Analysis of Sir2p Domains Required for rDNA and Telomeric Silencing in *Saccharomyces cerevisiae*

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ABSTRACT

Silent information regulator (Sir) 2 is a limiting component of the Sir2/3/4 complex, which represses transcription at subtelomeric and *HM* loci. Sir2p also acts independently of Sir3p and Sir4p to influence chromatin organization in the rDNA locus. Deleted and mutated forms of Sir2p have been tested for their ability to complement and/or to disrupt silencing. The highly conserved C-terminal domain of Sir2p (aa 199–562) is insufficient to restore repression at either telomeric or rDNA reporters in a *sir2*Δ background and fails to nucleate silencing when targeted to an appropriate reporter gene. However, its expression in an otherwise wild-type strain disrupts telomeric repression. Similarly, a point mutation (P394L) within this conserved core inactivates the full-length protein but renders it dominant negative for all types of silencing. Deletion of aa 1–198 from Sir2^{394L} eliminates its dominant negative effect. Thus we define two distinct functional domains in Sir2p, both essential for telomeric and rDNA repression: the conserved core domain found within aa 199–562 and a second domain that encompasses aa 94–198. Immunolocalization and two-hybrid studies show that aa 94–198 are required for the binding of Sir2p to Sir4p and for the targeting of Sir2p to the nucleolus through another ligand. The globular core domain provides an essential silencing function distinct from that of targeting or Sir complex formation that may reflect its reported mono-ADP-ribosyl transferase activity.

CHROMATIN-mediated repression at yeast subtelomeric regions and mating-type loci requires a multicomponent nucleosome-binding complex that contains a balanced complement of Sir2p, Sir3p, and Sir4p. These relatively abundant silent information regulators share no homology among themselves, yet both Sir3p and Sir4p can bind the N-terminal tails of histones H3 and H4 directly (Hecht *et al.* 1995). Extensive domain and deletion analysis has been carried out on Sir3p and Sir4p. In addition to being able to homodimerize, heterodimerize, and bind histones, they interact individually with a number of proteins involved in the nucleation step of telomeric and mating-type silencing (reviewed in Cockell *et al.* 1998a; Stone and Pillus 1998). For example, Sir4p binds to Rap1p, the Sir proteins 1, 2, and 3, Sif2p, Ubp3p, and yKu70p (Moretti *et al.* 1994; Cockell *et al.* 1995, 1998b; Moazed and Johnson 1996; Triolo and Sternglanz 1996; Tsukamoto *et al.* 1997), while Sir3p binds Rap1p, Rad7p, and Sir4p (Moretti *et al.* 1994; Paetkau *et al.* 1994; Strahl-Bolsinger *et al.* 1997). For both Sir3p and Sir4p, independent expression of their N- and C-terminal domains *in trans* can functionally complement the absence of the holoprotein (Marshall *et al.* 1987; Gotta *et al.* 1998). In contrast, little is known about how Sir2p functions or which components of the telomeric and *HM* silencing machinery it interacts with, other than Sir4p.

The mystery surrounding the role of Sir2p in chromatin-mediated repression is all the more surprising because *SIR2*, unlike *SIR3* or *SIR4*, is a member of a large family of genes that has been conserved from bacteria to humans. Among the four proteins *h*omologous to *s*ir *t*wo in budding yeast, elevated expression of *HST1* is able to restore mating type silencing in a *sir2*Δ strain while a *hst3 hst4* double mutant is partially deficient for telomeric position effect (TPE; Brachmann *et al.* 1995; Derbyshire *et al.* 1996). In addition to these budding yeast homologues, Sir2-like proteins exist in various bacteria, Trypanosoma, flies, worms, plants, mice, and humans (Brachmann *et al.* 1995; Frye 1999). A related gene from the pathogenic fungus *Candida albicans*, which can partially complement the loss of the budding yeast *SIR2* gene, has been implicated in the control of phenotypic switching and chromosome stability (Perez-Martin *et al.* 1999). In the fission yeast *Schizosaccharomyces pombe*, a gene closely related to *HST4* in budding yeast has recently been shown to influence silencing at both telomeres and centromeres (Freeman-Cook *et al.* 1999). These findings lend support to the hypothesis that other members of the *SIR2* family may affect chromatin organization.

Recent studies suggest a possible enzymatic function for this family: a *SIR2*-like gene from Salmonella was recently identified as an extragenic suppressor of a phosphoribosyl transferase mutant (Tsang and Escalante-

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Semerena 1998). It was unclear, however, whether overexpression of the Salmonella gene compensates for this mutation by supplying a similar enzymatic function or whether it acts indirectly by modifying the transcriptional regulation of other genes. More direct evidence is provided by a study of one of the human *SIR2* family members (hSirT2), which was shown to have a mono-ADP-ribosylation activity *in vitro* (Frye 1999). Further experiments indicate that inactivation of the enzymatic activity of the yeast Sir2p correlates with a loss of its silencing function (Tanny *et al.* 1999).

In addition to helping to repress *HM* and telomeric loci, Sir2p, unlike Sir3p or Sir4p, is highly enriched in the nucleolus and can be recovered efficiently crosslinked throughout the length of the 9-kb rDNA repeat unit (Gotta *et al.* 1997). On yeast chromosome XII, where there are 100–200 tandem copies of the rDNA repeat, Sir2p helps suppress homologous recombination (Gottlieb and Esposito 1989). Intriguingly, only about half of the ~200 copies of the 35S rRNA gene are transcribed at any one time (Dammann *et al.* 1993) and roughly one-fifth of the rDNA replication origins fire each cell cycle (Walmsley *et al.* 1984; Brewer and Fangman 1988). As shown for recombination, the efficiency of transcriptional (Smith and Boeke 1997) and origin firing activities (P. Pasero and M. Cockell, unpublished results) increases in the absence of Sir2p. Correlated with this general nucleolar activation, *sir2*Δ strains have an instability of the rDNA locus and a significant shortening in the average life-span (Sinclair and Guarente 1997).

The most commonly used assay for a condensed or repressed state within the nucleolus makes use of a Ty transposable element or another RNA Pol II-dependent reporter inserted in the rDNA repeats (Bryk *et al.* 1997; Smith and Boeke 1997). The Sir2p-dependent variegated repression of such reporters has been characterized by several laboratories. Nuclease, methylase, and psoralen accessibility assays reveal structural differences between the active and inactive copies of the rDNA repeat, which are dependent on Sir2p, Net1p, and a balanced dosage of the histone H2A/H2B dimer (Bryk *et al.* 1997; Fritze *et al.* 1997; Smith and Boeke 1997; Shou *et al.* 1999; Straight *et al.* 1999). Net1p, together with Nan1p and Cdc14p, forms a telophase regulatory complex that can be crosslinked to DNA with Sir2p throughout the rDNA repeat (Shou *et al.* 1999; Straight *et al.* 1999). Whereas Net1p associates with the rDNA in the absence of Sir2p, the converse is not true. Little else is known about the molecular basis of rDNA repression.

We initiated this study to determine whether different subdomains or amounts of Sir2p are required at its different sites of action. By examining the effects of overexpression of full-length Sir2p in strains carrying reporters for mating type, telomeric, and rDNA silencing, we conclude that Sir2p levels are normally limiting at both the rDNA and at telomeres, although not at *HM* loci. Others have also shown that Sir2p released from telomeres in *sir4*Δ strains contributes to enhanced rDNA repression, suggesting that the two loci compete for the same limiting pool of Sir2p (Smith *et al.* 1998). On the basis of the assumption that proteins interacting with different interfaces of Sir2p regulate its distribution among nuclear subcompartments, our aim was to examine the different roles played by Sir2p in modifying chromatin structure, by dissecting the protein into various subdomains that might mediate partial steps at telomeres or in the rDNA.

MATERIALS AND METHODS

The genotypes of the yeast strains and plasmids used in this study are indicated in Tables 1 and 2. Rich medium, minimal medium, amino acid supplements, and standard yeast genetic methods were used as described in Rose *et al.* (1990). Minimal medium was supplemented with either 2% (w/v) glucose or 2% (w/v) galactose and 1% (w/v) raffinose as indicated in the figure legends. Limiting adenine means 10 μg/ml. Recombinant DNA methods (Sambrook *et al.* 1989) and two-hybrid studies (Golemis *et al.* 1996) were carried out using published protocols.

Plasmid constructions: The plasmids pGal-Sir2, pGal-sir2^{394L}, pGal-sir2$^{94\text{-}562}$, pGal-sir2$^{199\text{-}562}$, pGal-sir2$^{263\text{-}562}$, and pGal-sir2$^{1\text{-}421}$ were constructed by in-frame ligation of *SIR2* fragments into the *Eco*RI and *Xho*I sites of the vector pJG45. The *Eco*RI-*Xho*I fragments encoding *SIR2* and parts of the protein were obtained by high-fidelity PCR using the relevant primer pairs on a plasmid template that contains a 4.6-kb genomic *Hin*dIII fragment encoding the full-length *SIR2* gene (pAR6, a gift of J. Broach). An *Eco*RI-*Xho*I fragment encoding *HST2* was also obtained from genomic template DNA by PCR and the plasmid pGal-Hst2 was created by in-frame ligation of this fragment into the vector pJG45 (called pGal in Table 2). Constructs were verified by DNA sequence analysis. Western blots on whole cell extracts of the yeast transformants verified the size of each fusion protein.

