Restoration of Silencing in *Saccharomyces cerevisiae* by Tethering of a Novel Sir2-Interacting Protein, Esc8

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ABSTRACT

We previously described two classes of *SIR2* mutations specifically defective in either telomeric/*HM* silencing (class I) or rDNA silencing (class II) in *S. cerevisiae*. Here we report the identification of genes whose protein products, when either overexpressed or directly tethered to the locus in question, can establish silencing in *SIR2* class I mutants. Elevated dosage of *SCS2*, previously implicated as a regulator of both inositol biosynthesis and telomeric silencing, suppressed the dominant-negative effect of a *SIR2-143* mutation. In a genetic screen for proteins that restore silencing when tethered to a telomere, we isolated *ESC2* and an uncharacterized gene, (*YOL017w*), which we call *ESC8*. Both Esc2p and Esc8p interact with Sir2p in two-hybrid assays, and the Esc8p-Sir2 interaction is detected *in vitro*. Interestingly, Esc8p has a single close homolog in yeast, the ISW1-complex factor Ioc3p, and has also been copurified with Isw1p, raising the possibility that Esc8p is a component of an Isw1p-containing nucleosome remodeling complex. Whereas *esc2* and *esc8* deletion mutants alone have only marginal silencing defects, cells lacking Isw1p show a strong silencing defect at *HMR* but not at telomeres. Finally, we show that Esc8p interacts with the Gal11 protein, a component of the RNA pol II mediator complex.

TRANSCRIPTIONAL silencing at the two cryptic mating-type loci *HMR* and *HML* in the budding yeast *Saccharomyces cerevisiae*, an example of "position-effect" gene regulation, plays an essential role in the mating behavior of haploid cells (reviewed in Lustig 1998). Silencing at the *HM* loci is initiated by flanking regulatory elements, called silencers, that consist of binding sites for the origin recognition complex (ORC) and one or both of two general regulatory factors, Rap1p and Abf1p. The silencer-binding proteins act to recruit a complex of *s*ilent *i*nformation *r*egulator (SIR) proteins that can propagate along adjacent chromatin through specific Sir3p- and Sir4p-histone tail interactions (reviewed in Gasser and Cockell 2001; Grunstein 1998). SIR protein assembly and spreading also appears to be promoted by a complex network of SIR-SIR, Rap1p-SIR, and ORC-SIR interactions (Moretti *et al.* 1994; Triolo and Sternglanz 1996; Moazed *et al.* 1997; Moretti and Shore 2001). A similar *SIR2*-, *SIR3*-, and *SIR4*-dependent form of silencing has been shown to propagate inward from the chromosome ends (telomeres) and is usually referred to as telomeric position effect (TPE; Aparicio *et al.* 1991). In this case, the telomeric $TG_{1\text{-}3}$ repeats themselves, which encode on average ~15–20 potential Rap1p-binding sites, function as silencer elements in conjunction with end-specific factors such as the yeast Ku protein (Laroche *et al.* 1998; Nugent *et al.* 1998; Polotnianka *et al.* 1998; Mishra and Shore 1999). More recently, transcriptional silencing of ectopic RNA polymerase II (Pol II)-transcribed genes has also been observed within the rDNA repeats (Bryk *et al.* 1997; Fritze *et al.* 1997; Smith and Boeke 1997). Unlike *HM* silencing or TPE, rDNA silencing relies on Sir2p but not Sir3p or Sir4p. Instead, Net1p, a protein involved in a mitotic exit checkpoint, is essential for Sir2p localization to the nucleolus and rDNA silencing (Shou *et al.* 1999; Straight *et al.* 1999).

