCA CAG).

DNA blot hybridization analyses and probe synthesis were carried out using the Genius system from Boehringer Mannheim (Indianapolis, IN) following the manufacturer's instructions. Probes for the Southern analysis were synthesized by PCR: 1 μl miniprep template DNA, 20 pmol of each primer, 1.5 μl 3 m KCl, 10 μl 25 mm $MgCl_2$, 1 μl 1 m Tris, pH 8.5, 10 μl 10× dig PCR mix (2 mm dGTP, 2 mm dATP, 2 mm dCTP, 1 mm dTTP, and 0.5 mm digoxigenin-11-dUTP), and 1 μl Taq enzyme were combined with water to bring the final volume to 100 μl. The reaction was then exposed to the following program of conditions: (1) 94° 5 min, (2) 94° 30 sec, (3) 50° 1 min, (4) 70° 2 min, and (5) 34 more repetitions of steps 2–4. To make the Y′ probe, T3 (5′-AGCGCGCAATTAACCCT CACTAAAG-3′) and T7 (5′-CGTAATACGACTCACTATAG GG-3′) primers were used in conjunction with plasmid pRS313/Y′RsaI template DNA. To make the $TG_{1\text{-}3}$ probe, M13 forward (5′-TGTAAAACGACGGCCAGT-3′) and reverse (5′-CAGGAAACAGCTATGACC-3′) primers were used in conjunction with YTCA-1 template DNA.

All gene disruptions were confirmed either by DNA blot hybridization analyses or by colony PCR (Adams *et al.* 1998).

RESULTS

A screen for overexpressed cDNAs that disrupt telomeric silencing: A *S. cerevisiae* strain was constructed (UCC3505) that provided an easy yet stringent assay for loss of telomeric silencing. UCC3505 has the *ADE2* gene located adjacent to the right telomere of chromosome *V* (V-R) and *URA3* next to the left telomere of chromosome *VII* (VII-L). Both of these genes are sensitive to telomeric silencing, and the combination provides a two-level filter for screening perturbations of telomeric silencing. This two-level screen inherently excludes gene-specific alterations, such as induction of *URA3* or *ADE2*, as well as single-telomere events, such as chromosomal rearrangements that move the marker gene (*URA3* or *ADE2*) away from the chromosome end [*e.g.*, spontaneous insertion of Y′ DNA elements between the marker gene and the telomere (Singer 1997)].

Telomeric silencing in UCC3505 was monitored using simple phenotypic assays of *ADE2* and *URA3* expression. Normally, colonies expressing *ADE2* are white, while those not expressing it are red (Roman 1956). Because of the epigenetic nature of telomeric silencing, strains with *ADE2* near a telomere give rise to genetically identical but phenotypically distinct clonal populations that are visible as red and white sectors within a single colony (Gottschling *et al.* 1990). The *URA3* gene located at a telomere also normally switches between transcriptional states (Gottschling *et al.* 1990). However, *URA3* gene expression in UCC3505 was weakened by deleting its transcriptional activator gene, *PPR1*, which caused the telomere-adjacent *URA3* gene to be completely silenced; the cells were thus unable to grow in the absence of uracil (Ura^-; Aparicio and Gottschling 1994).

To identify genes or gene fragments whose overexpression interferes with telomeric silencing, UCC3505 was transformed with a high-expression *S. cerevisiae* cDNA library. The expression of cDNA inserts in this library was controlled by the *GAL1* promoter, which is strongly induced by the presence of galactose in the medium (Johnston and Davis 1984; Elledge *et al.* 1991; Ramer *et al.* 1992). Of the 330,000 yeast transformants obtained, 48 displayed a plasmid- and galactose-dependent decrease in telomeric silencing. That is, when grown on medium containing galactose, the cells were able to grow in the absence of uracil (Ura^+) and gave rise to predominantly white colonies (Ade^+; Figure 1).

Identification and sequence analysis of the *DOT* cDNAs: On the basis of Southern analysis and DNA sequencing, we determined that these 48 clones represented 10 independent genes, which we refer to as the *DOT* (disruptor of telomeric silencing) genes. Of these genes, 5 had been identified previously: *SIR4*, *ASF1*, *DNA2*, *RPL32*, and *IFH1*. Four of the remaining genes are referred to as *DOT1*, *DOT4*, *DOT5*, and *DOT6*. We compared the sequences of our isolates of these genes to the genomic sequences that were generated by the *S. cerevisiae* sequencing project to assess the completeness of each of the clones. The final gene, which we named telomerase component 1 (*TLC1*), has been described elsewhere (Singer and Gottschling 1994).

Out of this collection, overexpression of two of the genes, *SIR4* and *ASF1*, was previously known to interfere with telomeric silencing (Marshall *et al.* 1987; Renauld *et al.* 1993; Cockell *et al.* 1995; Le *et al.* 1997). The fact that these two genes were also isolated in our screen reassured us of its efficacy.

Sir4p, a component of silent chromatin, is required for telomeric silencing (Aparicio *et al.* 1991). Eleven *SIR4*-containing plasmids were isolated, representing at least six independent clones. Only the C-terminal portion of the gene was present in these clones, consistent with earlier findings that overexpression of this region of Sir4p strongly interferes with silencing (Table 2; Cockell *et al.* 1995). Surprisingly, two of the plasmids (pTRP4 and pTRP58) had reversed inserts, suggesting that the *GAL1* promoter on the vector directed transcription of antisense RNA.

The role of *ASF1* in silencing is less clear. Nevertheless, 13 *ASF1*-containing plasmids were isolated in our screen, representing at least seven independent clones (Table 2). All contained the entire open reading frame (ORF) of the gene. One of the clones, pTRP30, was a fusion of RNA sequences from *ASF1* and ~75 nt in the 3′ region of the *SUM1* RNA, including the last 7 nt of the *SUM1* ORF. Coincidentally, *SUM1* encodes a nuclear protein of unknown function that has been implicated in silencing (Klar *et al.* 1985; Livi *et al.* 1990; Laurenson and Rine 1991; Chi and Shore 1996).

Gene fragments encoding the N-terminal third of Dna2p were isolated twice in our screen (Table 2). *DNA2* is an essential gene that encodes a 3′–5′ DNA helicase required during DNA replication (Budd and Campbell 1995; Budd *et al.* 1995). Dna2p is 1522 amino acids in

length, and its helicase motifs are all in the C-terminal half.

L32 is an essential ribosomal protein (Dabeva and Warner 1987). In our screen, its gene was identified in two plasmids, both of which included the entire open reading frame of *RPL32*, without its genome-encoded intron (Table 2). In one clone, pTRP54, the *RPL32* ORF was followed by the coding region of another ribosomal protein, S24.

The *IFH1* gene was originally isolated as a high-copy suppressor of a null allele of *FHL1*, a gene required for rRNA processing (Hermann-Le Denmat *et al.* 1994; Cherel and Thuriaux 1995). The function of *IFH1*, which has a predicted ORF of 1085 amino acids, is unknown. In our study, *IFH1*-containing clones were isolated six times, with only two of the clones being identical. Each clone encoded only the N-terminal portion of Ifh1p, terminating at residues 212, 213, 216, or 218 (Table 2).

The sole *DOT1* cDNA isolated was 1882 bp (pTRP28, Table 2) and found to contain an ORF encoding the entire 582-amino-acid-predicted protein. The predicted sequence of this protein suggests that it is hydrophilic and basic (pI = 9.03; charge at pH 7 = +17.29).

DOT4 was isolated once in our screen and the gene was predicted to encode a ubiquitin-specific hydrolase (reviewed in Hochstrasser 1996). The pTRP50 cDNA insert encoded a 789-amino-acid protein containing nearly all (residues 16–784) of the predicted 792-amino-acid Dot4 protein (Table 2). However, several minor differences exist between the GenBank Dot4p sequence and the predicted sequence of the cDNA-encoded protein (see Table 2).

DOT5 was isolated as the clone pTRP49, whose 807-bp insert included the entire ORF encoding a predicted protein of 215 amino acids.

The *DOT6* gene sequence predicts a 670-amino-acid protein with a single Myb-related motif between residues 78 and 116. In different proteins from a wide variety of eukaryotes, the Myb domain is involved in sequence-specific DNA binding (Lipsick 1996). Of the two *DOT6* cDNAs isolated, one clone (pTRP29) included a 5′ untranslated region and the first 286 amino acids of the Dot6p predicted protein. The second clone (pTRP23) lacked the extreme N terminus, but encoded the C-terminal 634 amino acids of the protein.

Effects of overexpressing the *DOT* cDNAs on telomeric silencing: To characterize phenotypes associated with overexpression, one clone was chosen as a representative from each of the 10 genes. Each selected clone

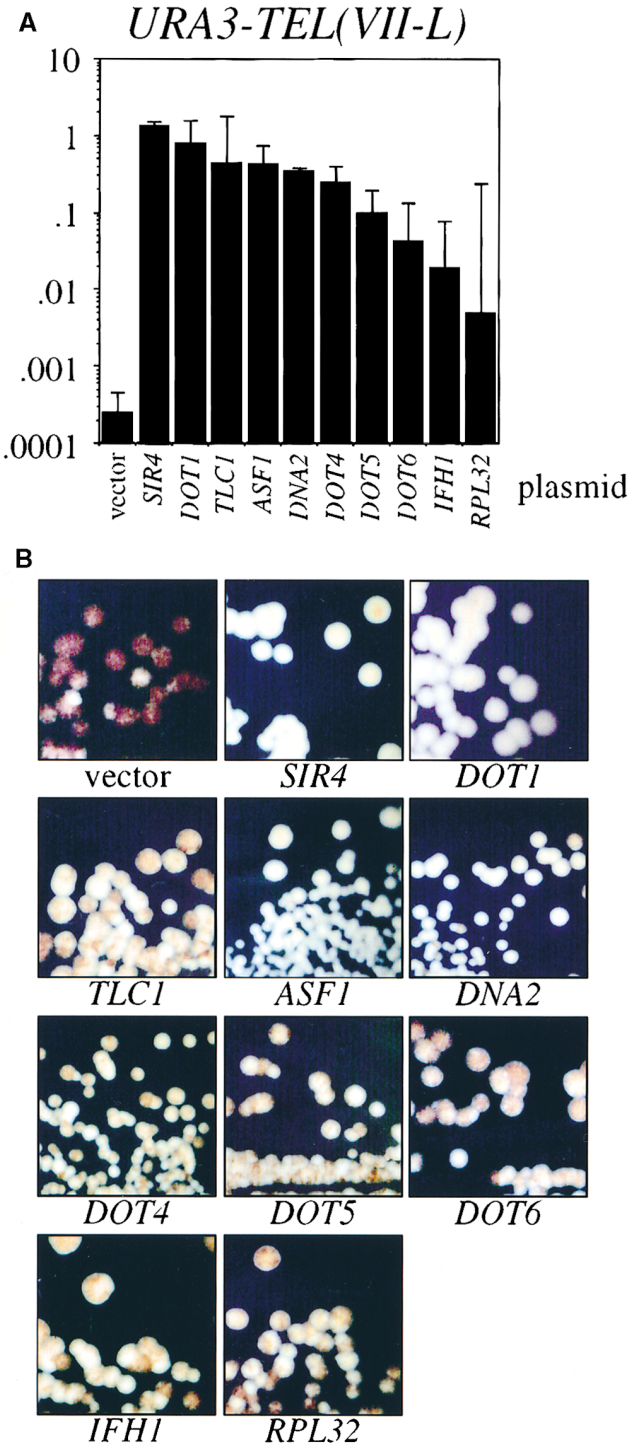

Figure 1.—Overexpressed cDNA genes disrupt silencing of telomeric *URA3* and *ADE2*. *S. cerevisiae* strain UCC3505, containing *URA3* at telomere VII-L and *ADE2* at telomere V-R, was transformed with a representative cDNA clone. Each clone contained a different gene identified in the screen for overexpressed cDNAs that disrupt telomeric silencing. Each transformant was grown on media lacking tryptophan and in the presence of galactose (3%) to induce cDNA overexpression. The clones used in this analysis were pTRP (vector), pTRP10 (*SIR4*), pTRP28 (*DOT1*), pTRP6 (*TLC1*), pTRP53 (*ASF1*), pTRP56 (*DNA2*), pTRP50 (*DOT4*), pTRP49 (*DOT5*), pTRP23 (*DOT6*), pTRP20 (*IFH1*), and pTRP54 (*RPL32*). (A) *URA3* expression was measured in terms of the viability of the strains on medium lacking uracil. Five independent transformants of each clone were tested, and the median value for viability in the absence of uracil is designated by the height of each bar. Each error bar indicates the difference between the median and the maximum value. (B) *ADE2* expression was reflected by the proportion of white (*ADE2* on) *vs.* red (*ADE2* off) sectors in the colonies of each strain.