An *Eco*RI-*Xho*I fragment encoding sir2^{394L} was generated by sequential PCR steps as described by Cormack (1991). Primer pairs were used on the plasmid template pAR6 to generate two fragments encompassing the mutation. These were annealed and extended with the flanking primers to generate a full-length *SIR2* fragment encoding leucine instead of proline at amino acid (aa) 394. The fragment thus generated was verified by DNA sequence analysis. Anti-HA epitope blots on extracts from transformed yeast confirm that Gal-Sir2p and Gal-sir2^{394L} migrate identically. The plasmid pGal-sir2core^{394L} was generated by exchanging the *Bgl*II-*Stu*I restriction fragment, which encodes aa 275–426 of pGal-Sir2 with the same fragment of pGal-sir2^{394L}. Insertion of the mutated fragment was verified by DNA sequencing.

The *Eco*RI-*Xho*I fragments encoding full-length *HST2*, *SIR2*, and parts of the *SIR2* gene were subcloned from the pGal-Sir2 series into the vectors p698 and p731 (pRS426-ADH and pRS416-ADH, respectively; Mumberg *et al.* 1995), pGBT9 and pGAD424 (Clontech Laboratories, Palo Alto, CA), and pEG202 (Golemis *et al.* 1996). Table 2 lists the plasmids created and the names used herein.

A *Bgl*II-*Xho*I fragment encoding aa 731–1358 of Sir4p was excised and inserted into the same sites of the vector pEG202 (Golemis *et al.* 1996) to give pEG202-sir4$^{731\text{–}1358}$. An *Eco*RI-*Xho*I fragment from pEG202-sir4$^{731\text{–}1358}$ was subcloned into the same

TABLE 1

Yeast strains used in this study

Strain	Genotype	Reference
GA426 (UCC3107)	*MAT***a** *ade2::hisG can1::hisG his3-11 leu2 trp1Δ ura3-52 TEL V-R::ADE2*	Stone and Pillus (1996)
GA427 (UCC3203)	*MAT***a** *ade2::hisG can1::hisG his3-11 leu2 trp1Δ ura3-52 TEL V-R::ADE2 sir2Δ::HIS3*	Gotta *et al.* (1997)
MC92 (EG5)	*MAT***a** *leu2-3, 112 ura3-52 trp1-289 his3 gal2 hml::LEU2″lacZ(+)EI*, pAAH5 (*LEU2, CEN, ARS)*	Maillet *et al.* (1996)
MC94 (EG30)	*MAT***a** *leu2-3, 112 ura3-52 trp1-289 his3 gal2 hml::LEU2″lacZ(+)EΔI*, +pAAH5 (*LEU2, CEN, ARS*)	Boscheron *et al.* (1996)
MC162 (EG139)	*MAT***a** *leu2-3, 112 ura3-52 trp1-289 his3 gal2 hml::LEU2″lacZ(+)EI sir4::HIS3*, +pAAH5 (*LEU2, CEN, ARS)*	Maillet *et al.* (1996)
GA503 (UCC3505)	*MAT***a** *ura3-52 lys2-801 ade2-101 trp1-63, his3Δ200 leu2-1 ppr1::HIS3 adh4::URA3-TEL VII-L; TEL V-R::ADE2*	Singer and Gottschling (1994)
GA758 (JS231)	*MAT***a** *his3Δ200 leu2Δ1 trp1Δ63 ura3-167 RDN1::mURA3/HIS3*	Smith *et al.* (1998)
GA760	*MAT***a** *his3Δ200 leu2Δ1 trp1Δ63 ura3-167 RDN1::mURA3/HIS3 sir2::kanMX4*	J. Smith
GA194 (GA185 × GA188)	*MAT***a**/*MATαade2/ADE2 trp1/trp1 his3-11/his3 ura3-1/ura3-52 can1-100/can1 leu2-3,112/lEU2 sir2::HIS3/sir2::HIS3*	Gotta *et al.* (1997)
GA225 (GA187 × GA184)	*MATa*/*MAT***α***ade2/ADE2 trp1/trp1his3-11,15/his3 ura3-1/ura3-52 can1-100/can1-100*	Gotta *et al.* (1997)
GA1034 (Ce76)	*MAT***a** *leu2-3, 112 ura3-52 trp1 his3Δ gal2 hml::GalUAS-URA3(+)EΔi*	C. Boscheron and E. Gilson
GA1035 (Ce77)	*MAT***a** *leu2-3, 112 ura3-52 trp1 his3Δ gal2 hml::GalUAS-URA3(+)ΔeΔi*	C. Boscheron and E. Gilson
GA1084 (Ce77 sir4Δ)	*MAT***a** *leu2-3, 112 ura3-52 trp1 his3Δ gal2 hml::GalUAS-URA3(+)ΔeΔi sir4Δ::HIS3*	Martin *et al.* (1999)
GA1210 (CTY10-5d sir3Δ)	*MAT***a** *ade2 trp1-901 leu2-3, 112 his3Δ200 gal4 gal80 URA3::lexAop-lacZ sir3Δ::TRP1*	
GA1209 (EGY48)	*MATα his3 trp1 ura3-52 leu2::pLEU2-lexAop6*	Golemis *et al.* (1996)

sites of the vector pJG45 to give pJG45-sir4$^{731–1358}$. A *Bam*HI-*Hin*dIII fragment encoding aa 838–1358 of Sir4p was excised from pBR-Sir4 (Marshall *et al.* 1987) and cloned into the *Bam*HI site of pEG202 after addition of a *Bam*HI linker to the 3′ end of the fragment to give pEG202-sir4$^{838–1358}$. The plasmid pGADsir4$^{838–1358}$ was a gift from Rolf Sternglanz and is derived from pCTC18 (Chien *et al.* 1991). The plasmid pNSir3N encodes a LexA DNA-binding domain fused 3′ of the DNA encoding aa 1–503 of the Sir3 N terminus. The Sir3N-lexA fusion protein localizes to the nucleolus and influences TPE, while fusions that are 5′ of the *SIR3* gene inactivate Sir3p functions (Gotta *et al.* 1998).

Repression assays: Liquid β-galactosidase assays on permeabilized yeast cells were performed as described in Boscheron *et al.* (1996). Transformed strains carrying the *ade2*-1 mutation and an intact *ADE2* gene integrated close to the telomere on the right arm of chromosome V were streaked onto selective media containing 10 μg/ml adenine. After growth for several days at 30°, colonies are stored for a week at 4° to allow pigment accumulation.

The *URA3* reporter gene was integrated at Tel VII-L to monitor TPE (Singer and Gottschling 1994) and the altered promoter version *mURA3* is integrated in the *RDN1* locus (Smith and Boeke 1997). Repression was monitored by determining the fraction of cells able to grow on medium lacking uracil. Serial dilutions are monitored for growth after 2–3 days at 30° on medium lacking the amino acid appropriate for the introduced plasmid, with or without uracil. The mean is calculated from multiple serial dilutions of at least four independent colonies. Error bars represent the spread of the values.

Immunofluorescence and preparation of antibodies: Immunofluorescence was performed as described previously (Gotta *et al.* 1996) using affinity-purified antibodies to the Sir4C terminus and to Rap1. Other antibodies used are anti-HA (HA.11, clone 16B12 monoclonal from BABCO), anti-Nop1 (A66, monoclonal yeast Nop1p, gift of John P. Aris, Miami), Cy5-coupled anti-mouse secondary antibody, and fluorescein-coupled anti-rabbit secondary antibody (both from Milan Analytica). Secondary antibodies were preabsorbed against fixed yeast spheroplasts prior to use. Confocal microscopy was performed on a Zeiss Axiovert 100 microscope (Zeiss laser scanning microscope 410) with a 63× Plan-Apochromat objective (1.4 oil) as previously described (Gotta *et al.* 1996). Under standard imaging conditions no signal from one fluorochrome could be detected on the other filter set. Standardized conditions for the image capture and subtraction of a background value taken from outside the yeast cells (∼15% of the maximum signal) were uniformly applied to all images.

RESULTS

Sir2p is limiting at telomeres and the rDNA, but not at *HML*: It has been established that the normal level of Sir3p in the nucleus is limiting for telomere proximal repression (Renauld *et al.* 1993), while elevated levels of Sir2p appeared to have no effect at telomeres (Braunstein *et al.* 1993; Renauld *et al.* 1993). On the other hand, increased *SIR2* dosage enhances repression