SIR2 is also unique among the *SIR* genes in that it is a member of an evolutionarily conserved gene family (Brachmann *et al.* 1995; Derbyshire *et al.* 1996; Frye 2000). Recently, several groups discovered that Sir2-family proteins constitute a novel class of NAD-dependent deacetylase enzymes (Imai *et al.* 2000; Landry *et al.* 2000; Smith *et al.* 2000). This important finding, coupled with an earlier report showing that *HM* loci and telomeres contain hypoacetylated nucleosomes and that *SIR2* overexpression leads to bulk histone deacetylation (Braunstein *et al.* 1993), points to histone deacetylation as a key silencing function of Sir2p. However, this hypothesis has yet to be tested directly and the existence of other important Sir2 substrates is not ruled out.

In a previous study (Cuperus *et al.* 2000), we isolated and characterized two distinct classes of *SIR2* mutants with locus-specific silencing defects. *SIR2* class II mutants are defective for rDNA silencing, and a subset of these mutants can be explained by the loss of a Sir2p-Net1p interaction and nucleolar Sir2p localization. Conversely, *SIR2* class I mutants are specifically defective in

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HMR and telomeric silencing. The molecular defect leading to a loss of silencing in the *SIR2* class I mutants is unknown, and we were surprised to find that none of these mutations seem to affect the Sir2p-Sir4p interaction (Cuperus *et al.* 2000). Instead, the class I mutants manifest an age-related phenotype where Sir3p and Sir4p relocalize to the nucleolus, apparently in association with the mutant Sir2 protein (Kennedy *et al.* 1997; Cuperus *et al.* 2000). This might be the result of a loss of interaction between Sir2p and another unidentified factor. Consistent with the notion of additional Sir2p-interacting factors (Cuperus *et al.* 2000; Perrod *et al.* 2001), Sir2p has recently been identified in two different high molecular weight complexes, called RENT and TEL, each of which contains several as yet unidentified proteins (Ghidelli *et al.* 2001).

In an effort to understand the molecular defect of *SIR2* class I mutants, we performed several suppressor screens designed to identify factors that might be involved in the telomeric/*HM* silencing pathway affected in these mutants. Here we report the isolation of *SCS2*, a gene-dosage suppressor of the dominant-negative effect of the *SIR2-143* mutation, and both *YOL017w* [here renamed *ESC8* (*e*stablishes *s*ilent *c*hromatin 8)] and *ESC2*. These two latter genes encode proteins capable of restoring silencing in *SIR2* class I mutants when physically tethered either to a telomere or to the *HMR* locus. Remarkably, both Esc8p and Esc2p interact with Sir2p in the two-hybrid system, a property that might explain their ability to suppress the *SIR2-143* mutation via direct recruitment of the Sir2/3/4 protein complex. However, Esc8p appears to have an additional function, since it is capable of suppressing a *sir2-424* mutant, where Sir complex recruitment fails to restore silencing (Cuperus *et al.* 2000). Although neither *ESC8* nor *ESC2* is required for silencing, their absence reduces the stability of the silenced state at *HMR*. Interestingly, both Esc8p and its single close homolog in yeast, the Ioc3 protein, interact with the imitation SWI one (*ISW1*) protein (Tsukiyama *et al.* 1999; Gavin *et al.* 2002), and Ioc3p has been shown directly to be part of an Isw1p-containing nucleosome-remodeling complex (Tsukiyama *et al.* 1999). We find that deletion of *ISW1* leads to a relatively severe reduction of *HMR* silencing and is epistatic to both *ESC8* and *IOC3* deletions. Finally, we identified Gal11p, a component of the mediator complex of Pol II holoenzyme, as a protein that interacts with Esc8p in the two-hybrid system. This observation may be relevant to the recent finding that RNA polymerase II appears to be bound to the *HMRa1* promoter in the silenced state (Sekinger and Gross 2001).

MATERIALS AND METHODS

Media and strains: Yeast strains used in this study are listed in Table 1. Growth and manipulation of yeast was done according to standard procedures (Adams *et al.* 1997). Full open reading frame (ORF) deletions of *ESC8*, *IOC3*, and *ISW1*, replaced by the *kanMX4* gene, were generated as described (Guldener *et al.* 1996). Deletion of the endogenous *ESC8* carboxy terminus was made by introducing a stop codon after amino acid 661 followed by the *kanMX4* gene. All gene disruptions were confirmed by colony PCR and/or Southern blotting. *ESC2* deletion was described earlier (Dhillon and Kamakaka 2000). Further details of strain constructions are available upon request.