TABLE 2

Overexpressed cDNA clones disrupt telomeric silencing

Gene (ORF size)	Clones	Insert size (kb)	Predicted expressed region (aa)	Comments
SIR4 (1358 aa)	pTRP2, pTRP3	1.27	1009–1358	
	pTRP4	0.77		*b*
	pTRP8, pTRP32	0.79	1242–1358	*c*
	pTRP10,[a] pTRP42, pTRP46, pTRP52	1.13	1071–1358	
	pTRP16	1.47	1009–1358	
	pTRP58	0.85		*d*
ASF1 (279 aa)	pTRP9	0.92	1–279	
	pTRP19, pTRP41	0.99	1–279	
	pTRP22, pTRP36, pTRP63	0.98	1–279	
	pTRP17, pTRP31, pTRP48, pTRP53[a]	1.04	1–279	
	pTRP30	1.00	1–279	*e*
	pTRP62	1.02	1–279	
	pTRP57	0.95	1–279	
IFH1 (1085 aa)	pTRP1, pTRP34	0.80	1–213	
	pTRP7	0.92	1–218	
	pTRP13	0.91	1–212	
	pTRP20[a]	0.92	1–216	
	pTRP27	0.92	1–212	
DNA2 (1522 aa)	pTRP35	1.07	1–338	
	pTRP56[a]	1.09	1–337	
RPL32 (105 aa)	pTRP24	0.46	1–105	
	pTRP54[a]	0.90	1–105	*f*
TLC1 (1.3-kb functional RNA)	pTRP6[a]	1.25		*g*
	pTRP61	1.25		
	pTRP14, pTRP47	1.21		
	pTRP33, pTRP39	1.22		
	pTRP55	1.21		
	pTRP59	1.21		
	pTRP60	1.00		
DOT1 (582 aa)	pTRP28[a]	1.88	1–582	
DOT4 (792 aa)[i]	pTRP50[a]	2.43	16–784 (with respect to GenBank Sequence)	*h*
DOT5 (215 aa)	pTRP49[a]	0.81	1–215	
DOT6 (670 aa)	pTRP29	0.89	1–286	
	pTRP23[a]	2.12	37–670	

[a] The plasmids used as representative clones for the overexpression studies.

[b] Antisense. RNA sequence is complementary to part of the *SIR4* mRNA: the last 593 nt of the *SIR4* ORF and 172 nt of the 3′ untranslated region.

[c] The first ORF in the cDNA is a 35-amino-acid-encoding sequence in a different reading frame.

[d] Antisense. RNA sequence is complementary to part of the *SIR4* mRNA: the last 658 nt of the *SIR4* ORF and 189 nt of the 3′ untranslated region.

[e] Fused to 75-nt fragment of *SUM1* antisense RNA.

[f] 3′ end of *RPL32* RNA is fused to *RPS24* RNA (starting at nucleotide 30 of the *RPS24* ORF).

[g] All the *TLC1* clones have been described previously (Singer and Gottschling 1994).

[h] pTRP50 has four point mutations and encodes a different C-terminal tail compared to the *DOT4* sequence contained in GenBank. After amino acid 784, the GenBank protein sequence terminates with the peptide NKKRKFTK. The protein encoded by pTRP50 terminates with the sequence IKKGSSPNEKTRYSWIFLFS.

[i] GenBank sequence.

had the strongest effect of disrupting telomeric silencing within its gene group.

The effect that overexpression of the clones had on telomeric silencing was quantitatively evaluated. Each representative plasmid was retransformed into UCC 3505. The transformants were pregrown on selective medium containing galactose to induce transcription of the cDNAs, and then assayed for derepression of the telomeric *URA3* and *ADE2* genes. Expression of *URA3* was measured as the viability of the transformed strain on medium lacking uracil (Figure 1A). Transcription of the *ADE2* gene was assessed qualitatively in terms of colony color, with white sectors reflecting *ADE2* expression, and red sectors representing *ADE2* repression (Figure 1B). The results from these two assays of telomeric silencing were consistent in all cases. Overexpression of clones containing *SIR4*, *DOT1*, *TLC1*, *ASF1*, *DNA2*, and *DOT4* each had a strong effect of disrupting telomeric silencing. There was a ≥1000-fold increase in the ability of plasmid-bearing strains to grow in the absence of uracil compared to a strain carrying vector without a cDNA insert. Similarly, colonies of strains that contained these overexpressed cDNAs had more prominent white sectors than those seen with the strain carrying vector alone. Overexpression of *DOT5*, *DOT6*, *IFH1*, and *RPL32* had a weaker but still significant ability to interfere with telomeric silencing; smaller fractions of the colonies were white, and there was only a 20–400-fold increase in the ability of the plasmid-bearing strain to grow on medium lacking uracil.

Effects of overexpressing the *DOT* cDNAs on *HM* silencing: To determine whether the action of each gene was limited to telomeric silencing, the effect of overexpression on nontelomeric silenced loci *HML* and *HMR* was assayed. The 10 cDNA overexpression plasmids were transformed into yeast strains that had the *URA3* gene inserted into the *HML* or *HMR* locus. (*URA3* is silenced much better at *HML* than at *HMR* because of the difference in the way the gene was inserted within the two *HM* loci. Thus, silencing at the *HMR* locus was more sensitive to perturbation than silencing at *HML*.) These transformed strains were pregrown on galactose, and derepression of *URA3* was measured as the viability of the transformed strains on medium lacking uracil (Figure 2, A and B). Consistent with previously published data, *SIR4* and *ASF1* overexpression derepressed both these silenced loci, causing the strains to have ~100% viability on media lacking uracil (Renauld *et al.* 1993; Cockell *et al.* 1995; Le *et al.* 1997). Similarly, overexpression of clones containing *DOT1*, *DOT4*, and *IFH1* caused a dramatic decrease in silencing at the *HML* and *HMR* loci. *RPL32* overexpression had a weak effect at both loci. *DNA2*, *DOT5*, and *DOT6* had a weak effect at *HML* and a stronger effect at *HMR*. Finally, *TLC1* had no effect at *HML* and a weak effect at *HMR*. [This weak effect was highly variable between transformants (see Figure 2B).] Thus, *TLC1* had a primarily telomere-specific effect, while the rest of the genes affected silencing both at telomeres and the *HM* loci, though they varied widely in the potency of their effects.

Effects of overexpressing the *DOT* cDNAs on silencing within the rDNA locus: Silencing within the tandemly duplicated repeats of rDNA is qualitatively different than at telomeres and the *HM* loci (see Introduction). Therefore, the effects of *DOT* cDNA overexpression were also examined at the rDNA, using strains having a single *URA3* gene inserted within the *RDN1* locus. Changes in *URA3* expression were assessed by examining both the strains' abilities to grow on media lacking uracil and their growth on medium containing 5-fluoro-orotic acid (5-FOA), which is converted to a toxic compound by the *URA3* gene product (Boeke *et al.* 1987; Gottschling *et al.* 1990). Analyzing *URA3* expression through the combination of both these assays provided a greater range of sensitivity for the degree of rDNA silencing.

The effects of overexpressing the *DOT* cDNAs on rDNA silencing can be divided into four classes (Figure 2C). Overexpression of *DNA2*, *ASF1*, *DOT4*, *DOT6*, and *IFH1* modestly reduced rDNA silencing, causing increases in sensitivity to 5-FOA and a commensurate increase in growth on media lacking uracil. Overexpression of *DOT1* also caused a loss of rDNA silencing, as indicated by a significant increase in 5-FOA sensitivity. Curiously, there was no corresponding increase in growth on media lacking uracil. This may indicate that overexpression of *DOT1* causes a higher rate of switching between repressed and active states, with the average fraction of active cells in the population remaining constant. *TLC1*, *DOT5*, and *RPL32* had essentially no effect on rDNA silencing. In contrast to its effect at telomeres and the *HM* loci, the data suggest that SIR4 overexpression, if anything, caused a subtle increase in rDNA silencing (note the lower level of growth in the second from the left spot on the plate labeled −Uracil in Figure 2C).

Effects of overexpressing the *DOT* cDNAs on a nonsilenced gene: For those cases in which overexpression of a *DOT* cDNA disrupted silencing at all four loci, it was possible that the apparent increased expression was not caused by a defect in silencing, but by an unrelated mechanism, such as active induction of the marker genes or stabilization of their protein products. To determine whether the effects of the *DOT* cDNAs were restricted to derepressing genes in silenced loci, we examined their action on a nonsilenced *URA3* gene. We used an assay employing 6-AU, a competitive inhibitor of the *URA3*-encoded enzyme orotidine 5′-phosphate decarboxylase (Loison *et al.* 1980). 6-AU is readily taken up by yeast from the medium. Hence, the ability of a strain to grow in the absence of exogenously provided uracil and in the presence of 6-AU reflects the level of *in vivo* activity of the *URA3*-encoded enzyme. That is, the greater the cellular levels of active *URA3* gene prod-