TABLE 2

Plasmids used in this study

Plasmid	Description	Reference
pGal	Equivalent to pJG45 (*2*μARS, *TRP1*, expresses B42 activation domain-NLS-HA under control of UAS_G)	Golemis *et al.* (1996)
pGal-Sir2	B42-NLS-HA fused to N-ter of Sir2p	
pGal-sir2$^{94–562}$	B42-NLS-HA fused to N-ter of sir2$^{94–562}$	
pGal-sir2$^{199–562}$	B42-NLS-HA fused to N-ter of sir2$^{199–562}$	
pGal-sir2$^{348–562}$	B42-NLS-HA fused to N-ter of sir2$^{348–562}$	
pGal-sir2$^{1–421}$	B42-NLS-HA fused to N-ter of sir2$^{1–421}$	
pGal-sir2^{394L}	B42-NLS-HA fused to N-ter of Sir2p carrying a P to L mutation at aa 394	
pGal-sir2core^{394L}	B42-NLS-HA fused to N-ter of sir2$^{199–562}$ carrying a P to L mutation at aa 394	
pGal-Hst2	B42-NLS-HA fused to N-ter of Hst2p	
p698	Equivalent to pRS424-ADH, (*2*μARS, *TRP1*, proADH1)	Mumberg *et al.* (1995)
p698-Sir2	Sir2p expressed under proADH1	
p731	Equivalent to pRS416-ADH, (*CEN*, *TRP1*, proADH1)	Mumberg *et al.* (1995)
p731-Sir2	Sir2p expressed under proADH1	
pGBD	Equivalent to pGBT9, Clontech (*2*μARS, *TRP1*, expresses GAL4 DNA-binding domain under proADH1)	
pGBD-Sir2	GBD fused to N-ter of Sir2p	
pGBD-sir2$^{94–562}$	GBD fused to N-ter of sir2$^{94–562}$	
pGBD-sir2$^{199–562}$	GBD fused to N-ter of sir2$^{199–562}$	
pGBD-sir2^{394L}	GBD fused to N-ter of Sir2p carrying a P to L mutation at aa 394	
pEG202	(2μm, HIS3, expresses LexA DNA-binding domain under proADH1)	Golemis *et al.* (1996)
pEG202-Sir2	LexA DNA-binding domain fused to Sir2p	
pEG202-sir2$^{94–562}$	LexA DNA-binding domain fused to sir2$^{94–562}$	
pEG202-sir2$^{199–562}$	LexA DNA-binding domain fused to sir2$^{199–562}$	
pEG202-sir2^{394L}	LexA DNA-binding domain fused to Sir2p carrying a P to L mutation at aa 394	
pEG202-Hst2	LexA DNA-binding domain fused to Hst2p	
pEG202-sir4$^{838–1358}$	LexA DNA-binding domain fused to sir4$^{838–1358}$	
pEG202-sir4$^{731–1358}$	LexA DNA-binding domain fused to sir4$^{731–1358}$	
pNsir3N$^{1–503}$	sir3N$^{1–503}$ inserted upstream of the LexA DNA-binding domain	Gotta *et al.* (1998)
pGAD	(*2*μARS, *LEU2*, expresses GAL4 activation domain and NLS under proADH1)	Clontech
pGAD-sir4$^{838–1358}$	GAL4 activation domain fused to sir4$^{838–1358}$ (derived from pCT18)	Chien *et al.* (1991)
pGAD-Sir2	GAL4 activation domain fused to Sir2p	
pGAD-sir2^{394L}	GAL4 activation domain fused to Sir2p carrying a P to L mutation at aa 394	

NLS, nuclear localization signal; UAS_G, upstream activating sequence of the GAL1-10 promoter; proADH1, promoter sequence of the ADH1 gene; HA, hemoagglutinin epitope.

of RNA pol II reporters in the rDNA (Smith and Boeke 1997). Since this might indicate that Sir2p functions differently at different sites, we examined more closely whether Sir2p is limiting for repression at telomeres and *HM* loci by overexpressing the protein at several different levels in strains carrying the appropriate subtelomeric reporters. All full-length *SIR2* constructs are able to complement *sir2*Δ strains, indicating that in the cases where Sir2p is fused to bacterial domains, these latter do not interfere with Sir2p function (Figure 1A).

To monitor TPE, we use strains carrying an *ADE2* reporter integrated next to the telomeric repeat of chromosome V-R, such that the accumulation of red pigment in sectors or throughout individual colonies reflects the extent of *ADE2* repression (Figure 1). In the *SIR2* strain (GA426) carrying an empty vector, the metastable repression at telomeres produces a sectored phenotype, while the isogenic *sir2*Δ strain (GA427) is uniformly white, due to efficient *ADE2* expression. Low levels of Sir2p or of a fusion protein, Gal-Sir2p, restore TPE to the *sir2*Δ strain and also improve silencing significantly in an isogenic *SIR2* strain (see Sir2 and Gal-Sir2, labeled CEN and glu, respectively, Figure 1A). This indicates that Sir2p is normally limiting for maximal telomeric repression. On the other hand, when Gal-*SIR2* is induced on galactose-containing medium, telomere proximal silencing is disrupted (gal, Figure 1A). A 10^3-fold derepression of a *URA3* reporter inserted at Tel VII-L was also measured upon induction of Gal-*SIR2*, indicating that this effect is not reporter specific (Figure 1C, left). Thus, even though Sir2p may initially be limiting at telomeres, there is a threshold beyond which excess

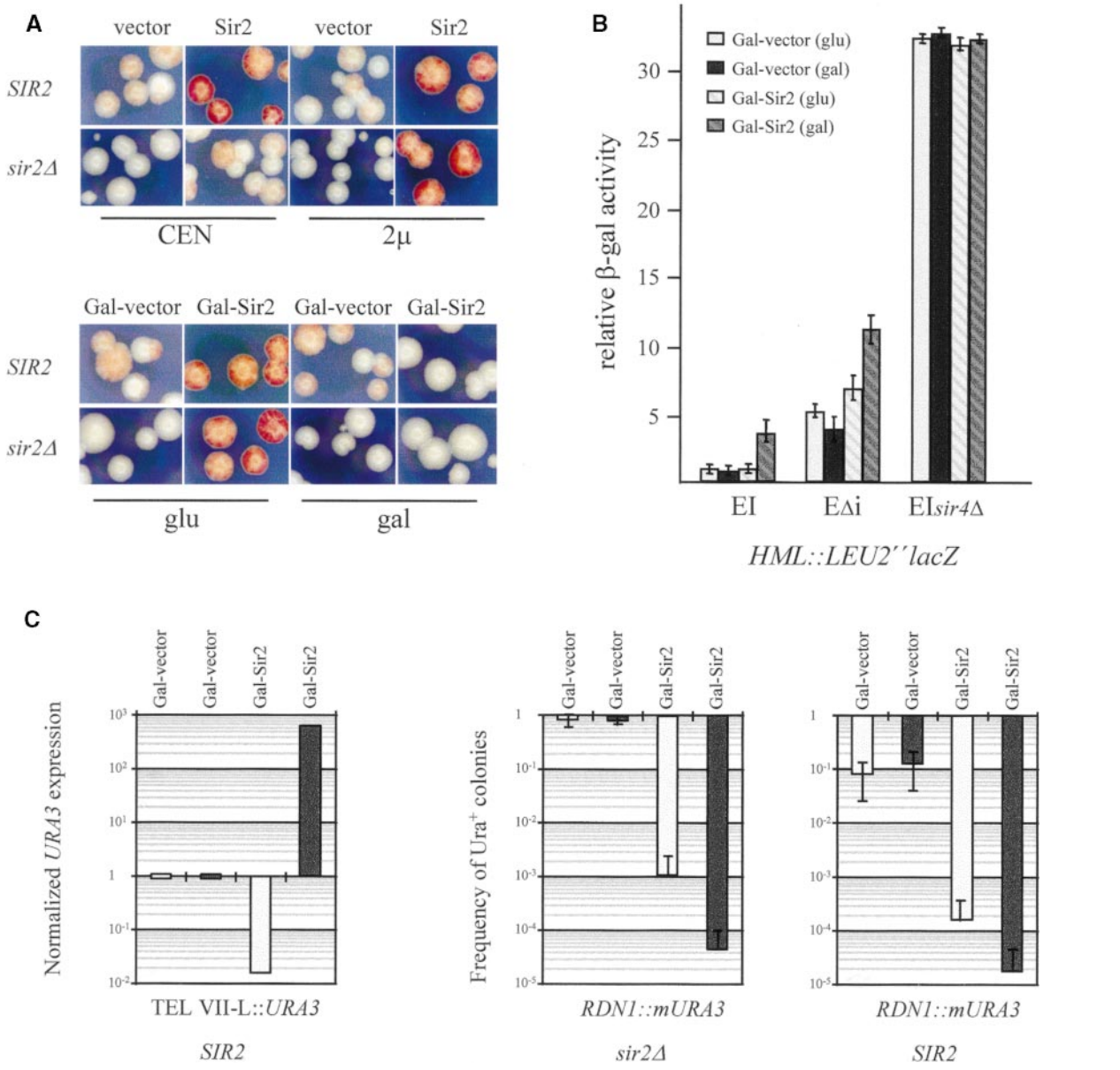

Figure 1.—Sir2p is limiting at telomeres and rDNA, but not at *HML*. (A) In isogenic *SIR2* and *sir2*Δ strains, colony color reflects the extent of repression at TELV-R::*ADE2*. The metastable switching of telomeric silencing in the wild-type strain results in pink/white sectored colonies, while red or white colony color correlates with strong repression or derepression, respectively. (Top) Strains GA426 (*SIR2*) and GA427 (*sir2*Δ) were transformed with low-copy (CEN, p731) or 2μ-based (p698) plasmids encoding full-length Sir2p (Sir2) or only the selectable marker (vector). The transformed strains were grown for 2 days on glucose media and then stored at 4° to facilitate pigment visualization. (Bottom) The same strains were transformed with a high-copy galactose-inducible vector (pGal), encoding a peptide containing the bacterial activation domain (B42), a nuclear localization signal (NLS), and the HA-epitope (Gal-vector), with or without an in-frame fusion to the Sir2p N terminus (labeled Gal-Sir2). Growth on glucose (labeled glu) allows low-level expression and complementation of a *sir2*Δ deficiency, while high-level expression (growth on galactose/raffinose, labeled gal) derepresses the telomere proximal *ADE2* gene. (B) Repression at the *HML* locus was monitored using the bacterial *lacZ* gene under control of a truncated *LEU2* promoter (*LEU2′′lacZ*) that is inserted between the silencers E and I of the *HML* locus (Boscheron *et al.* 1996; Maillet *et al.* 1996). Reporter strains all carry a *LEU2* plasmid and either intact E and I silencers (EI, EG5), a deleted I silencer (*EΔt*, EG30), or both silencers and a *sir4*::*HIS3* disruption (EI*sir4*Δ, EG139). β-Galactosidase activity was measured in triplicate for each strain transformed either with the parental vector (pGal, labeled Gal-vector) or the same encoding Sir2p (pGal-Sir2), after growth on media containing either glucose (glu) or galactose/raffinose (gal) (Boscheron *et al.* 1996). The β-galactosidase level in strain EG5 carrying the vector alone was normalized to 1 and all other values are given relative to this. The standard deviation and mean were calculated from the results of three independent experiments. (C) Strain UCC3505 carrying *URA3* at TEL VII-L was transformed with the vector alone (Gal-vector) or the same encoding full-length Sir2p (Gal-Sir2p). *URA3* repression was monitored by growing serial dilutions of the transformants on selective media with and without uracil, either under conditions of low-level expression (glucose, open bars) or high-level expression (galactose/raffinose medium, solid bars). In strain UCC3505, the fraction of colonies expressing *URA3* in the presence of the vector plasmid alone is low (<0.1%). This was normalized to 1 for each medium, and the fraction of Ura$^+$ colonies expressing Gal-Sir2p is given relative to this. Three independent experiments gave similar results. To monitor the effects of Sir2p expression on rDNA repression fusion protein, the same plasmids were introduced into GA760 (*sir2*Δ) and GA758 (*SIR2*), which carry *URA3* with a modified promoter introduced at *RDN1* (*RDN1*::*mURA3*; Smith *et al.* 1998). Standard deviations and the mean were calculated from the results of at least three independent colonies for growth either on glucose (open bars) or galactose/raffinose (solid bars).