Plasmids: GLC575, -577, -579, -581, -583, and -585 were constructed by PCR amplification of GLC520–525 (rescued from the suppressor screen), respectively, using primers YOL017-delta661 (5′ aaagtcgacctaatcttcacctttttcttg-tcaatgt 3′) and pGBT9-Gbd (5′ ggggatccgtcgacctgc 3′). The PCR product was digested and recloned in pGBT9 (Bartel and Fields 1995) using the unique *Sal*I and *Bam*HI restriction sites. Plasmid GLC525 was recloned in pBTM116 (a LexA fusion vector; Bartel and Fields 1995) using *Sma*I and *Pst*I. *ESC8* was fused to glutathione S-transferase (GST) by digesting GLC525 with *Sma*I and *Pvu*II and recloning the fragment in pGEX4-T1 (Pharmacia, Piscataway, NJ) digested with *Sma*I. 9xMyc-tagged *SIR2* plasmids were described previously (Cuperus *et al.* 2000). GLC547, encoding GAD-Gal11p [amino acids (aa) 902–1081], was recloned in pBTM116 using *Eco*RI and *Pst*I. Gbd-Ioc3p fusions were made by PCR amplification using primer ioc3_5004-27 (5′ aatgaattcgattctccatccaattctatccag 3′) for the full-length fusion and primer ioc3_6837-62 (5′ aaagaattccat gatgttgcacgaggcagaaatac 3′) for the carboxy-terminal fusion, in combination with primer ioc3_7650-26 (5′ aaactgcagaacca gaggggaaggataccaaaac 3′). The PCR products were digested with *Eco*RI and *Pst*I and cloned in pGBT9. Gbd-Sir3p and Gbd-Sir4p plasmids were described previously (Marcand *et al.* 1996).

Libraries and screening: Strains GCY185 and GCY186 were used in a high-copy suppressor screen. The library was made in YEplac181 (a 2μ-*LEU2* plasmid) and was a generous gift of P. Linder. From a total of ~250,000 transformants, six 5-fluoroorotic acid (5-FOA)-resistant clones were identified, all of which carried a plasmid containing the complete *SIR2* ORF. A high-copy suppressor screen in GCY190 was carried out with the same library. A telomeric *ADE2* marker was used in this case due to a low 5-FOA^R background in a telomeric *URA3* reporter strain (GCY206). From ~100,000 transformants, we identified five potential suppressors, four of which carried plasmids containing *SIR2*. The fifth clone contained a plasmid with a 7.3-kb fragment from chromosome V (400942–408255). Recloning of each predicted ORF present on this fragment identified the *SCS2* gene as being responsible for the restoration of telomeric silencing (data not shown).

A Gal4p DNA-binding domain (Gbd)-fusion library (a generous gift of S. Fields), made from sheared genomic DNA cloned into pGBT-CYH (a *CEN*, *TRP1*, *CYH2*, pADH1-Gbd vector derived from pGBT9C), was used in a "one-hybrid" screen as follows. The plasmid library was transformed into both GCY212 and GCY213, and Trp$^+$ transformants were replica plated onto 5-FOA-containing plates to identify those in which telomeric silencing had been restored. We isolated 23 independent clones with plasmids containing the *SIR2* gene and 20 other clones containing in-frame fusions between the Gbd and one of three different loci: the uncharacterized open reading frame *YOL017w* (hereafter named *ESC8*), the *ESC2* gene, and the 2μ plasmid-encoded *REP1* gene. The *ESC8* gene was isolated nine times in GCY212 and five times in GCY213 for a total of eight different fusion end points (see Figure 2). *ESC2* and *REP1* were both isolated three times in GCY212, but