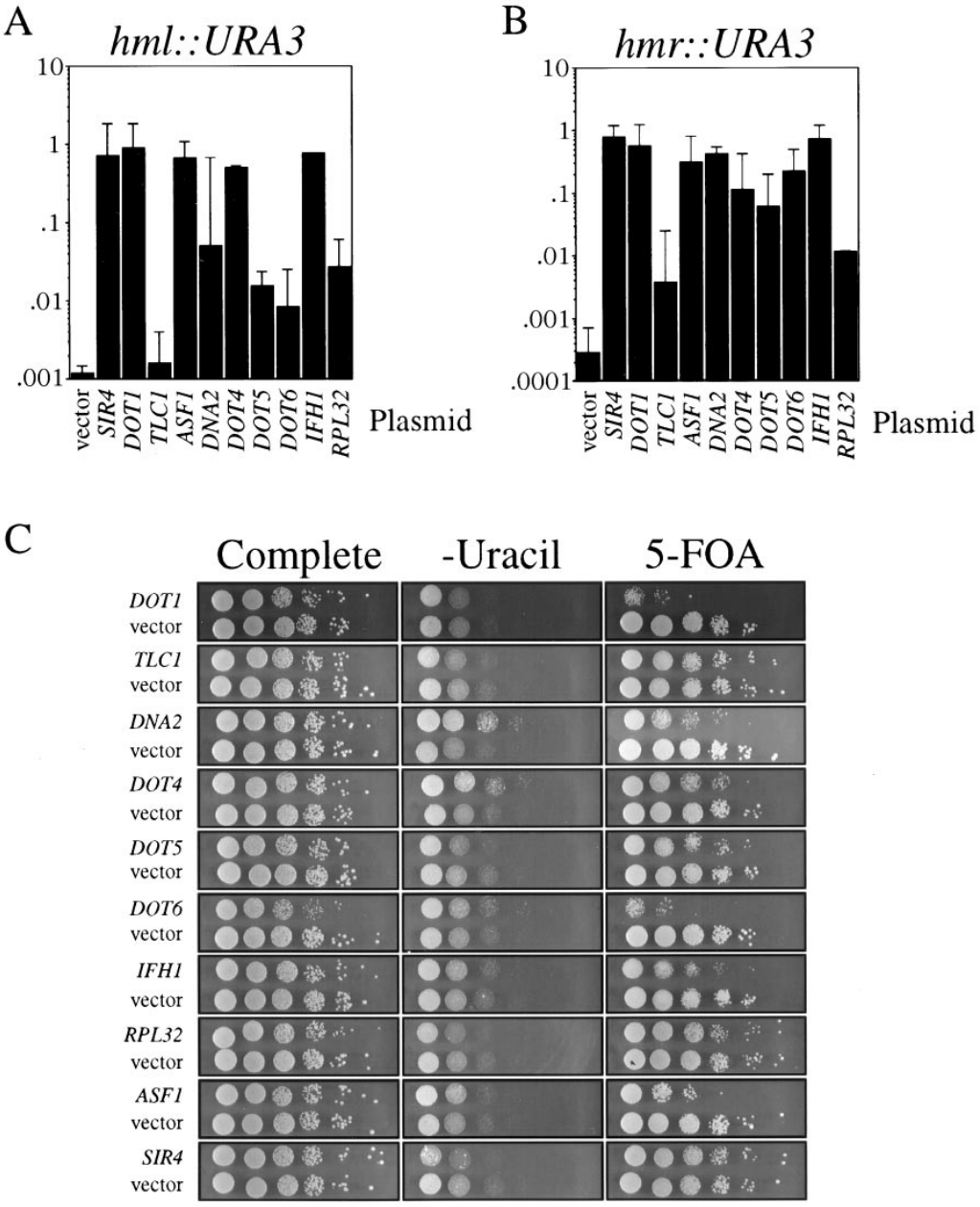

Figure 2.—Overexpressed *DOT* cDNAs have varying effects on silencing at *HML*, *HMR*, and within the rDNA locus. Viability on medium lacking uracil was measured for *S. cerevisiae* strains containing *URA3* inserted into (A) the *HML* locus (*hml::URA3*; UCC3515) or (B) the *HMR* locus (*hmr::URA3*; UCC4564) and overexpressing *DOT* cDNA clones. Three (UCC3515) or five (UCC4564) independent transformants of each strain were tested. The data are presented and the strains were grown as described in Figure 1A. (C) A strain with *URA3* inserted within the rDNA (JS125) was transformed with the *DOT* cDNA clones and grown as in Figure 1A. Tenfold serial dilutions of each transformant were then spotted onto medium lacking tryptophan (Complete), lacking tryptophan and uracil (−Uracil), or lacking tryptophan but containing 5-FOA (+5-FOA). Four independent transformants for each clone were examined, and one representative of each clone is shown with a vector control on the same plate.

uct, the greater the resistance of the strain to 6-AU. We used this assay because it provides greater sensitivity to changes in *URA3* expression than measuring message levels by Northern analysis (Losson and Lacroute 1981; Aparicio and Gottschling 1994).

The assay was carried out in a strain with the *URA3* gene inserted into *ADH4*, a nonsilenced locus on chromosome *VII*. Under conditions in which expression of the *DOT* cDNA was induced (galactose-containing medium), the ability of the strain to grow in the presence of 6-AU was tested (Figure 3). Two positive controls were included in the analysis, the overexpression of *URA5* and *PPR1*. Cells overexpressing the *URA5* gene product, which creates the normal substrate for orotidine 5′-phosphate decarboxylase (Ura3p), had improved growth on this medium compared to cells with vector alone (Figure 3). Also, cells overexpressing Ppr1p, which induces *URA3* transcription, were resistant to the effects of the 6-AU (Figure 3; Losson and Lacroute 1981). In contrast, none of the overexpressed *DOT* cDNAs, with the possible exception of *DOT6*, caused a significant improvement in growth of the transformed strain on 6-AU medium, compared to the strain carrying empty vector. Thus, the improvement of marker gene (*URA3*) expression caused by the high levels of each *DOT* cDNA, except perhaps *DOT6*, appeared to occur through a defect in silencing.

Effect of *DOT* cDNA overexpression on telomeric DNA tract length: An effect on telomeric silencing may well be accompanied by effects on other aspects of telo-

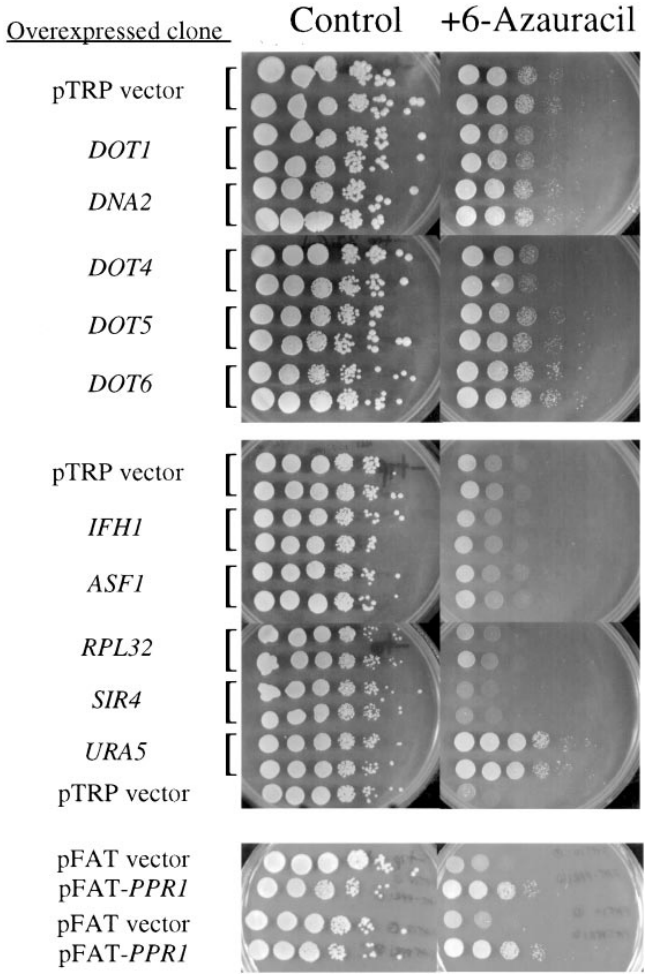

Figure 3.—Overexpressed *DOT* cDNAs have little effect on the expression of a nonsilenced *URA3* marker. *S. cerevisiae* strain UCC4567, containing *URA3* at an internal (nontelomeric) chromosomal locus, was transformed with and induced to overexpress the *DOT* cDNA clones or *URA5* (pTRP21) or empty vector (pTRP). Similarly, UCC4567 was transformed with a different high-copy vector (pFAT) or the same vector into which the *PPR1* gene was inserted (pFAT-*PPR1*). Tenfold serial dilutions of a colony from each strain were spotted onto medium lacking tryptophan (control), as well as the same medium lacking uracil and containing 3 mg/l of 6-AU. Galactose (3%) was present in all media. The variation in growth observed between the first and second sets of plates of pTRP plasmid-bearing strains reflects the fact that these experiments were carried out at different times. The relevant comparison is to the vector-harboring strain within each set.

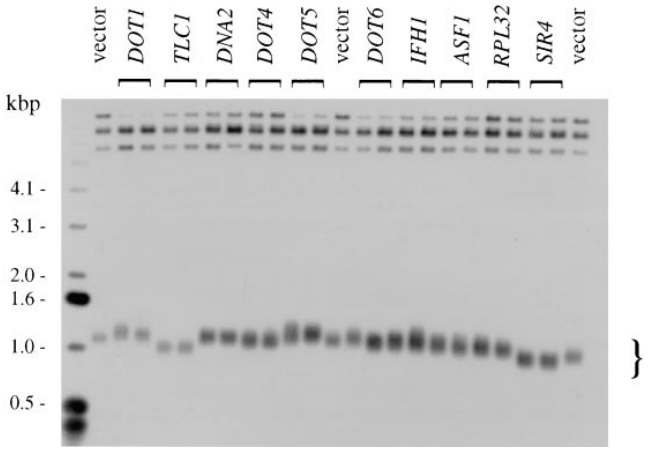

Figure 4.—Overexpressed *DOT* cDNAs have varying effects on telomere length. *S. cerevisiae* strain UCC3505 was transformed with and induced to overexpress cDNA clones of the genes isolated in the screen as high-copy suppressors of telomeric silencing. The clones used in this analysis are those listed in Figure 1. Genomic DNA was prepared from two independent transformants of each plasmid after culturing cells for 100 generations in galactose. DNA was digested with *Xho*I, separated by electrophoresis through a 1% agarose gel, and blotted onto a nylon membrane. The membrane was probed with Y′ DNA, which is present in the telomere-proximal region of several chromosomes. The telomeric fragments are indicated by the brace.

mere structure or metabolism. For example, overexpression of *TLC1*, the telomerase RNA gene, causes the telomere DNA tract at the end of the chromosome to shorten (Singer and Gottschling 1994). To see if this was true for the other *DOT* cDNAs, each representative clone was overexpressed and telomere DNA length was measured. Because changes in telomere length can take many generations to manifest themselves (Lustig and Petes 1986), plasmid-bearing transformants of UCC 3505 were cultured on galactose medium for ~100 generations before genomic DNA was collected for Southern analysis of telomere length (Figure 4). As expected, the typical heterogeneity in telomere length of a population of cells was observed, even when examining a unique chromosome end. Only *TLC1* and *SIR4* overexpression had significant and reproducible effects of shortening telomere length, consistent with earlier reports for *TLC1* (Singer and Gottschling 1994). In contrast, *DOT5* overexpression caused a modest increase in the average telomere length. Thus, overexpression of only three *DOT* genes, *TLC1*, *SIR4*, and *DOT5*, affected telomere length regulation and telomeric silencing.

Effects of deleting the *DOT* genes on telomeric silencing: When overexpression of a gene or gene fragment interferes with a biological process or structure, such as the one described here for telomeric silencing, it may be because the wild-type gene product normally participates in the process or is a component of the structure. The overexpressed gene product may act at an inappropriate time or place and, thus, interact with its partner protein(s) to create a futile complex that interferes with the normal cellular process. For example, both deletion and overexpression of *SIR4* result in the same phenotype, loss of silencing (Ivy *et al.* 1986; Marshall *et al.* 1987; Aparicio *et al.* 1991). To ascertain whether any of the other *DOT* genes are important for telomeric silencing, the genomic copy of *DOT1*, *DOT4*, *DOT5*, *DOT6*, *TLC1*, or *ASF1* was deleted and the effect on silencing was examined. This analysis could not be done,

however, for *RPL32*, *IFH1*, or *DNA2*, which are essential for viability (Dabeva and Warner 1987; Budd and Campbell 1995; Cherel and Thuriaux 1995; data not shown).

The gene deletions were made in strains in which telomeres V-R and VII-L were labeled with *ADE2* and *URA3*, respectively. In addition, two versions of these strains were made, one that was wild type for *PPR1*, the transcriptional activator that is responsible for *URA3*'s inducible transcription, and one that was mutant (*ppr1*). We analyzed telomeric *URA3* expression, as reflected by growth on media lacking uracil and resistance to 5-FOA, in both *PPR1* and *ppr1* cells to provide a greater range of phenotypic sensitivity to differences between wild-type and *dot* strains.