Sir2p derepresses TPE. This may be due to disruption of the Sir complex by altering the balance of Sir2p, Sir3p, and Sir4p in the nucleus or may reflect the titration of another component essential for their assembly. Consistent with the loss of repression, we note that Sir3p and Sir4p are delocalized from telomeric foci when wild-type *SIR2* is overexpressed (see Figure 7 and S.P., data not shown).

We next examined the effects of increasing Sir2p amounts on a *lacZ* reporter inserted at the *HML* locus (Figure 1B). In this case, low levels of Gal-Sir2p have no effect on a reporter flanked by two intact silencer elements (EI), and rather than improving repression, Gal-Sir2p derepresses slightly when one silencer element is present (EΔi). At high levels, the loss of silencing is more pronounced, but derepression is not equivalent to that of a *sir4*Δ strain (EI *sir4*Δ). Thus, in contrast to the situation at telomeres, normal Sir2p levels are not limiting for silencing at *HML*, perhaps reflecting the redundancy of nucleation sites present within silencer elements.

The mechanism of repression within the rDNA repeats is clearly different from that at telomeres or *HM* loci, since it requires *SIR2*, but not *SIR3* or *SIR4* (Bryk *et al.* 1997; Fritze *et al.* 1997; Smith and Boeke 1997; Smith *et al.* 1998). Nonetheless, if nucleolar Sir2p interacts with other limiting components to compact rDNA chromatin, then very high levels of the protein might also titrate the limiting components of such a complex. To determine whether this was the case, we monitored the effects of Gal-Sir2p levels in a strain carrying a *URA3* reporter inserted at the rDNA locus.

Low levels of Gal-Sir2p improve repression of the rDNA reporter up to 10^3-fold in isogenic *sir2*Δ (GA759) and *SIR2* strains (GA758; Figure 1C, middle and right, respectively). This is consistent with data from Smith *et al.* (1998) who observed an increase in rDNA repression when wild-type levels of Sir2p were increased 2- to 3-fold. Surprisingly, when pGal-*SIR2* is induced by growth on galactose, silencing in the rDNA is even stronger (increased up to 10^5-fold), although the same induction conditions compromise subtelomeric and *HM* repression. The fact that we see no loss of rDNA repression at very high levels of Gal-Sir2p suggests that there is no essential nucleolar ligand that is readily titrated by an excess of Sir2p. Immunofluorescence confirms that Gal-Sir2p is exclusively nuclear (see Figure 7) and is concentrated in the nucleolus when present at more moderate levels.

The N-terminal 93 aa of Sir2p are dispensable for TPE and rDNA silencing: Because Sir2p appears to be limiting at more than one genomic locus, we next asked whether we could identify domains of Sir2p specifically required for telomeric or rDNA silencing. We constructed a series of N- and C-terminal deletions based on an alignment of Sir2p with the proteins encoded by the yeast *HST* family (Figure 2). The alignments indicate that Sir2p has a unique domain at its extreme N terminus. This is followed by a region sharing 50% identity with the N terminus of Hst1p. Motifs distributed throughout the C-terminal two-thirds of the Sir2p sequence, on the other hand, are found conserved in all Sir2-like proteins ("core" domain, shaded in Figure 2). Finally, a short C-terminal extension is again shared between Hst1p and Sir2p. The fragments of the fusion proteins used in this study are aligned below full-length Sir2p in Figure 2. We also constructed full-length and N-terminally truncated Sir2p fusion proteins carrying the mutation P394L (called $sir2^{394L}$ and $sir2\ core^{394L}$, respectively). Proline 394 is a conserved residue situated just before the second cysteine pair of a four-cysteine cluster that is predicted to form a Zn^{2+} finger (Rhodes and Klug 1993). In one of the five hSir2 homologues (Frye 1999), mutation of the equivalent site is responsible for its recognition as a melanoma antigen (S. Perrod, M. Cockell, T. Woefel and S. M. Gasser, data not shown), suggesting that the mutation may affect an important function of the protein.

Low-level expression of these truncated proteins in *sir2*Δ strains identifies the minimal region that is able to complement either TPE or rDNA silencing (Figure 3). Western blots confirm that all constructs produce equivalent amounts of protein (data not shown). Like the full-length Sir2p, low-level expression of Gal-$sir2^{94–562}$ (glucose medium) is sufficient to restore TPE, although galactose induction is necessary to obtain significant rDNA repression in *sir2*Δ strains (Figure 3, A and B). The truncated protein, however, functions less efficiently than full-length Gal-Sir2p in both assays, suggesting that the extreme N terminus either facilitates repression or helps promote the correct folding of Sir2p. Further deletion of the Sir2 N terminus (Gal-$sir2^{199–562}$) completely eliminates silencing at both sites, as does a C-terminal deletion (Gal-$sir2^{1–421}$), and the point mutation (Gal-$sir2^{394L}$). At high levels (induction by galactose) Gal-$sir2^{94–562}$ derepresses TPE slightly less efficiently than full-length Sir2p, consistent with a minor loss of silencing activity. However, since no other truncation restores repression in a *sir2*Δ strain, we conclude that the only domain that is even partially dispensable for Sir2p function is first 93 aa.

Sir2p encodes distinct subdomains as defined by dominant negative effects: To examine whether any of the noncomplementing Gal-Sir2p fragments nonetheless define independent structural domains, we tested whether these truncated fusion proteins can compete with wild-type Sir2p for interacting components. Low levels of Gal-$sir2^{94–562}$, like the full-length protein, improve TPE, whereas low levels of the other deletion fragments have no effect (glu, Figure 4A). However, at induced levels, the C-terminal domain Gal-$sir2^{199–562}$ derepresses TPE even more efficiently than full-length Sir2p or Gal-$sir2^{94–562}$ (gal, Figure 4A). Other deletions

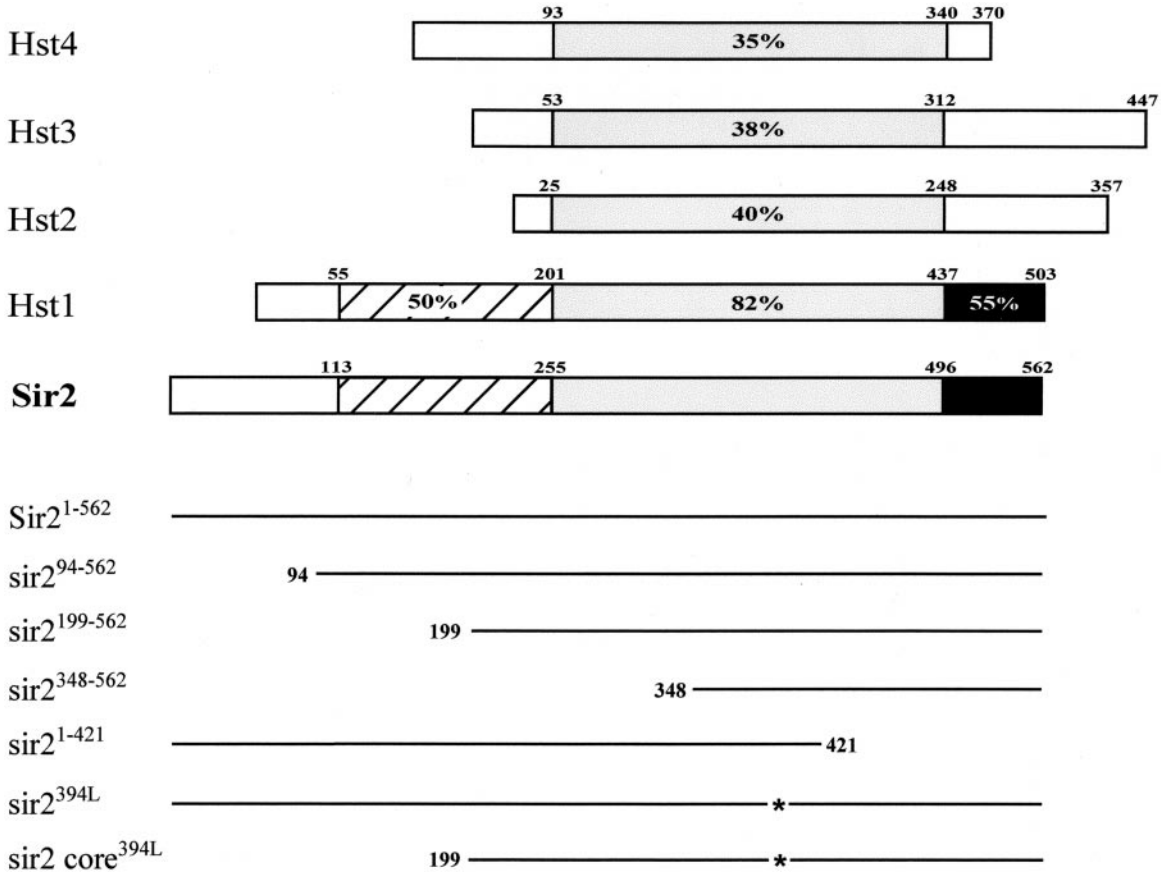

Figure 2.—Comparative alignment of the yeast *SIR2* family. Schematic representations of the *h*omologous to sir *t*wo (HST) family aligned with respect to their conserved core domains (shading). The amino acid identity between the core domains of individual homologues and Sir2p is indicated. Only the N- (cross-hatching) and C-terminal (solid) regions of the Hst1p also show significant identity with N- and C-terminal domains of Sir2p. Unshaded blocks indicate regions of sequence unique to individual *SIR2* family members. Below Sir2p, Sir2p fragments are aligned with respect to the full-length Sir2p. The first or last aa of the truncated version is indicated. The proteins sir2^{394L} and sir2 core^{394L} harbor a proline-to-leucine transition at the position indicated by the asterisk.