Comparing *dot1* and *DOT1* strains, the *DOT1* gene product was found to be important for telomeric silencing (Figure 5A). In *DOT1 PPR1* strains, the telomeric *URA3* gene was silenced in a large fraction of the cells, as evidenced by the high frequency of growth on medium containing 5-FOA. However, when *DOT1* was deleted, the resistance of the strain to 5-FOA declined by $\sim 10^5$-fold. Similarly, in strains in which telomeric silencing of the *URA3* gene was made stronger by the absence of PPR1, the deletion of *DOT1* still reversed this repression, resulting in an $\sim$1000-fold increase in viability of the strain on medium lacking uracil (Figure 5A). The loss of telomeric repression in *dot1* strains also occurred for the *ADE2* gene located at telomere V-R. Whereas wild-type colonies had prominent red sectors representing cells in the population in which the *ADE2* gene was silenced, the colonies of *dot1* strains were almost completely white (data not shown).

Disrupting *DOT4* also had a strong effect on telomeric silencing. The colonies of *dot4* strains were less red than their wild-type counterparts, consistent with a decreased repression of the telomeric *ADE2* gene (data not shown). Moreover, there was a 1000-fold increase in the ability of *ppr1* strains to grow in the absence of uracil when *DOT4* was deleted, suggesting a decrease in silencing of the telomeric *URA3* gene (Figure 5B). However, there was no increased 5-FOA sensitivity in *dot4 PPR1* strains. At present, it is difficult to interpret the significance of this difference because there are pleiotropic defects in *dot4* strains, including slowed growth (note colony size in Figure 5B). It is possible that the *dot4* mutation may also affect 5-FOA utilization or uptake.

Examining telomeric silencing in a *TLC1* deletion strain presents an unusual circumstance in the analysis. As a result of losing TLC1 function and, consequently, telomerase activity, the ($TG_{1\text{-}3}$) DNA tracts at the ends of the chromosomes shorten with each cell division. Therefore, the level of silencing *URA3* at the VII-L telomere was determined in a population of cells while the average length of their terminal ($TG_{1\text{-}3}$) repeats was examined. As can be seen in Figure 5C, a population of cells with an average VII-L telomeric tract that is about half the length of wild-type cells still silences the telomeric gene very efficiently. Thus, *TLC1* is not directly required for telomeric silencing. These results also demonstrate that telomeric DNA tracts as short as 180 bp can efficiently silence genes.

Deletion of *DOT5* or *DOT6* had no detectable effects on telomeric silencing (data not shown). However, disruption of *ASF1*, which also caused cells to be slow growing, resulted in a modest telomeric silencing defect (Figure 5D).

Taken together, we conclude that the *DOT1* and *DOT4* gene products are important factors for telomeric silencing while *ASF1* may play a minor role. *TLC1* does not appear to play a direct role in telomeric silencing. Also, the *DOT5* and *DOT6* gene products are not required for telomeric silencing.

Effects of deleting the *DOT* genes on *HM* silencing: To determine whether the *DOT* genes were important for silencing at the *HML* and *HMR* loci, a test similar to the one described above for telomeric silencing was conducted. Strains in which the *URA3* gene was inserted into either *HML* or *HMR* were constructed, and the genomic copy of *DOT1*, *DOT4*, *DOT5*, *DOT6*, *TLC1*, or *ASF1* was deleted. These strains were compared for their ability to grow on media lacking uracil and their resistance to 5-FOA.

Consistent with the effect at telomeres, deletion of *DOT1* caused a decrease in silencing at the *HML* and *HMR* loci (Figure 6A). In the wild-type strain, silencing of *URA3* at the *HMR* locus resulted in a high level of 5-FOA resistance. When the *DOT1* gene was deleted, however, the ability of the strain to grow in the presence of 5-FOA decreased dramatically, indicating increased expression of the *URA3* marker gene. In *DOT1* strains in which the *URA3* gene was located at *HML*, the repression of *URA3* caused poor viability on media lacking uracil. However, when *DOT1* was deleted, plating efficiency on media lacking uracil was increased.

DOT4 was also found to be involved in silencing at both *HM* loci (Figure 6B). As with the strains in which *URA3* was located at a telomere, decreased silencing at *HML* and *HMR* in the *dot4* mutants was observed as increased viability on media lacking uracil compared to wild-type strains.

Even though they had shorter telomeres, strains without *TLC1* showed no change in silencing at *HML* or *HMR* compared to strains with *TLC1* (Figure 6C). As was true for telomeric silencing, deletion of *DOT5* and *DOT6* had no detectable effect on silencing at *HML* and *HMR* (data not shown), and deletion of *ASF1* caused a weak derepression at both *HML* and at *HMR* (Figure 6D). This weak effect was not reported in earlier work on *ASF1* and may reflect a difference in the assays used (Le *et al.* 1997).

Effects of deleting the *DOT* genes on rDNA silencing: Deletion of the *DOT* genes had a somewhat different spectrum of effects on rDNA silencing than on the telo-

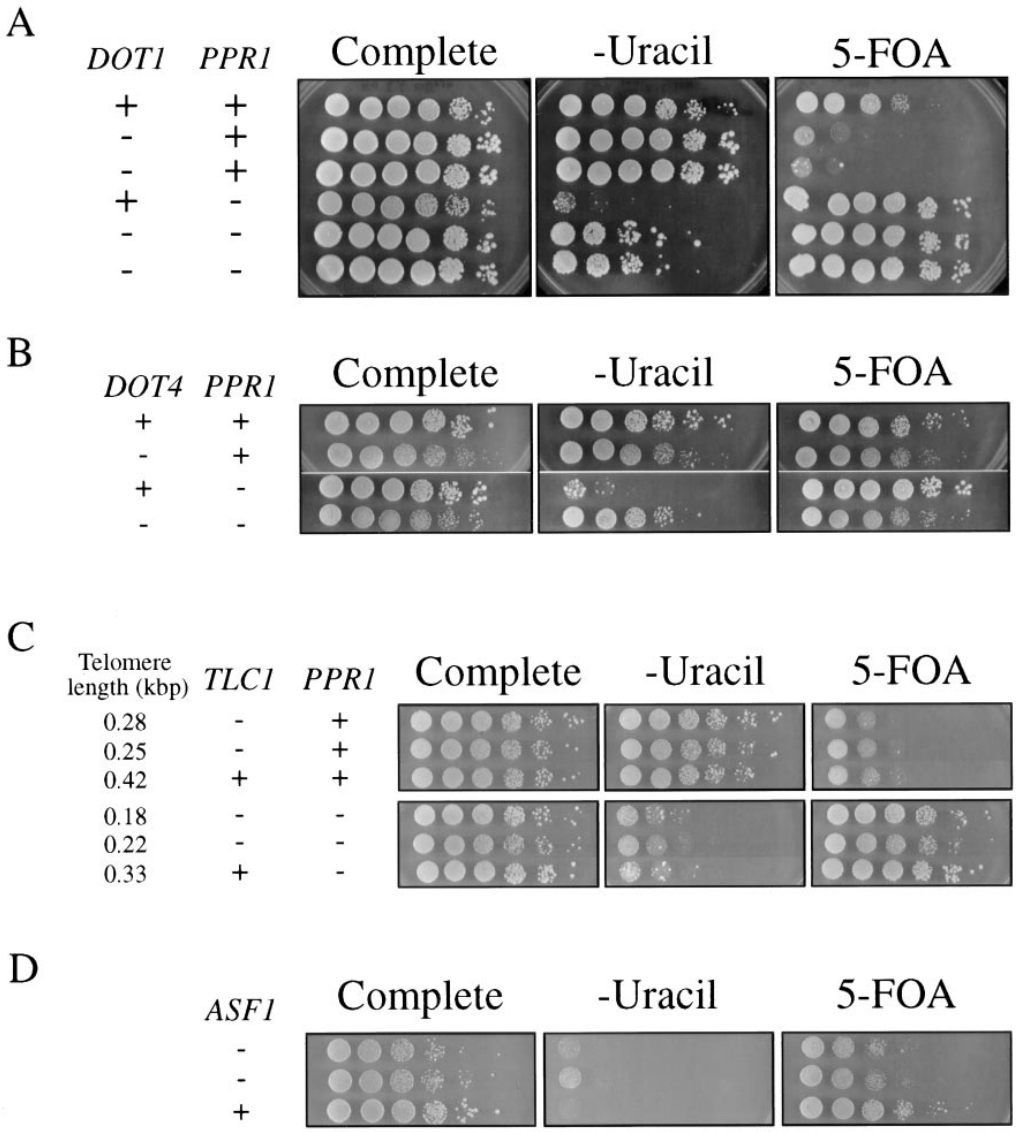

Figure 5.—Effects of mutant *DOT1*, *DOT4*, *TLC1*, and *ASF1* on telomeric silencing. Strains with *URA3* at telomere VII-L and either wild-type or null alleles of (A) *DOT1*, (B) *DOT4*, (C) *TLC1*, or (D) *ASF1* were compared for their ability to grow in complete medium, in the same medium lacking uracil, or in the presence of 5-FOA, a compound that is lethal to cells expressing *URA3*. Strains containing or lacking *PPR1* were examined. (D) Only *ppr1* strains are presented because no difference was seen between *PPR1* and *ppr1* strains (data not shown). Tenfold serial dilutions of a colony from each strain were spotted onto each medium. (A) Strains (from top to bottom) are UCC4562, UCC4554, UCC4555, UCC4563, UCC4560, and UCC4561. (B) Strains are UCC4602, UCC4591, UCC4595, and UCC4594. (C, top) UCC3503 (*TLC1*) with two independent *tlc1* transformants. Similarly, the lower panel is UCC3504 with two independent *tlc1* derivatives. (Strain names were not given to these derivatives as they eventually die as a result of telomere erosion with each cell doubling.) The average length (in kilobase pairs) of terminal ($TG_{1\text{-}3}$) tracts adjacent to the telomeric *URA3* gene on VII-L was determined by Southern analysis. In D, there are two colonies of UCC6562 and one of UCC6555.

meric or *HM* loci (Figure 7). While *dot4* cells had slightly less rDNA silencing than wild-type cells, as judged by sensitivity to 5-FOA, deletion of *DOT1*, *DOT5*, *DOT6*, and *TLC1* had no effect. Deletion of *ASF1* and *SIR4* resulted in a subtle increase of rDNA silencing, as judged by decreased growth in the absence of uracil. This subtle change in rDNA silencing when *SIR4* was deleted is consistent with an earlier study (Smith and Boeke 1997).

Effects of deleting the *DOT* genes at a nonsilenced locus: To have a clearer understanding of the effects of *DOT* gene deletions on silencing of *URA3* at telomeres, *HML*, *HMR*, and within the rDNA cluster, we examined the expression of *URA3* at a nonsilenced locus in a set of *dot*$^-$ strains. To assay the expression of this *URA3* marker, the ability of the strain to grow in the presence of 6-AU was measured. As mentioned above, cells in which *URA3* expression is increased are better able to grow in the presence of 6-AU. Under the conditions chosen for this assay, the parental (*ppr1*) strain grows poorly on plates containing 6-AU (Figure 8). If *URA3* transcription is improved by the presence of the *PPR1* gene product, resistance to 6-AU rises sharply. As expected, deletion of the *SIR4* gene, a recognized component of silencing chromatin, had no effect on the expression of the nonsilenced *URA3* gene. Similarly, deletion of *DOT1* had no effect. *ASF1*, which was observed to have very weak effects at telomeres and *HML*,

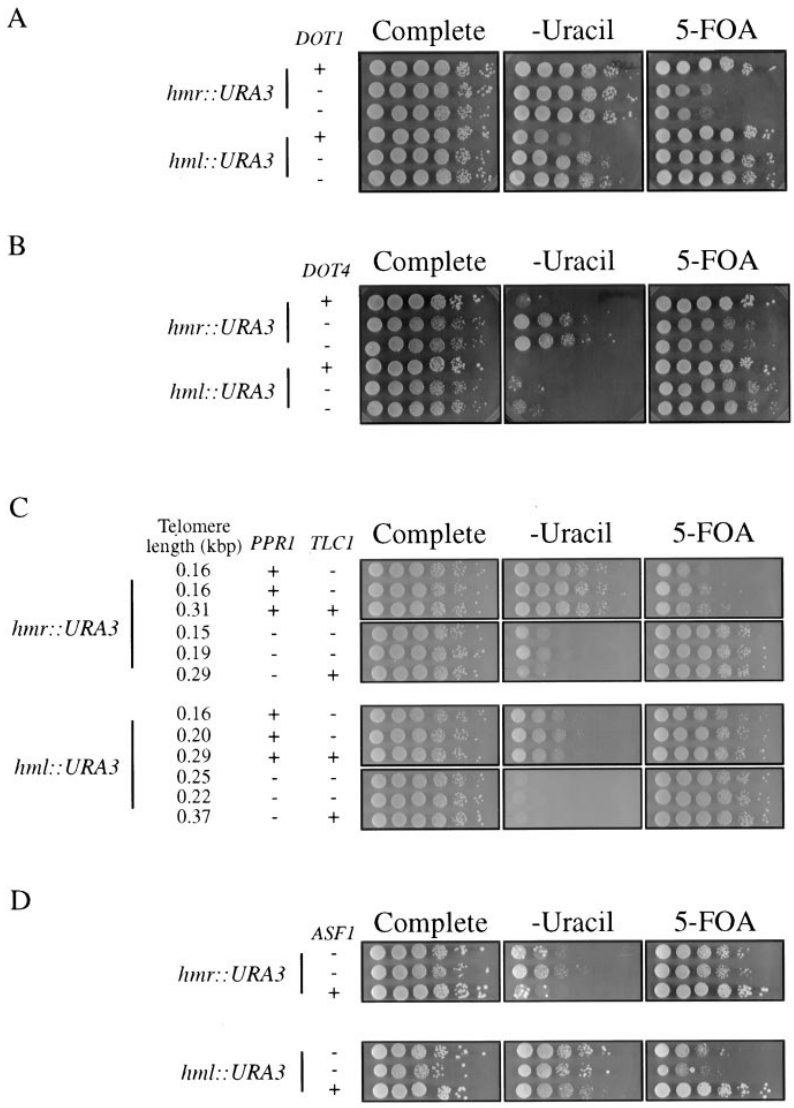

Figure 6.—Effects of mutant *DOT1*, *DOT4*, *TLC1*, and *ASF1* on the silent mating type loci. Strains with *URA3* inserted into *HML* or *HMR* and either wild-type or null alleles of (A) *DOT1*, (B) *DOT4*, (C) *TLC1*, or (D) *ASF1* were compared for their ability to grow on medium lacking uracil or containing 5-FOA, as described in Figure 5. In A, the strains (from top to bottom) are UCC3511, two colonies of UCC4586, UCC3515, and two colonies of UCC4574; they are all *PPR1*. In B, the strains are UCC4564, two colonies of UCC4579, UCC4565, and two colonies of UCC4580; they are all *ppr1*. In C, each of the *tlc1* strains is derived from the parent strain in the same panel. The parent strain in each panel is UCC3511, UCC4564, UCC3515, and UCC4565. The average length (in kbp) of terminal ($TG_{1\text{-}3}$) tracts adjacent to Y′ elements was determined by Southern analysis. In D, the strains in the top panel are *ppr1*: two colonies of UCC3615 and one of UCC4564; the bottom panel are *PPR1*: two colonies of UCC3612 and one of UCC3515.

also failed to discernibly improve the resistance of the cells to 6-AU. In contrast, deletion of *DOT4* caused a significant resistance to the presence of 6-AU. This result may represent improved transcription of the unsilenced *URA3* in *dot4* strains, or (as with the 5-FOA experiments described above) it may reflect an unrelated mechanism of 6-AU resistance, such as a defect in uptake of the 6-AU compound.

Effects of deleting the *DOT* genes on telomeric DNA tract length: Finally, to assess whether the *DOT* genes have a role in maintaining normal telomeric DNA structure, telomere length was measured using a $TG_{1\text{-}3}$ probe that detected all telomeres in the cell in strains deleted for one of the nonessential genes isolated in the screen [*DOT1*, *DOT4*, *DOT5*, *DOT6*, *ASF1*, and *SIR4* (*TLC1* results were published earlier in Singer and Gottschling 1994)]. Deletion of *SIR4* caused a modest telomere length decrease (Figure 9), as had been reported earlier (Palladino *et al.* 1993). The only other reproducible difference was a result of deleting *DOT1*; *dot1* cell telomeres were somewhat more heterogeneous in length than wild-type cells.

DISCUSSION

We have identified a group of 10 genes involved in telomeric silencing, based on the ability of either a full-length or partial cDNA clone of each gene to disrupt telomeric silencing when overexpressed. The *DOT* genes include two genes that had previously been known to disrupt telomeric silencing when overexpressed: silent chromatin component *SIR4* as well as *ASF1*, whose role in silencing is not known. In an earlier report, we described the defect in telomeric silencing when the telomerase RNA template gene *TLC1* is overexpressed (Singer and Gottschling 1994). The remaining 7 genes have not been reported previously as having an effect on telomeric silencing: ribosomal protein gene *RPL32*, DNA helicase gene *DNA2*, *IFH1*, and the newly identified genes *DOT1*, *DOT4*, *DOT5*, and *DOT6*.

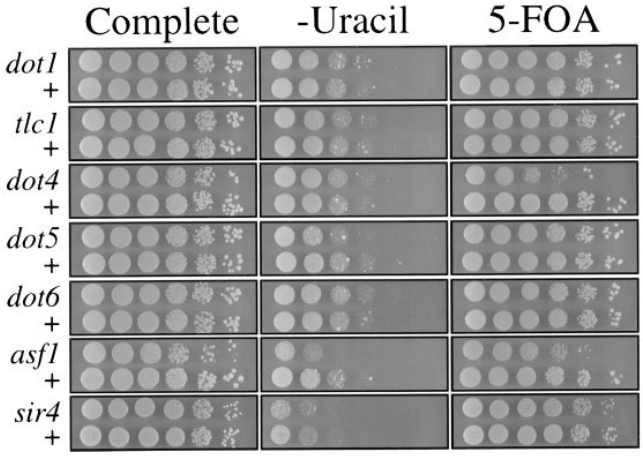

Figure 7.—Null mutations in *DOT* genes have little or no effect on rDNA silencing. *DOT1* (UCC6605), *DOT4* (UCC 6606), *DOT5* (UCC6607), *DOT6* (UCC6608), *TLC1*, or *ASF1* (UCC6609) were deleted in strain JS125, which has *URA3* inserted into the rDNA locus. *SIR4* (UCC6616) was deleted in a similar strain, JS128. The parent (+) and mutant pairs were compared for their ability to grow on medium lacking uracil or containing 5-FOA, as described in Figure 5.

Overexpression of a subset of the *DOT* genes also altered silencing at *HML*, *HMR*, and the *RDN1* locus. All of the genes except for *TLC1* reduced silencing at the *HM* loci, and all except *TLC1* and *RPL32* reduced rDNA silencing. *SIR4* overexpression resulted in a very mild increase in silencing at *RDN1*. These different effects of the *DOT* genes reflect the qualitative similarities and differences between the four silencing loci.

To model how overexpression of the *DOT* genes might disrupt telomeric silencing, it is worth reviewing a few aspects of our current understanding of silent telomeric chromatin.

The ability to silence a gene requires the coordinated assembly of a complex set of molecules (*e.g.*, histones, Rap1p, Sir3p, Sir4p, etc.) onto a scaffold of telomere-

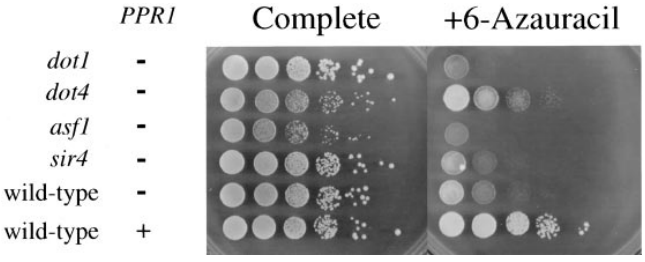

Figure 8.—Only *DOT4* deletion confers increased resistance to 6-AU for a strain with an internally located URA3 gene. *DOT1* (UCC4571), *DOT4* (UCC4576), *ASF1* (UCC3617), or *SIR4* (UCC6541) were deleted in UCC4567 (wild type, *ppr1*), which contains an internally located *URA3* gene. As a positive control, UCC4566 (wild type, *PPR1*), which contains a transactivator of the *URA3* gene, was used. Serial dilutions of a colony from each strain were spotted onto complete synthetic medium (HC, complete synthetic), as well as the same medium lacking uracil and containing 20 g/l 6-AU.

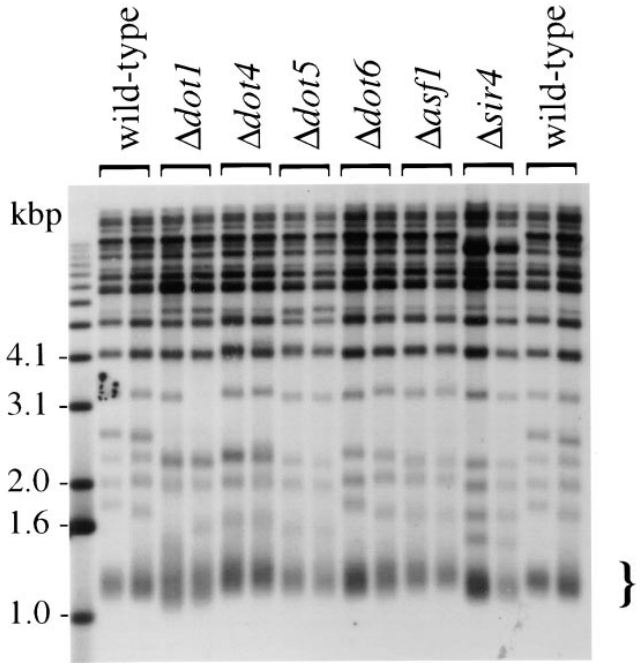

Figure 9.—The newly identified *DOT* genes have little effect on telomere length maintenance. Southern analysis was used to examine the telomere lengths of UCC3504 (wild type) and derivative strains lacking *DOT1* (UCC6008), *DOT4* (UCC 4583), *DOT5* (UCC6550), *DOT6* (UCC6552), *ASF1* (UCC 3611), or *SIR4* (UCC6542). Genomic DNA was prepared from two colonies of each strain, digested with *Xho*I, separated by electrophoresis through a 1.25% agarose gel, and blotted onto a nylon membrane. The membrane was probed with $TG_{1\text{-}3}$ sequences. The telomeric fragments are indicated by the brace.

proximal DNA. To achieve silencing, this assembly must occur in the face of a challenge by transcriptional machinery attempting to assemble onto the same DNA scaffold. It must also be reproduced every time the chromosome is duplicated.