(*i.e.*, Gal-sir2$^{348–562}$ or Gal-sir2$^{1–421}$) eliminate the dominant negative effect, indicating that an intact C-terminal domain (*i.e.*, aa 199–562, containing the conserved core of aa 255–493) is both necessary and sufficient to disrupt silencing *in trans.* These data are consistent with the prediction that the conserved core of Sir2p folds into an integral structural domain, a proposal reinforced by the fact that this region is highly conserved among all known Sir2p family members (see Figure 2).

Despite the fact that the full-length Sir2p carrying a single point mutation in the core domain (Gal-sir2^{394L}) is inactive for silencing (Figure 3), overexpression of this point mutant at either low or high levels efficiently derepresses TPE in the wild-type background (Figure 4A). This highly efficient dominant negative effect could have different explanations. The simplest is that the N-terminal 199 aa of Sir2p contain an important site of interaction for a limiting ligand. Alternatively, the inactive core domain itself may contain an additional binding site that competes for a limiting ligand. Finally, the sir2^{394L} mutation could be dominant negative because it sequesters a ligand or substrate by binding it more tightly than the wild-type domain. To test these possibilities, we examined the effect of expressing only the C-terminal domain (aa 199–562) of the mutated sir2^{394L} allele. In contrast to the wild-type core domain, low- or high-level expression of this domain (Gal-sir2 core^{394L}) no longer derepresses TPE (Figure 4A). Thus, the dominant negative effect of Gal-sir2^{394L} requires an intact N terminus, suggesting that the first option is correct. The simplest interpretation of these results is that there are two domains of Sir2p, each dominant negative for silencing when expressed individually. One lies within the conserved C-terminal domain of the protein, and a second requires both N- and C-terminal portions of the protein.

To examine if the dominant negative character of the Sir2p constructs applies to rDNA silencing, the same fragments were introduced into a *SIR2* strain carrying the *mURA3* reporter within a rDNA repeat. Like the full-length fusion protein, high levels of Gal-sir2$^{94–562}$ enhance rDNA silencing (Figure 4B for galactose; data

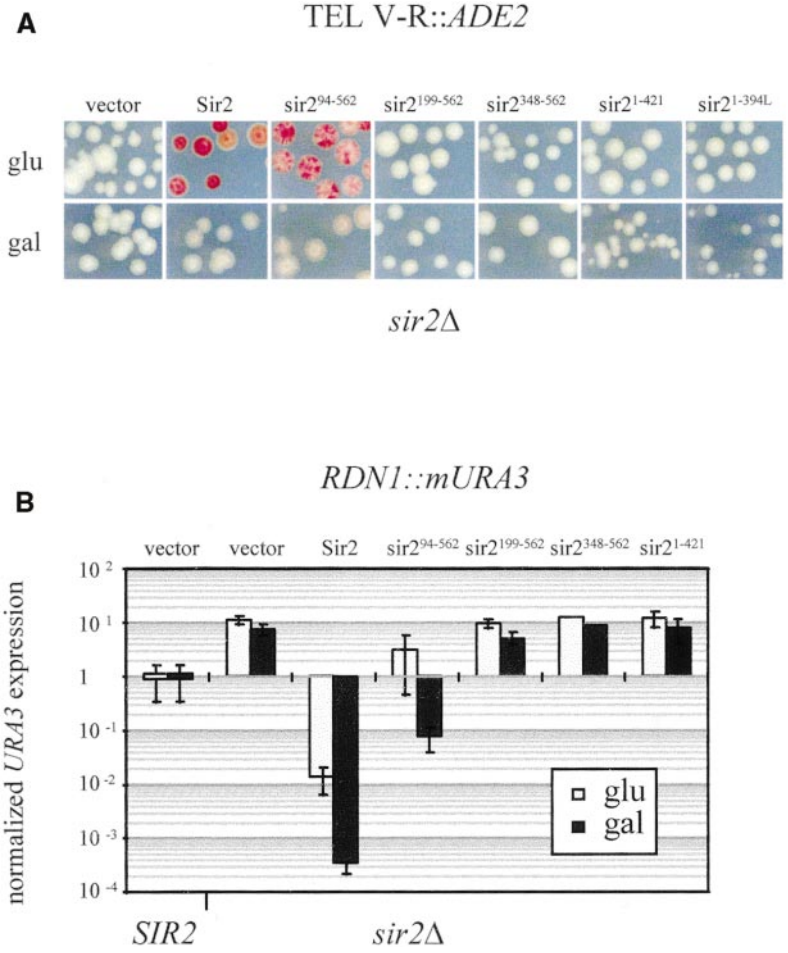

Figure 3.—Both N and C termini of Sir2p are necessary for complementation of TPE and rDNA silencing. (A) The effects of Gal-Sir2 fusion proteins on telomeric silencing phenotypes were determined after growth on glucose (glu, low-level expression) and galactose/raffinose (gal, high-level expression) in strain GA427 (*sir2*Δ), transformed with either the vector pGal, or the same plasmid expressing the various Sir2p fusion proteins noted above each panel under control of the *GAL1* upstream activation sequence (UAS). (B) The efficiency of expression of the *mURA3* gene inserted in the rDNA repeat in strain GA758 (*SIR2*) or strain GA760 (*sir2*Δ), transformed with the same series of plasmids as described in A, is shown on logarithmic scale. *URA3* repression was monitored by growing fivefold serial dilutions of the transformants on selective media with and without uracil, either on glucose (open bars) or galactose (solid bars) media. The efficiency of *RDN1::mURA3* expression in the $Sir2^+$ strain has been normalized to 1, and all other values are given relative to this. Standard deviations and means were calculated from at least three independent colonies.

not shown for glucose media). None of the other partial Sir2p domains, including the wild-type C-terminal domain (Gal-$sir2^{199–562}$), affects rDNA silencing in wild-type cells. On the other hand, Gal-$sir2^{394L}$ is dominant negative for rDNA silencing at both low and high levels of expression, while the N-terminally truncated form is not (Gal-sir2 $core^{394L}$, Figure 4B; data not shown for glucose media). In summary, we find that the core domain is able to saturate or titrate components leading to derepression at subtelomeric sites, while it is unable to do so in the nucleolus, even though it is required for both types of repression. A second domain of Sir2p, which must include the N-terminal aa 94–198, titrates or disrupts silencing complexes at both nucleolar and subtelomeric regions, particularly when bearing a point mutation near the Zn^{2+} finger motif.

Nucleolar localization of Sir2p requires aa 94–198: In summary, we find that whereas $sir2^{94–562}$ restores rDNA silencing in a *sir2*Δ strain and enhances repression in a $Sir2^+$ strain, $sir2^{199–562}$ does not (Figures 3 and 4, data on glucose not shown). To see if this reflects restrictions on their subnuclear localization, we localized these fusion proteins by anti-Sir2 immunofluorescence in a *sir2*Δ strain (Figure 5).

The immunostaining results show clearly that GBD-$sir2^{94–562}$, GBD-$sir2^{394L}$, and full-length GBD-Sir2p are strongly enriched in the nucleolus, colocalizing with Nop1p, an abundant nucleolar protein (see merge, Figure 5). In contrast, GBD-$sir2^{199–562}$ is detected as a diffuse staining throughout the nucleus, confirming that aa 94–198 are required for nucleolar accumulation of Sir2p. Indeed, the inability of this core domain to accumulate in the nucleolus may contribute to its lack of dominant negative effect on rDNA silencing. The fact that the $sir2^{94–562}$ construct is efficiently enriched in the nucleolus, yet fails to fully complement rDNA repression in a *sir2*Δ strain (Figure 3), suggests that the extreme N-terminal 93 aa contribute a function other than targeting, which influences the efficiency of both TPE and rDNA repression.