Just as assembly of the Sir proteins is a requisite for establishing and maintaining silent chromatin, duplication of silent chromatin likely requires that it be transiently remodeled or taken apart. For instance, silent chromatin components may be modified in coordination with DNA replication to permit passage of the replication fork (Bradbury 1992; Ito *et al.* 1997), or the replication machinery may have an intrinsic ability to dissociate silent chromatin as it polymerizes new DNA strands along the chromosome (Bonne-Andrea *et al.* 1990).

Silent chromatin is limited to a subset of loci in the yeast genome, yet some silent chromatin components, such as histones H3 and H4, are present along the entire chromosome, and others, such as Rap1p, are present at a multitude of nonsilenced loci (Shore 1994). The mechanism(s) by which specificity for silencing is imparted upon these nonspecific proteins is not clear. Moreover, there are multiple silencing loci—telomeres, *HML*, *HMR*, *RDN1*—that are distinct physically and

structurally. Coordination between these different loci must be achieved to maintain the appropriate level of silencing at each locus.

Given these challenges to its formation, it is not surprising that telomeric silencing is semistable (Gottschling *et al.* 1990). Moreover, perturbations of any one of the many conditions required for silent telomeric chromatin formation could easily shift the balance in favor of silencing disruption. This would lead to the strong phenotype of gene expression that we selected in our screen. Perturbations that may arise from overexpressed cDNAs may have a dominant positive or negative effect with respect to their normal gene function. As such, they may disrupt silencing if their gene products normally participate in the process of disassembly or assembly of any silent locus. By the model mentioned earlier, in which a defective or overabundant subunit may poison an entire complex, the *DOT* genes may themselves encode part of the silencing structure. Alternatively, they may include genes whose products are not intimately associated with silent telomeric chromatin, but, rather, affect its assembly in a more indirect way, such as modulating the synthesis or turnover of silent chromatin components.

Another possibility is that overabundance of a truncated or full-length gene product may cause it to associate with a new set of molecules and, thus, involve it in telomeric silencing even though it normally has no role in this process. These new interactions may result from the production of a gene product that is improperly regulated (because of the production of an incomplete gene product or an abnormally high level of synthesis) or from the sheer excess of the overproduced protein, which increases the frequency of a low-affinity interaction. According to this model, the illegitimate interactions that result from this cross-reaction would preclude normal productive interactions and, thus, disrupt the formation of silencing chromatin at the telomere.

In light of these ideas, we offer speculation about each of the *DOT* genes and why they were identified in our screen.

***SIR4*:** It had previously been observed that overexpression of the entire *SIR4* gene or overproduction of just the C-terminal region of the protein results in a loss of silencing at *HM* loci and telomeres (Ivy *et al.* 1986; Marshall *et al.* 1987; Renauld *et al.* 1993). Hence, our identification of *SIR4* (Table 3) served primarily as a positive control for our screen.

The mechanism by which *SIR4* overexpression disrupts silencing has been studied by others. Sir4p interacts via its C-terminal region with Sir3p (Moretti *et al.* 1994; Strahl-Bolsinger *et al.* 1997), suggesting that overexpression of *SIR4* may titrate Sir3p, a required silencing factor, away from chromatin. This model is supported by the finding that overexpression of *SIR3* suppresses the loss of silencing caused by overexpression of *SIR4* (Marshall *et al.* 1987).

A protein interaction model might not apply to all the *SIR4* clones we identified. Surprisingly, pTRP4 and pTRP58 were inserted into the expression vector in the reverse orientation with respect to the *GAL1*-promoter such that they could produce RNA that is antisense to *SIR4* sequence. We speculate that such an antisense RNA reduces the level of Sir4p in the cell. It is noteworthy that while antisense technology works well in many organisms, our results represent one of the rare cases in which antisense RNA expression produces a phenotype in *S. cerevisiae* (Nasr *et al.* 1995; Kho *et al.* 1997; Machado *et al.* 1997).

***ASF1*:** Overexpression of *ASF1* strongly derepressed both telomeric and *HM* loci and had a significant effect on the rDNA locus (Table 3; Le *et al.* 1997). As has been suggested by others, the presence of acidic stretches within Asf1p and the upregulation of its gene before and during S phase suggest that it is involved in replication or chromatin assembly (or disassembly) (Le *et al.* 1997). Regardless of how *ASF1* may normally function, it is likely that the loss of cell cycle regulation when its cDNA is under *GAL1*-directed expression plays an important role in the disruption of silencing.

***IFH1*:** *IFH1* is an essential gene whose connection to silencing has not been recognized previously (Cherel and Thuriaux 1995). However, clones encoding the N-terminal region of Ifh1p were isolated six times in our screen. Overexpression of this region weakened silencing not only at the telomeres, but also at the *HM* loci and rDNA (Table 3).

The highly acidic domain in the N terminus of *IFH1*, like that found in *ASF1*, may mediate interaction with chromatin proteins. Because *IFH1* is proposed to normally interact with *FHL1* (Hermann-Le Denmat *et al.* 1994; Cherel and Thuriaux 1995), a member of the *fork head* family of proteins of which mammalian histone H5 is also a member (Kaufmann and Knochel 1996), *IFH1* may be particularly suited for counteracting the repressive nature of analogous DNA-binding proteins at silenced loci. It is not known at this point whether *IFH1* normally plays a role at the silent loci or whether it affected these loci by virtue of its overexpression.

***DNA2*:** DNA2 is an essential gene that encodes a 3′–5′ DNA helicase whose function is required during DNA replication (Budd and Campbell 1995; Budd *et al.* 1995). Gene fragments encoding the N-terminal region, which does not include the helicase domains, were isolated twice in our screen and found to diminish silencing at telomeres, *HM* loci, and rDNA (Table 3). The N terminus of Dna2p has no motifs that indicate its function; however, the importance of the region has been underscored by the finding that deletions and point mutations within it are lethal (Budd and Campbell 1995). Genetic and biochemical data suggest that Dna2p acts at the replication fork. Thus, its overexpression may cause a defect in DNA replication that indirectly affects silent chromatin assembly (Laman *et al.* 1995), as men-

TABLE 3

Disruption of silencing by *DOT* genes

	Overexpression			Deletion		
Gene	Telomeres	*HM*	rDNA	Telomeres	*HM*	rDNA
DOT1 (YDR440W; *PCH1*)	++++	++++	++[b]	+++	+++	NC
TLC1[a]	++++	NC	NC	NC	NC	NC
DNA2 (YHR164C)	+++	++	++	NA	NA	NA
DOT4 (YNL186W)	+++	+++	++	++[c]	++[c]	+[c]
DOT5 (YIL010W)	++	++	NC	NC	NC	NC
DOT6 (YER088C)	++	++	++	NC	NC	NC
IFH1 (YLR223C)	++	++++	+	NA	NA	NA
ASF1 (YJL115W)	++++	++++	++	+	+	−
RPL32 (YBL092W)	+	+	NC	NA	NA	NA
SIR4 (YDR227W)	++++	++++	−	++++	++++	−

A summary of the silencing phenotypes of the *DOT* genes at telomeres, *HM* loci, and rDNA. +, increase in *URA3* expression caused by the disruption of silencing; −, a decrease in *URA3* expression caused by an enhancement of silencing; NC, no change; NA, not applicable because the gene is essential for viability.

[a] *TLC1* is located on the Watson strand between YBR035C and YBR036C.

[b] Increased FOA sensitivity, but no change in growth on media lacking uracil.

[c] *dot4*Δ strains show an increase in resistance to 6-AU.

tioned for *ASF1*. Alternatively, *DNA2* may be more directly involved in chromatin assembly or disassembly at the replication fork. The helicase may have a dual role of loosening chromatin structure in combination with separating the DNA strands.

***RPL32*:** L32 is an essential ribosomal protein. Overexpression of its cDNA clone caused a subtle but reproducible loss of silencing at telomeres, *HML*, and *HMR*, but had no effect on expression of a marker in the rDNA locus (Table 3).

Unlike most yeast genes, *RPL32* contains an intron. The L32 protein negatively regulates its own expression by two mechanisms: L32 binds its pre-mRNA and inhibits its splicing, and it binds its own spliced transcript and inhibits translation (Dabeva *et al.* 1986; Eng and Warner 1991; Dabeva and Warner 1993). Because the *RPL32* cDNAs we isolated contained no intron, the first form of regulation could not prevent the high level of induction of mRNA synthesis directed from the *GAL1* promoter. However, it is not clear how much L32 protein was actually synthesized, given the inhibition of translation that normally comes into play. Therefore, at this point, it is formally possible that it is the mRNA of *RPL32* rather than the protein that is causing a disruption of silencing.

If the protein is actually overexpressed and is the active component, the interactions that cause it to weaken silencing may be in the context of its function in the ribosome. Overexpressing L32 may cause a translational defect that lowers the level of a critical silencing factor. Another possibility is that L32 has a function apart from the ribosome that is much more closely related to chromatin structure. It has been proposed that many ribosomal proteins originated as proteins with a different function (often involving nucleic acid interaction) and were co-opted for use in the ribosome. In accordance with this model, ribosomal proteins have been found to participate in a variety of cellular functions, including transcription, RNA processing, and DNA repair (Wool 1996). Hence, L32 might have a direct role in silencing gene transcription. Finally, it is possible that the overexpressed L32 binds and suppresses translation of mRNAs other than its own; one of these could be the message for a critical silent chromatin component or regulator.

***DOT1*:** *DOT1* is a previously unidentified gene whose overexpression disrupted silencing at telomeres, the *HM* loci, and rDNA (Table 3), but had no effect on an unsilenced locus. Moreover, deleting *DOT1* also reduced silencing at the telomeres and *HM* loci (Table 3). In addition, the deletion of *DOT1* caused increased heterogeneity in telomere length.

DOT1 was identified recently in a mutant screen for genes involved in a meiotic checkpoint and referred to as *PCH1* (pachytene checkpoint; S. Roeder, personal communication). By immunostaining, it was found to be associated with chromosomes in meiosis and present within the mitotic nucleus. Taken together with our findings, it seems very likely that *DOT1* is a chromatin protein, and, like *SIR4*, it is normally important for the formation of repressive chromatin.

***DOT4*:** *DOT4*, another previously unidentified gene, caused a loss of silencing at telomeres and the *HM* loci and a weak effect at the rDNA locus when overexpressed (Table 3), either as the truncated clone isolated in this work or as a full-length genomic clone (A. Kahana and D.E. Gottschling, unpublished results). Overexpression of *DOT4* had no effect on the expression of an

unsilenced marker gene, suggesting that *DOT4* overexpression specifically reversed the effects of repressive chromatin rather than generally increasing gene transcription.

Sequence analysis suggested that *DOT4* encodes a ubiquitin-processing protease (Ubp), 1 of 17 predicted to be in *S. cerevisiae* (Hochstrasser 1996). These enzymes cleave ubiquitin moieties from proteins. Conjugation of ubiquitin to proteins can target them for degradation by the 26S proteosome; removal of ubiquitin from a protein substrate by a Ubp would, therefore, be expected to result in stabilization of the protein. Conversely, Ubps can also act to enhance protein degradation by increasing the pool of free ubiquitin monomers or by helping to clear the proteosome of proteolytic fragments attached to ubiquitin. Aside from its role in regulating protein stability, ubiquitin conjugation to a protein substrate has also been implicated in macromolecular protein complex assembly (Finley *et al.* 1989; Davie and Murphy 1990; Chen *et al.* 1996; Hicke 1997).

Other components of the ubiquitin-dependent proteolytic pathway have been associated with silencing. Ubp3p was found to bind to a Sir4p affinity column, and deletion of *UBP3* results in an increase in telomeric silencing (Moazed and Johnson 1996). Also, deletion of the ubiquitin-conjugating enzyme *RAD6* weakens silencing at telomeres, the *HM* loci, and the *RDN1* locus (Bryk *et al.* 1997; Huang *et al.* 1997).

Although the effects of overexpressing *DOT4* suggest a connection between the protein and silencing chromatin, directly testing this connection was complicated because, in addition to causing defects in silencing, deletion of *DOT4* caused a growth defect. Thus, at this point, it is difficult to conclude what role *DOT4* might normally play in silencing.

***DOT5*:** Overexpression of *DOT5* had a relatively strong disruptive effect on telomeric silencing, a more modest effect on *HML* and *HMR* silencing, and no effect on rDNA silencing (Table 3). Its overexpression also caused an increase in telomere length. However, deletion of this gene had no effect on any of the silent loci (Table 3) and did not change telomere length.

Dot5p itself may not be required for normal telomere structure, but may interact with some required factor. Overexpression of *DOT5* may shift the steady state of that interaction, causing a decrease in the concentration of silencing factor available for telomere binding. Curiously, *DOT5* maps immediately adjacent to *EST3*, a gene required for replication of telomeric DNA (Morris and Lundblad 1997). While it is not known if *EST3* affects silencing, it may be that high levels of *DOT5* expression affect *EST3* expression. A change in EST3p levels may explain why *DOT5* overexpression caused a change in telomere length (Figure 4).

***DOT6*:** The *DOT6* sequence predicts a protein with a single Myb-related motif. The Myb domain comprises ~50 amino acids and is involved in sequence-specific DNA binding (Lipsick 1996). Intriguingly, it was recently found that various telomere sequence-binding proteins, including the telomere repeat-binding factors from human cells and *Schizosaccharomyces pombe*, contain a single repeat of a Myb-related sequence (Bilaud *et al.* 1996). The *S. cerevisiae* telomere repeat-binding protein Rap1p has two motifs related to this sequence. It is interesting to note that both cDNAs of *DOT6* isolated in our screen contained the motif.

Overexpression of the *DOT6* cDNA caused moderate disruption of telomeric and *RDN1* silencing, but had only a small effect at *HML* and *HMR* (Table 3). Overexpression of the clone had no effect, however, on telomere length. Moreover, deletion of *DOT6* had no effect in any of these assays (Table 3). The simplest explanation of these results is that *DOT6* normally has no role in silencing, but that overexpression of its Myb-like sequence competed with the related region in Rap1p for DNA binding. This competition would have to be limited, however, because it did not result in an effect on telomere length, which is sensitive to telomeric Rap1p levels (Conrad *et al.* 1990; Lustig *et al.* 1990; Kyrion *et al.* 1992; Marcand *et al.* 1997).

***TLC1*:** As described in our earlier work, overexpression of the telomerase RNA gene *TLC1* disrupts telomeric silencing specifically and causes shortening of the telomeric DNA tract (Singer and Gottschling 1994). In the present study, we found that *TLC1* was not directly required for silencing; strains without *TLC1* and with only half the normal length of telomeric DNA at the end of the chromosome were still silenced (Table 3). From these results, we suggest that the loss of telomeric silencing when *TLC1* is overexpressed is not the consequence of telomere DNA shortening, but rather, that *TLC1* RNA is interacting with a telomere-specific silencing factor. Furthermore, *TLC1* interferes with the telomeric silencing factor when overexpressed, but *TLC1* is not normally required for its telomeric silencing function. From this, we propose that this putative factor is not only important in telomeric silencing, but that it also serves as an anchor for telomerase to localize near the end of the chromosome.

The study of telomeric silencing has yielded insights both specific to telomere structure and generalizable to the larger, interacting collection of repressive loci in the genome. The genes affecting telomeric silencing identified in this work include both newly studied genes and previously known genes whose wider roles had not before been recognized. Although the function of several of these genes is still not known, it appears likely that most of these genes affect silencing through very different mechanisms. This finding reinforces the notion that epigenetic regulation in the cell is the result of an intricate and dynamic system that may be affected and regulated at multiple levels.

We thank J. Broach, J. Choy, S. Diede, R. Esposito, M. Hochstrasser, E. Louis, J. Rine, R. Schiestl, and J. Stevenson for plasmids; J. Boeke and J. Smith for strains; S. Elledge for the cDNA library; and S. Diede and M. Dubois for comments on the manuscript. This work was supported by a Medical Scientist National Research Service Award 5T32 GM07281 (A.K. and M.S.S.), a National Defense Science and Engineering Graduate Fellowship (M.S.S.), a Pew Charitable Trust Biomedical Scholars Fellowship, a Cancer Research Foundation Fletcher Scholarship, and National Institutes of Health grant GM43893 (D.E.G.).

LITERATURE CITED

Adams, A., D. E. Gottschling, C. A. Kaiser and T. Stearns, 1998 *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Aparicio, O. M., and D. E. Gottschling, 1994 Overcoming telomeric silencing: a *trans*-activator competes to establish gene expression in a cell cycle-dependent way. Genes Dev. **8:** 1133–1146.

Aparicio, O. M., B. L. Billington and D. E. Gottschling, 1991 Modifiers of position effect are shared between telomeric and silent mating-type loci in *S. cerevisiae*. Cell **66:** 1279–1287.

Ausubel, F. M., R. Brent, R. E. Kingston, D. O. Moore, J. G. Seidman *et al.* (Editors), 1995 *Current Protocols in Molecular Biology*. John Wiley & Sons, New York.

Axelrod, A., and J. Rine, 1991 A role for CDC7 in repression of transcription at the silent mating-type locus HMR in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11:** 1080–1091.

Beeler, T., K. Gable, C. Zhao and T. Dunn, 1994 A novel protein, CSG2p, is required for Ca^{2+} regulation in *Saccharomyces cerevisiae*. J. Biol. Chem. **269:** 7279–7284.

Berben, G., J. Dumont, V. Gilliquet, P.-A. Bolle and F. Hilger, 1991 The YDp plasmids: a uniform set of vectors bearing versatile gene disruption cassettes for *Saccharomyces cerevisiae*. Yeast **7:** 475–477.

Bilaud, T., C. E. Koering, E. Binet-Brasselet, K. Ancelin, A. Pollice *et al.*, 1996 The telobox, a Myb-related telomeric DNA binding motif found in proteins from yeast, plants, and humans. Nucleic Acids Res. **24:** 1294–1303.

Boeke, J. D., J. Trueheart, G. Natsoulis and G. R. Fink, 1987 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. Methods Enzymol. **154:** 164–75.

Bonne-Andrea, C., M. L. Wong and B. M. Alberts, 1990 In vitro replication through nucleosomes without histone displacement. Nature **343:** 719–726.

Brachmann, C. B., A. Davies, G. J. Cost, E. Caputo, J. Li *et al.*, 1998 Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast **14:** 115–132.

Bradbury, E. M., 1992 Reversible histone modifications and the chromosome cell cycle. Bioessays **14:** 9–16.

Bryk, M., M. Banerjee, M. Murphy, K. E. Knudsen, D. J. Garfinkel *et al.*, 1997 Transcriptional silencing of Ty1 elements in the *RDN1* locus of yeast. Genes Dev. **11:** 255–269.

Buck, S. W., and D. Shore, 1995 Action of a RAP1 carboxy-terminal silencing domain reveals an underlying competition between *HMR* and telomeres in yeast. Genes Dev. **9:** 370–384.

Budd, M. E., and J. L. Campbell, 1995 A yeast gene required for DNA replication encodes a protein with homology to DNA helicases. Proc. Natl. Acad. Sci. USA **92:** 7642–7646.

Budd, M. E., W.-C. Choe and J. L. Campbell, 1995 *DNA2* encodes a DNA helicase essential for replication of eukaryotic chromosomes. J. Biol. Chem. **270:** 26766–26769.

Chen, Z. J., L. Parent and T. Maniatis, 1996 Site-specific phosphorylation of IκBα by a novel ubiquitination-dependent protein kinase activity. Cell **84:** 853–862.

Cherel, I., and P. Thuriaux, 1995 The *IFH1* gene product interacts with a fork head protein in *Saccharomyces cerevisiae*. Yeast **11:** 261–270.

Chi, M.-H., and D. Shore, 1996 *SUM1-1*, a dominant suppressor of *SIR* mutations in *Saccharomyces cerevisiae*, increases transcriptional silencing at telomeres and *HM* mating-type loci and decreases chromosome stability. Mol. Cell. Biol. **16:** 4281–4294.

Christianson, T. W., R. S. Sikorski, M. Dante, J. H. Shero and P. Hieter, 1992 Multifunctional yeast high-copy-number shuttle vectors. Gene **110:** 119–122.

Cockell, M., F. Palladino, T. Laroche, G. Kyrion, C. Liu *et al.*, 1995 The carboxy termini of Sir4 and Rap1 affect SIR3 localization: evidence for a multicomponent complex required for yeast telomeric silencing. J. Cell Biol. **129:** 909–924.

Conrad, M. N., J. H. Wright, A. J. Wolf and V. A. Zakian, 1990 RAP1 protein interacts with yeast telomeres *in vivo*: overproduction alters telomere structure and decreases chromosome stability. Cell **63:** 739–750.

Dabeva, M. D., and J. R. Warner, 1987 The yeast ribosomal protein L32 and its gene. J. Biol. Chem. **262:** 16055–16059.

Dabeva, M. D., and J. R. Warner, 1993 Ribosomal protein L32 of *Saccharomyces cerevisiae* regulates both splicing and translation of its own transcript. J. Biol. Chem. **268:** 19669–19674.

Dabeva, M. D., M. A. Post-Beittenmiller and J. R. Warner, 1986 Autogenous regulation of splicing of the transcript of a yeast ribosomal protein gene. Proc. Natl. Acad. Sci. USA **83:** 5854–5857.

Davie, J. R., and L. C. Murphy, 1990 Level of ubiquitinated histone H2B in chromatin is coupled to ongoing transcription. Biochemistry **29:** 4752–4757.

Elledge, S. J., J. T. Mulligan, S. W. Ramer, M. Spottswood and R. W. Davis, 1991 Lambda YES: a multifunctional cDNA expression vector for the isolation of genes by complementation of yeast and *Escherichia coli* mutations. Proc. Natl. Acad. Sci. USA **88:** 1731–1735.

Eng, F. J., and J. R. Warner, 1991 Structural basis for the regulation of splicing of a yeast messenger RNA. Cell **65:** 797–804.

Enomoto, S., P. D. McCune-Zierath, M. Gerami-Nejad, M. A. Sanders and J. Berman, 1997 RLF2, a subunit of yeast chromatin assembly factor I, is required for telomeric chromatin function in vivo. Genes Dev. **11:** 358–370.

Finley, D., B. Bartel and A. Varshavsky, 1989 The tails of ubiquitin precursors are ribosomal proteins whose fusion to ubiquitin facilitates ribosome biogenesis. Nature **338:** 394–401.

Fox, C. A., A. E. Ehrenhofer-Murray, S. Loo and J. Rine, 1997 The origin recognition complex, *SIR1*, and the S phase requirement for silencing. Science **276:** 1547–1551.

Fritze, C. E., K. Verschueren, R. Strich and R. E. Esposito, 1997 Direct evidence for *SIR2* modulation of chromatin structure in yeast rDNA. EMBO J. **16:** 6495–6509.

Gottschling, D. E., 1992 Telomere-proximal DNA in *Saccharomyces cerevisiae* is refractory to methyltransferase activity *in vivo*. Proc. Natl. Acad. Sci. USA **89:** 4062–4065.