Tethered GBD-Sir2p promotes Sir4p-dependent repression of an adjacent reporter: To monitor the ability of a protein subdomain to recruit and nucleate a repressive chromatin structure, we used a third repression assay, that of tethered silencing, to analyze wild-type and mutant domains of Sir2p (Figure 6). In this assay a protein domain is targeted to a *URA3* reporter inserted at the *HML* locus by the Gal4p DNA binding domain (GBD), in an otherwise wild-type background. This allows one to monitor the protein domain's potential to

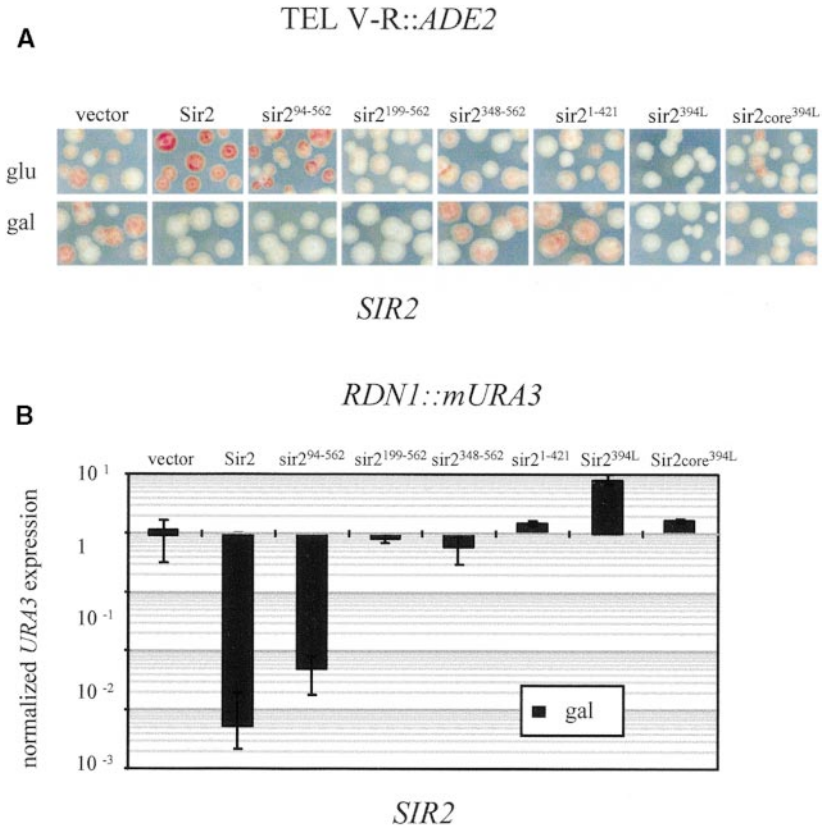

Figure 4.—Dominant negative phenotypes identify two distinct domains of Sir2p. (A) Strain GA426 (*SIR2)* carrying the *ADE2* gene inserted adjacent to Tel V-R was transformed with either the vector pGal or the same vector expressing fusion proteins encoding wild-type or mutated fragments of Sir2p as indicated above each panel. The transformants were grown on medium containing glucose (glu) or galactose/raffinose (gal) under limiting adenine conditions such that red pigment accumulates when *ADE2* is repressed. White colonies indicate a dominant negative effect of the overexpressed protein. (B) The efficiency of expression of the *mURA3* gene inserted in the rDNA repeat in strain GA758 (*SIR2*), transformed with the same series of plasmids as described in A, is shown on a logarithmic scale for growth on galactose-containing medium. A similar dominant negative effect was obtained for the sir2^{394L} mutant at low-level expression on glucose (data not shown). *URA3* repression was monitored by growing fivefold serial dilutions of the transformants on selective media with and without uracil; the *mURA3* expression in the presence of empty vector has been normalized to 1, and all other values are given relative to this. Standard deviations and means were calculated from at least three independent colonies.

nucleate repression, either in the absence of a silencer (ΔeΔi) or in the presence of one silencer (EΔi). Specific subdomains of Rap1p, Orc1p, Sir1p, Sir3p, and Sir4p (Buck and Shore 1995; Boscheron *et al.* 1996; Lustig *et al.* 1996; Marcand *et al.* 1996; Triolo and Sternglanz 1996; Gardner *et al.* 1999; Martin *et al.* 1999), have all been shown to efficiently nucleate Sir-dependent silencing when targeted in multiple copies to a reporter gene. Here we show that full-length Sir2p fused to GBD also results in efficient transcriptional repression, whether the reporter gene is adjacent to a conventional silencer or not (Figure 6, see EΔi and ΔeΔi). GBD-Sir2p and GBD-Orc1p nucleate Sir4p-dependent silencing equally well, suggesting that both are able to recruit the Sir2/3/4 complex. Since rDNA repression is Sir3p and Sir4p independent, we might have expected GBD-Sir2p to be able to nucleate a Sir4p-independent silencing of the reporter. As this is not the case, we propose that a *cis*-acting sequence, the repetitive array, or another nucleolar factor is necessary to establish the characteristic rDNA repression.

When mutated domains of Sir2p are targeted to the reporter at *HML*, we find that GBD-sir2$^{94–562}$, but not the core domain (GBD-sir2$^{199–562}$), shorter truncations, nor the GBD-sir2^{394L} point mutant, is competent for promoting repression in the absence of one or both silencer elements (Figure 6). Thus, loss of the N terminus, as well as the presence of an internal point mutation, eliminates the ability of Sir2p to seed Sir2/3/4-mediated silencing. Similarly, the tethering of the full-length Sir2p homologue, Hst2p, which contains primarily the core domain, is unable to confer repression. In conclusion, the N-terminal domain of Sir2p does not simply ensure accurate subnuclear distribution, but appears to be necessary for the assembly and/or propagation of silent chromatin itself.

Sir4p binds the N-terminal domain of Sir2p in two-hybrid assays: It appears likely that the targeted Sir2p nucleates Sir4-dependent silencing through a recruitment of Sir4p and the Sir2/3/4 complex. Since the Sir2p core domain is unable to nucleate silencing, it would follow that the N-terminal 198 aa must be important for Sir4p interaction. To map the site of Sir2p interaction and examine other potential partners for Sir2p among components of the silencing machinery, we performed two-hybrid assays (Golemis *et al.* 1996) using the subdomains that we characterized functionally above. To avoid repression of the reporter gene by the tethering of wild-type Sir2p, we performed the assays in both *SIR*$^+$ and *sir3*Δ strains.

Table 3 summarizes the results of our two-hybrid studies. First we show that the region of Sir4p that is necessary for binding Sir2p lies within its C-terminal 621 amino acids (sir4$^{731–1358}$), while a fragment that is 100 aa shorter (sir4$^{838–1358}$) is not sufficient for the same interaction. We detect no interaction between Sir2p bait and either Sir2p or Sir3p, although the constructs used for these two-hybrid studies are fully functional in silencing

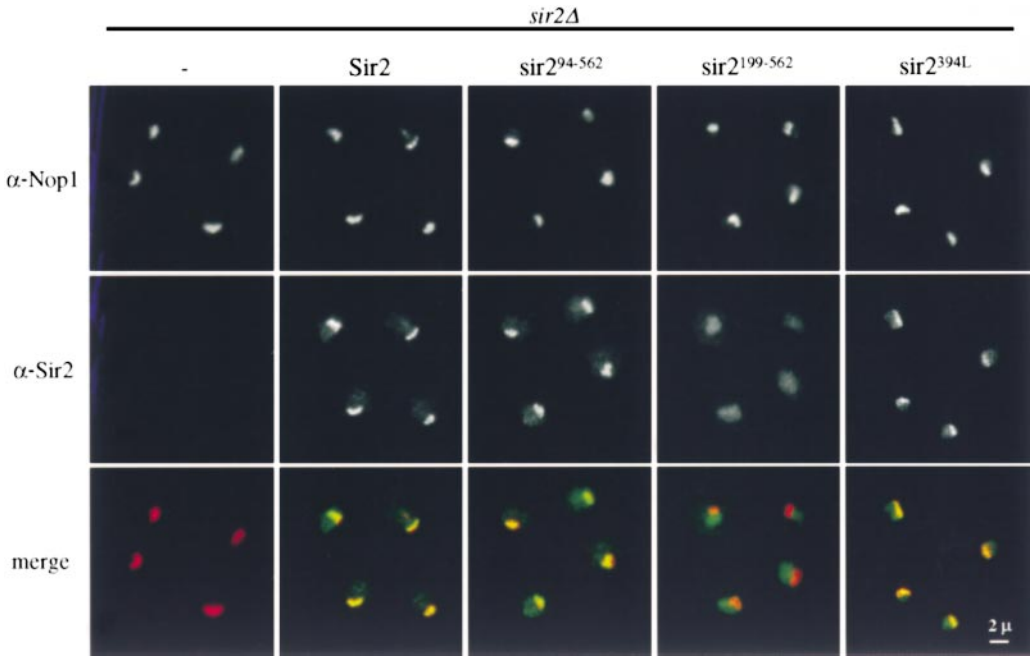

Figure 5.—Nucleolar targeting of Sir2p requires sequence between aa 94–199. The diploid strain GA194 (*sir2*Δ) was immunostained with both rabbit anti-Sir2 (detected by a fluorescein-conjugated secondary antibody) and mouse-anti-Nop1p (detected by a Cy5-conjugated secondary antibody) either before transformation (−) or after transformation with plasmids derived from the pGBT9 vector that encode GBD fusions to the fragments of Sir2p indicated above the panels. In the merge of the two patterns, Nop1p staining is shown in red, Sir2p staining is shown in green, and the coincidence of the signals is yellow. Analogous results were obtained with the same truncations and mutants expressed from the pGal vector.

assays (Figures 1 and 3 and data not shown). This indicates that the interaction of Sir2p with Sir4p does not require an intact Sir complex and is thus likely to be direct. Finally we find that both sir2$^{94–562}$ and sir2^{394L} bind Sir4p efficiently, while sir2$^{199–562}$ and sir2$^{1–421}$ do not. Thus, we conclude that both the N-terminal domain between aa 94–198 and a region between aa 422–562 are required for interaction with Sir4p.

sir2^{394L} expression disrupts TPE without delocalizing telomeric foci: It was somewhat surprising that the point mutant sir2^{394L} fails to target silencing in a wild-type background, since it is able to bind Sir4p (*cf.* Figure 6 and Table 3). This may indicate that the tethered sir2^{394L} protein acts locally in a dominant manner to interfere with either Sir protein assembly or the recruitment of a novel silencing factor, rather than disrupting the Sir complex itself. To investigate this possibility further we followed the behavior of endogenous Sir4p and Rap1p foci in strains derepressed for TPE due to overexpression of either Sir2p or sir2^{394L} (Figure 4). As expected, we found that strong overexpression of the functional Sir2p partially delocalizes the endogenous Sir4p from telomeric foci as it disrupts silencing (Figure 7). On the other hand, equivalent levels of the sir2^{394L} fusion protein leaves the foci of telomeric proteins intact, despite a significant loss in subtelomeric repression (Figures 7 and 4A).