Gottschling, D. E., O. M. Aparicio, B. L. Billington and V. A. Zakian, 1990 Position effect at *S. cerevisiae* telomeres: reversible repression of Pol II transcription. Cell **63:** 751–762.

Grunstein, M., 1997 Molecular model for telomeric heterochromatin in yeast. Curr. Opin. Cell Biol. **9:** 383–387.

Hecht, A., S. Strahl-Bolsinger and M. Grunstein, 1996 Spreading of transcriptional repressor SIR3 from telomeric heterochromatin. Nature **383:** 92–96.

Henikoff, S., and M. K. Eghtedarzadeh, 1987 Conserved arrangement of nested genes at the Drosophila *Gart* locus. Genetics **117:** 711–725.

Hermann-Le Denmat, S., M. Werner, A. Sentenac and P. Thuriaux, 1994 Suppression of yeast RNA polymerase III mutations by *FHL1*, a gene coding for a *fork head* protein involved in rRNA processing. Mol. Cell. Biol. **14:** 2905–2913.

Herskowitz, I., 1987 Functional inactivation of genes by dominant negative mutations. Nature **329:** 219–222.

Hicke, L., 1997 Ubiquitin-dependent internalization and down-regulation of plasma membrane proteins. FASEB J. **11:** 1215–1226.

Hochstrasser, M., 1996 Ubiquitin-dependent protein degradation. Annu. Rev. Genet. **30:** 405–439.

Hoffman, C. S., and F. Winston, 1987 A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. Gene **57:** 267–272.

Horn, D., and G. A. Cross, 1995 A developmentally regulated position effect at a telomeric locus in *Trypanosoma brucei*. Cell **83:** 555–561.

Huang, H., A. Kahana, D. E. Gottschling, L. Prakash and S. W. Liebman, 1997 The ubiquitin-conjugating enzyme Rad6 (Ubc2) is required for silencing in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **17:** 6693–6699.

Ito, T., J. K. Tyler and J. T. Kadonaga, 1997 Chromatin assembly factors: a dual function in nucleosome formation and mobilization? Genes Cells **2:** 593–600.

Ivy, J. M., A. J. Klar and J. B. Hicks, 1986 Cloning and characterization of four *SIR* genes of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **6:** 688–702.

Johnston, M., and R. W. Davis, 1984 Sequences that regulate the divergent *GAL1-GAL10* promoter in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **4:** 1440–1448.

Kaufmann, E., and W. Knochel, 1996 Five years on the wings of fork head. Mech. Dev. **57:** 3–20.

Kaufman, P. D., R. Kobayashi and B. Stillman, 1997 Ultraviolet radiation-sensitivity and reduction of telomeric silencing in *Saccharomyces cerevisiae* cells lacking chromatin assembly factor-I. Genes Dev. **11:** 345–357.

Kennedy, B. K., M. Gotta, D. A. Sinclair, K. Mills, D. S. McNabb *et al.*, 1997 Redistribution of silencing proteins from telomeres to the nucleolus is associated with extension of life span in *S. cerevisiae*. Cell **89:** 381–391.

Kho, C. J., G. S. Huggins, W. O. Endege, C. M. Hsieh, M. E. Lee *et al.*, 1997 Degradation of E2A proteins through a ubiquitin-conjugating enzyme, UbcE2A. J. Biol. Chem. **272:** 3845–3851.

Kimmerly, W. J., and J. Rine, 1987 Replication and segregation of plasmids containing cis-acting regulatory sites of silent mating-type genes in *Saccharomyces cerevisiae* are controlled by the *SIR* genes. Mol. Cell. Biol. **7:** 4225–4237.

Klar, A. J., S. N. Kakar, J. M. Ivy, J. B. Hicks, G. P. Livi *et al.*, 1985 *SUM1*, an apparent positive regulator of the cryptic mating-type loci in *Saccharomyces cerevisiae*. Genetics **111:** 745–758.

Kyrion, G., K. A. Boakye and A. J. Lustig, 1992 C-terminal truncation of RAP1 results in the deregulation of telomere size, stability, and function in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **12:** 5159–5173.

Kyrion, G., K. Liu, C. Liu and A. J. Lustig, 1993 RAP1 and telomere structure regulate telomere position effects in *Saccharomyces cerevisiae*. Genes Dev. **7:** 1146–1159.

Laman, H., D. Balderes and D. Shore, 1995 Disturbance of normal cell cycle progression enhances the establishment of transcriptional silencing in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **15:** 3608–3617.

Laurenson, P., and J. Rine, 1991 *SUM1-1*: a suppressor of silencing defects in *Saccharomyces cerevisiae*. Genetics **129:** 685–696.

Laurenson, P., and J. Rine, 1992 Silencers, silencing, and heritable transcriptional states. Microbiol. Rev. **56:** 543–560.

Le, S., C. Davis, J. B. Konopka and R. Sternglanz, 1997 Two new S-phase-specific genes from *Saccharomyces cerevisiae*. Yeast **13:** 1029–1042.

Levis, R., T. Hazelrigg and G. M. Rubin, 1985 Effects of genomic position on the expression of transduced copies of the white gene of Drosophila. Science **229:** 558–561.

Lipsick, J. S., 1996 One billion years of myb. Oncogene **13:** 223–235.

Livi, G. P., J. B. Hicks and A. J. Klar, 1990 The *sum1-1* mutation affects silent mating-type gene transcription in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10:** 409–412.

Lloyd, V. K., D. A. Sinclair and T. A. Grigliatti, 1997 Competition between different variegating rearrangements for limited heterochromatic factors in *Drosophila melanogaster*. Genetics **145:** 945–959.

Loison, G., R. Losson and F. Lacroute, 1980 Constitutive mutants for orotidine-5-phosphate decarboxylase and dihydroorotic acid dehydrogenase in *Saccharomyces cerevisiae*. Curr. Genet. **2:** 39–44.

Loo, S., and J. Rine, 1995 Silencing and heritable domains of gene expression. Annu. Rev. Cell Dev. Biol. **11:** 519–548.

Losson, R., and F. Lacroute, 1981 Cloning of a eukaryotic regulatory gene. Mol. Gen. Genet. **184:** 394–399.

Lustig, A. J., and T. D. Petes, 1986 Identification of yeast mutants with altered telomere structure. Proc. Natl. Acad. Sci. USA **83:** 1398–1402.

Lustig, A. J., S. Kurtz and D. Shore, 1990 Involvement of the silencer and UAS binding protein RAP1 in regulation of telomere length. Science **250:** 549–553.

Machado, C. R., U. M. Praekelt, R. C. de Oliveira, A. C. Barbosa, K. L. Byrne *et al.*, 1997 Dual role for the yeast *THI4* gene in thiamine biosynthesis and DNA damage tolerance. J. Mol. Biol. **273:** 114–121.

Maillet, L., C. Boscheron, M. Gotta, S. Marcand, E. Gilson *et al.*, 1996 Evidence for silencing compartments within the yeast nucleus: a role for telomere proximity and Sir protein concentration in silencer-mediated repression. Genes Dev. **10:** 1796–1811.

Marcand, S., E. Gilson and D. Shore, 1997 A protein-counting mechanism for telomere length regulation in yeast. Science **275:** 986–990.

Marshall, M., D. Mahoney, A. Rose, J. B. Hicks and J. R. Broach, 1987 Functional domains of *SIR4*, a gene required for position effect regulation in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **7:** 4441–4452.

Moazed, D., and A. D. Johnson, 1996 A deubiquitinating enzyme interacts with SIR4 and regulates silencing in *S. cerevisiae*. Cell **86:** 667–677.

Moretti, P., K. Freeman, L. Coodly and D. Shore, 1994 Evidence that a complex of SIR proteins interacts with the silencer and telomere-binding protein RAP1. Genes Dev. **8:** 2257–2269.

Morris, D. K., and V. Lundblad, 1997 Programmed translational frameshifting in a gene required for yeast telomere replication. Curr. Biol. **7:** 969–976.

Nasr, F., A. M. Becam, S. C. Brown, D. De Nay, P. P. Slonimski *et al.*, 1995 Artificial antisense RNA regulation of *YBR1012* (*YBR136w*), an essential gene from *Saccharomyces cerevisiae* which is important for progression through G1/S. Mol. Gen. Genet. **249:** 51–57.

Palladino, F., T. Laroche, E. Gilson, A. Axelrod, L. Pillus *et al.*, 1993 SIR3 and SIR4 proteins are required for the positioning and integrity of yeast telomeres. Cell **75:** 543–555.

Pryde, F. E., and E. J. Louis, 1997 Saccharomyces cerevisiae telomeres. A review. Biochemistry **62:** 1232–1241.

Ramer, S. W., S. J. Elledge and R. W. Davis, 1992 Dominant genetics using a yeast genomic library under the control of a strong inducible promoter. Proc. Natl. Acad. Sci. USA **89:** 11589–11593.

Renauld, H., O. M. Aparicio, P. D. Zierath, B. L. Billington, S. K. Chhablani *et al.*, 1993 Silent domains are assembled continuously from the telomere and are defined by promoter distance and strength, and by *SIR3* dosage. Genes Dev. **7:** 1133–1145.

Roman, H., 1956 Studies of gene mutation in *Saccharomyces*. Cold Spring Harbor Symp. Quant. Biol. **21:** 175–185.

Runge, K. W., and V. A. Zakian, 1989 Introduction of extra telomeric DNA sequences into *Saccharomyces cerevisiae* results in telomere elongation. Mol. Cell. Biol. **9:** 1488–1497.

Shore, D., 1994 RAP1: a protean regulator in yeast. Trends Genet. **10:** 408–412.

Sikorski, R. S., and P. Hieter, 1989 A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:** 19–27.

Singer, M. S., 1997 Genetic studies of telomeric position effect and the identification of the telomerase template RNA in *Saccharomyces cerevisiae*. Ph.D. Thesis, The University of Chicago.

Singer, M. S., and D. E. Gottschling, 1994 *TLC1*: template RNA component of *Saccharomyces cerevisiae* telomerase. Science **266:** 404–409.

Smith, J. S., and J. D. Boeke, 1997 An unusual form of transcriptional silencing in yeast ribosomal DNA. Genes Dev. **11:** 241–254.

Strahl-Bolsinger, S., A. Hecht, K. Luo and M. Grunstein, 1997 SIR2 and SIR4 interactions differ in core and extended telomeric heterochromatin in yeast. Genes Dev. **11:** 83–93.

Thompson, J. S., X. Ling and M. Grunstein, 1994 Histone H3 amino terminus is required for telomeric and silent mating locus repression in yeast. Nature **369:** 245–247.

Triolo, T., and R. Sternglanz, 1996 Role of interactions between the origin recognition complex and SIR1 in transcriptional silencing. Nature **381:** 251–253.

van Steensel, B., A. Smogorzewska and T. de Lange, 1998 TRF2 protects human telomeres from end-to-end fusions. Cell **92:** 401–413.

Wool, I., 1996 Extraribosomal functions of ribosomal proteins. Trends Biochem. Sci. **21:** 164–165.

Zakian, V. A., 1996 Structure, function, and replication of *Saccharomyces cerevisiae* telomeres. Annu. Rev. Genet. **30:** 141–172.

Zomerdijk, J. C., R. Kieft, M. Duyndam, P. G. Shiels and P. Borst, 1991 Antigenic variation in *Trypanosoma brucei*: a telomeric expression site for variant-specific surface glycoprotein genes with novel features. Nucleic Acids Res. **19:** 1359–1368.

Communicating editor: F. Winston