On the basis of these observations, we propose that sir2^{394L} interferes with an essential step in repression that occurs after the recruitment of Sir proteins into foci. To examine whether the mutant protein allows formation of telomere-associated silencing complexes in the absence of wild-type Sir2p, we compared Rap1p immunostaining in *sir2*Δ strains expressing either sir2^{394L} or a functional Sir2p. Cells with Sir2p show a perinuclear focal pattern of Rap1p, while Rap1p staining is diffuse in sir2^{394L} cells (Figure 7), suggesting that the mutant sir2^{394L} fails to assemble chromatin-bound Sir complexes, even though it binds Sir4p in a two-hybrid assay. Thus, while sir2^{394L} cannot compete for Sir complex formation, it also fails to promote formation of a telomere-localized complex. In conclusion, we propose that the core domain plays a role in both Sir complex assembly and the maintenance of repression once Sir2/3/4 complexes form.

DISCUSSION

Analysis of domain function: Sequence alignment and structure prediction analyses for the large Sir2-like gene family (Clustal X, Higgins *et al.* 1996) predicts that the highly conserved C-terminal core of ~250 aa folds into a single globular domain (aa 255–496). In yeast Sir2p, the N-terminal 198 aa and a short C-terminal extension share significant homology only with Hst1p (Figure 2). Since these are the only family members that can functionally substitute for each other, it appeared likely that the N-terminal extension might be providing an essential, silencing-specific function. To examine these predictions, we have monitored the activity of a series of deletion mutants and a full-length Sir2p carrying a point mutation within the core. Our results

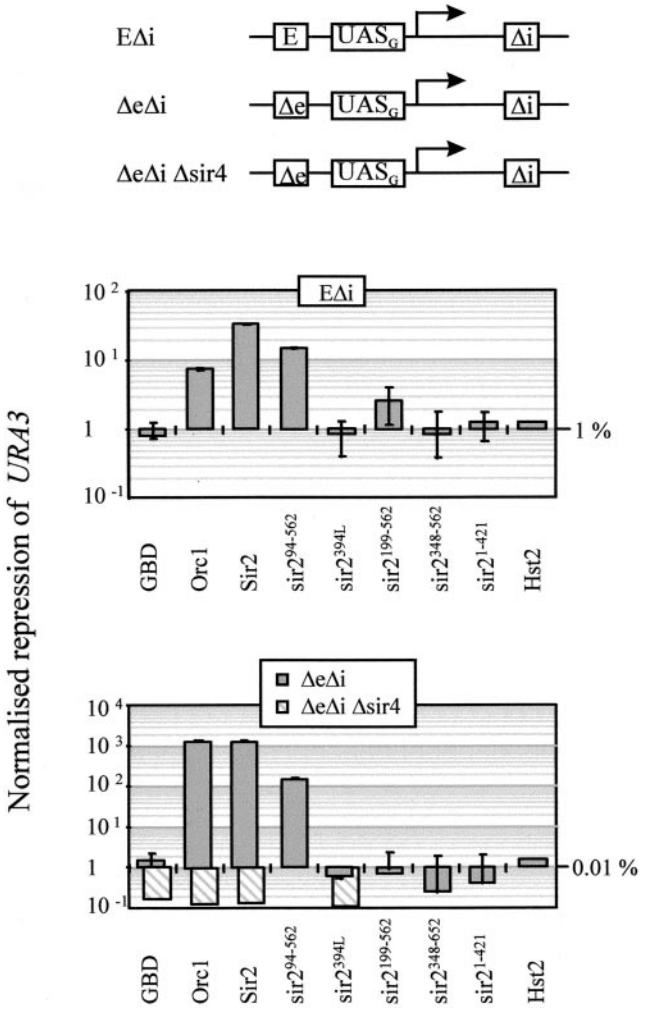

Figure 6.—Tethered Sir2p, but not its core domain, is able to nucleate Sir-dependent repression. The reporter strains for tethered silencing contain a *URA3* gene inserted at the *HML* locus flanked by eight binding sites for the Gal4p DNA-binding domain (UAS_G) and either one intact silencer (EΔi, GA1034) or no silencers (ΔeΔi, GA1035). As a control for Sir dependence, a *SIR4* deletion was also created in the latter strain (ΔeΔi *sir4*Δ, GA1084). Silencing of the *URA3* gene was monitored by growing 10-fold serial dilutions of the transformants on selective media with and without 1 mg/ml 5-fluoroorotic acid (5-FOA), in the strain indicated at the top of each graph, transformed with plasmids encoding either the DNA-binding domain of Gal4p alone (GBD), or Orc1N, Hst2p, or fragments of Sir2p, all fused in frame to GBD. Quantitation of the serial dilution assays using the strains described was performed on at least three independent colonies of each transformant and the mean of the ratios of cells growing on +FOA and −FOA is shown. The *x* axis represents the efficiency of *URA3* repression (percentage of colonies growing on 5-FOA) in log scale after normalizing the repression detected in the presence of GBD alone to 1 (the absolute value is indicated to the right of each graph). To test whether the silencing mediated by Sir2p is Sir4 dependent, identical targeted silencing assays were performed in GA1084 (hatched bars, bottom graph). In all cases <3 in 10^5 cells from the tested transformants grew on 5-FOA when *SIR4* was deleted.

demonstrate that aa 94–198 are involved in targeting Sir2p to rDNA and binding Sir4p at telomeres. The fact that the rDNA and telomeres compete for a limiting pool of Sir2p suggests that site-specific ligand(s) may bind Sir2p in a mutually exclusive manner. The globular C-terminal domain contained within aa 199–562 has a separate function that is also essential for both types of silencing. The exact nature of this activity is unclear because this domain alone does not dimerize or bind any of the other Sir proteins by two-hybrid analysis.

Recent reports suggest that the Sir2p core has a conserved enzymatic activity capable of a phosphoribosyl transferase reaction *in vitro* and indicate that this activity correlates with its repression competence (Frye 1999; Tanny *et al.* 1999). Our data indicate that the silencing function of this globular domain is inactivated if deleted from either end or mutated near the Zn^{2+} finger motif. Thus, if this domain represents an enzyme or an enzyme's cofactor, our data implicate that activity in silencing at both telomeres and rDNA.

It was previously reported that Sir2p binds the C-terminal half of Sir4p (Moazed *et al.* 1997; Strahl-Bolsinger *et al.* 1997). Our two-hybrid studies implicate the Sir2 N terminus in Sir4 interaction and suggest that one role of the N-terminal domain is to target Sir2p to telomeres. We cannot rule out that either the short C-terminal extension (aa ~493–562) of Sir2p or its conserved core also contributes to this interaction. The fact that the Sir2p N-terminal domain is required for nucleolar accumulation may reflect interaction between Sir2p and Net1p, a nucleolar protein required to target Sir2p to the nucleolus (Straight *et al.* 1999). Indeed, consistent with localization studies using *sir2* mutants and *net1* mutants (Figure 5, and Straight *et al.* 1999), two-hybrid results indicate that the N terminus of Sir2p can bind Net1p (G. Cuperus and D. Shore, personal communication). We find that deletion of the first 93 aa of Sir2p only slightly impairs its silencing functions, *i.e.*, sir2$^{94-562}$ still mediates telomeric and rDNA repression and the proper localization of Sir2p within the nucleus, while C-terminal and more extensive N-terminal deletions destroy Sir2p activity. Therefore we propose that the N-terminal aa 94–198 mediates essential interactions with Sir4p and Net1p and thus is as important as the core for both telomeric and rDNA silencing. Tethering data confirm that the interaction of Sir2p with Sir4p is not only important for recruitment but is also required for assembly or stability of the Sir complex.

In view of our inability to isolate complementing fragments of Sir2p, most of our information on function is deduced from the dominant negative effects of ectopically expressed subdomains. For instance, the sir2$^{199-562}$ truncation alone has a dominant negative effect on TPE. This has also been observed for high levels of Hst2p (S. Perrod, M. Cockell, T. Woelfel and S. M. Gasser, data not shown), indicating that the conserved domain of at least some Sir2-family members compete for a

TABLE 3

Summary of two-hybrid interactions

	Prey										
Bait	pGal	pGal-sir4$^{731–1358}$	pGal-Sir2	pGal-sir2$^{94–562}$	pGal-sir2$^{199–562}$	pGal-sir2^{394L}	pGal-Hst2	pGAD	pGAD-sir4$^{831–1358}$	pGAD-sir2	pGAD-sir2^{394L}
pEG202-sir4$^{838–1358}$	–	+	–	–	–	–	–	–	+	–	–
pEG202-sir4$^{731–1358}$	–	+	+	+	–	+	–	–	+	+	+
pEG202-Sir2	–	–	–	–	–	–	–	–	–	–	–
pEG202-sir2^{394L}	–	+	–	–	–	–	–	–	–	–	–
pEG202-sir2$^{94–562}$	–	+	–	–	–	–	–	–	–	–	–
pEG202-sir2$^{199–562}$	–	–	–	–	–	–	–	–	–	–	–
pEG202-sir2$^{1–421}$	–	–	–	–	–	–	–	–	–	–	–
pEG202-Hst2	–	–	–	–	–	–	–	–	–	–	–
pNsir3N-sir3$^{1–503}$	–	–	–	–	–	–	–	–	–	–	–
				EGY48 (*SIR3*)					GA329 (*sir3*Δ)		

The Sir2 N terminus is required for Sir4p binding. Two-hybrid interactions between the fusion proteins indicated were tested by cotransformation of plasmids expressing bait and prey fusion proteins into the strains EGY48 (*SIR3*) and GA329 (*sir3*Δ). Bait constructs constitutively express domains or mutated forms of Sir2p, Sir3p, Sir4p, and full-length Hst2, as indicated by the amino acid superscripts fused to the LexA DNA-binding domain. Prey constructs express full-length or subdomains of Sir2p, Sir4p, and Hst2p fused to either the galactose-inducible bacterial activation domain pJG45 (pGal) or the constitutively expressed Gal4 activation domain (pGAD). Bait–prey combinations that give rise to a strong, reproducible transcriptional activation of a *lacZ* reporter gene are indicated by +, while combinations giving no activation are indicated by –.

common ligand or substrate. The inability of sir2$^{199–562}$ to affect rDNA silencing reflects in part its lack of accumulation in the nucleolus, but also suggests that the core domain has no ligand that is limiting in the rDNA. This may also explain why high levels of full-length Sir2p improve rDNA repression, while they are dominant negative for TPE. These results underscore differences in how Sir2p functions at telomeres and in the rDNA, even though N-terminal and core domains are required for both.

The most striking phenotype we detect is the strong dominant negative effect of the point mutation P394L, which is found near the Zn^{2+} finger motif of the core domain. This mutated form requires the Sir2 N terminus for its dominant negative effects, suggesting that the core alone must be targeted either to a substrate or to a site, to disrupt silencing. It is therefore unlikely that sir2^{394L} simply sequesters a coenzyme or ligand from sites of repression. Moreover, when tethered to a reporter gene in an otherwise wild-type background, this mutant fails to promote silencing, despite the fact that it binds Sir4p in a two-hybrid assay. This leads us to propose that the function of the core domain that is disrupted by the mutation is a critical activity necessary for maintenance of Sir-mediated states of repression. Such a hypothesis is supported by the observation that even low levels of the mutated form (sir2^{394L}) interfere with silencing without disrupting the clustered phenotype of Sir complexes at telomeres. The activity lost in this mutant may well be the monoribosyltransferase activity recently attributed to various Sir2-family members (Frye 1999).

Strong overexpression of *SIR2* has been reported to cause a severe growth defect (Holmes *et al.* 1997), yet in GA426 or GA427 strains we do not observe significant effects on viability due to high levels of Gal-Sir2 fragments. On the other hand, high levels of full-length Sir2p or certain subfragments of the protein do result in a slow growth phenotype. Such colonies are smaller after 2 days' growth, although they become indistinguishable from those of control strains after longer periods of growth. The magnitude of the growth defects induced by *SIR2* overexpression seem to be strain specific, yet in our hands plating efficiency is never decreased more than 10-fold (data not shown). We do note that in some strains elevated levels of Sir2p provoke an intriguing pseudohyphal-like appearance (data not shown). In all strains examined both the slow growth and pseudohyphal phenotypes correlate with overexpression of the intact core domain of Sir2p, rather than with a change in silencing activity *per se*, suggesting that the effects of Sir2p on growth rate and morphology may be associated with the putative enzymatic activity of the core domain.

Is Sir2p function conserved? Multiple Sir2-like genes have been identified in yeast and in many other species (Brachmann *et al.* 1995; Yahiaoui *et al.* 1996; Perez-

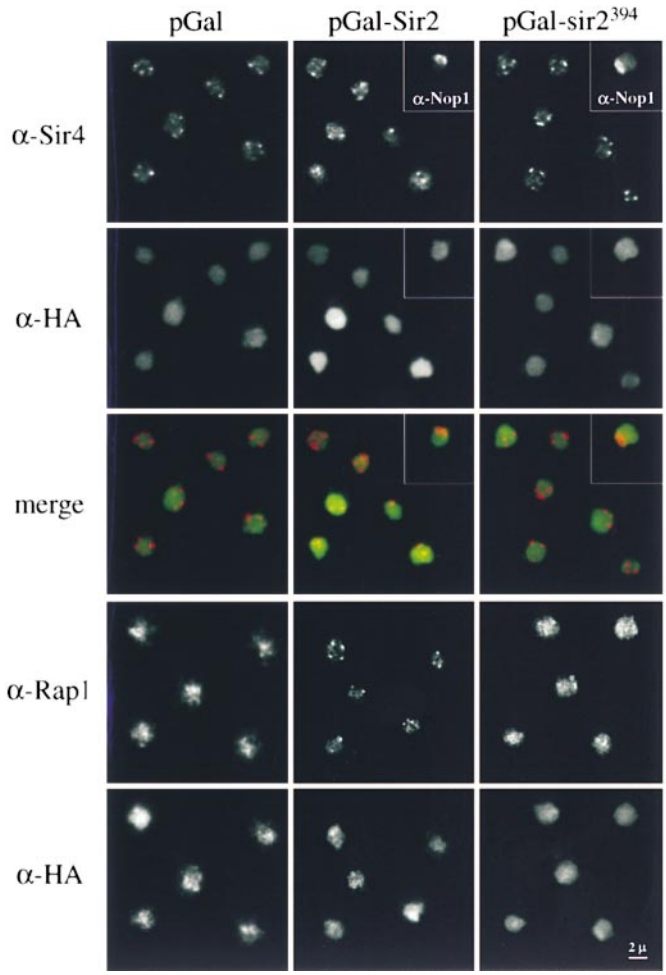

Figure 7.—High levels of Sir2p, but not of sir2^{394L}, disrupt Sir4p foci. Congenic diploid strains GA225 (*SIR2*) and GA194 (*sir2*Δ) were transformed either with vector alone (pGal), which encodes a short bacterial activation domain fused to the HA epitope, or with the same plasmid containing fusion proteins encoding Sir2p and sir2^{394L}, as indicated at the top. All were grown on 2% galactose for 8 hr to induce the plasmid-borne gene maximally. The GA225 transformants were stained with affinity-purified anti-Sir4p (α-Sir4, red in merge) to detect endogenous Sir4p through a fluorescein-conjugated secondary antibody and with HA.11 (α-HA, green in merge) to detect expression of the induced fusion proteins using a CY-5-conjugated secondary antibody. In the inset double labeling with anti-Nop1 and anti-HA show that at high levels Sir2p becomes dispersed throughout the nucleus. The GA194 transformants (*sir2*Δ) were stained with rabbit anti-Rap1 to detect endogenous Rap1p and with HA.11 (α-HA) to detect expression of the plasmid-encoded fusion proteins, as above. A diffuse pattern for Sir4p and Rap1p was expected for both *SIR2* overexpression and disruption, since TPE is lost in both cases. Intriguingly, high sir2^{394L} levels do not delocalize Sir4p despite disrupting telomeric silencing (see Figure 4A). Bar, 2 μm.

Martin *et al.* 1999), engendering speculation that the role of Sir2p as a direct modifier of chromatin structure has been conserved. Indeed, a *SIR2* homologue from *S. pombe* influences both telomeric and centromeric silencing (Freeman-Cook *et al.* 1999) and deletion of the *Candida albicans SIR2* gene results in chromosome rearrangements and a higher frequency of phenotypic switching (Perez-Martin *et al.* 1999). Recent database searches with a multiple alignment algorithm (Clustal X, Higgins *et al.* 1996) demonstrate that, as for yeast, families of *SIR2*-like genes are present in the genomes of mammals, flies, and worms. However, *SIR2*-like genes are also common in bacterial genomes, suggesting the preservation of an ancient function. Interestingly, all of the proteins encoded by bacterial *SIR2*-like genes contain only the conserved core, while every eukaryote examined contains both short variants and Sir2-like proteins with unique N- or C-terminal extensions. This raises the question whether all Sir2-like proteins can bind chromatin or DNA or whether the role of the conserved domain requires recruitment by a separate structural domain. Our data are consistent with the latter.

Initial speculation that Sir2p mediates an enzymatic activity important for transcriptional repression was based on the observation that *SIR2* overexpression led to global hypoacetylation of histones (Braunstein *et al.* 1993). There was, however, no resemblance between the conserved motifs of the Sir2 core domain and the signature motifs of HDAC family members whose structures are known (Leipe and Landsman 1997). Recently a recombinant version of hSir2, a related protein from Salmonella, and Sir2p from yeast were shown to have a mono-ADP ribosylation activity *in vitro* (Frye 1999; D. Moazed, personal communication). It should be noted, however, that *in vivo* targets for the putative ADP-ribosylation activity are unlikely to be the same for all Sir2-like proteins. Consistently, chimeric Sir2 proteins with small core domain substitutions show locus-specific complementation of Sir2p function (Sherman *et al.* 1999). Among potential substrates for mono-ADP-ribosylation are histones (Boulikas 1991) and RNA PolI (Mishima *et al.* 1993), modification of which correlates with enhanced rDNA transcription.

The strong dominant negative phenotype that correlates with ectopic expression of full-length Sir2p carrying a mutation near the Zn^{2+} finger motif is not consistent with the effect expected from overexpression of an

inactive enzyme. This argues rather that the mutant form of Sir2p releases a ligand, alters its specificity, or sequesters a ligand from its function. For any of these scenarios the Zn^{2+} finger could either serve as a homo- or heterodimerization site, as it does in the casein kinase 2β subunit dimer (Chantalat *et al.* 1999), or coordinate a heavy metal necessary for catalytic activity. The fact that overexpression of the core domain is also dominant negative for silencing as long as this motif is intact suggests that it binds a ligand that is present in limiting amounts. Extragenic suppressors of the point mutation should lead us either to the substrates of the putative "sirtuin" enzyme (Frye 1999) or to a structural partner whose association with Sir2p is necessary for efficient repression.

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