TABLE 1

Yeast strains used in this study

Strain	Genotype	Reference
W303-1A	*MAT***a** *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1*	Thomas and Rothstein (1989)
BUY674	*MAT*α *esc2::HIS3*	Dhillon and Kamakaka (2000)
GCY23	*MAT***a** *RDN1::MET15* Δ*Ahmr::TRP1 adh4::URA3* Tel (VII-L) *sir2 met15 ADE2*	Cuperus *et al.* (2000)
GCY185	*MAT*α Δ*Ahmr::TRP1 adh4::URA3* Tel (VII-L) *SIR2-143*	This study
GCY186	*MAT*α Δ*Ahmr::TRP1 adh4::URA3* Tel (VII-L) *sir2-424*	This study
GCY190	*MAT*α Δ*Ahmr::TRP1 adh4::ADE2* Tel (VII-L) *HIS3::SIR2-143*	This study
GCY206	*MAT*α Δ*Ahmr::TRP1 adh4::URA3* Tel (VII-L) *HIS3::SIR2-143*	This study
GCY212	*MAT***a** *adh4::4UASg-URA3* Tel (VII-L) *SIR2-143*	This study
GCY213	*MAT***a** *adh4::4UASg-URA3* Tel (VII-L) *sir2-424*	This study
GCY245	*MAT*α *Aeb::UASg hmr::URA3 adh4::ADE2* Tel (VII-L) *sir2-424*	This study
GCY247	*MAT*α *Aeb::UASg hmr::URA3 adh4::ADE2* Tel (VII-L) *SIR2-143*	This study
GCY310	*MAT***a** Δ*Ahmr::ADE2 esc8::KanMX*	This study
GCY311	*MAT***a** Δ*Ahmr::ADE2 esc2::HIS3*	This study
GCY312	*MAT***a** Δ*Ahmr::ADE2 ioc3::KanMX*	This study
GCY314	*MAT***a** Δ*Ahmr::ADE2 esc8::KanMX ioc3::KanMX*	This study
GCY315	*MAT***a** Δ*Ahmr::ADE2 esc2::HIS3 ioc3::KanMX*	This study
GCY316	*MAT***a** Δ*Ahmr::ADE2 esc2::HIS3 esc8::KanMX ioc3::KanMX*	This study
GCY317	*MAT***a** Δ*Ahmr::ADE2*	This study
GCY340	*MAT***a** *RDN1::MET15* Δ*Ahmr::TRP1 adh4::URA3* Tel (VII-L*) ADE2 sir2 met15 esc8::KanMX*	This study
GCY341	*MAT***a** *RDN1::MET15* Δ*Ahmr::TRP1 adh4::URA3* Tel (VII-L) *ADE2 sir2 met15 esc8*Δ*ct::KanMX*	This study
GCY367	*MAT***a** Δ*Ahmr::ADE2 adh4::URA3* Tel (VII-L) *isw1::KanMX*	This study
GCY373	*MAT***a** Δ*Ahmr::ADE2 adh4::URA3* Tel (VII-L) *esc8*Δ*ct::KanMX*	This study
GCY388	*MAT*α Δ*Ahmr::TRP1 adh4::URA3* Tel (VII-L) *esc8::KanMX*	This study
GCY389	*MAT***a** Δ*Ahmr::TRP1 adh4::URA3* Tel (VII-L) *ioc3::KanMX*	This study
GCY390	*MAT***a** Δ*Ahmr::TRP1 adh4::URA3* Tel (VII-L) *isw1::KanMX*	This study
GCY391	*MAT*α Δ*Ahmr::TRP1 adh4::URA3* Tel (VII-L) *esc8::KanMX isw1::KanMX*	This study
GCY392	*MAT***a** Δ*Ahmr::TRP1 adh4::URA3* Tel (VII-L) *ioc3::KanMX isw1::KanMX*	This study
GCY393	*MAT*α Δ*Ahmr::TRP1 adh4::URA3* Tel (VII-L)	This study
YEA80	*MAT*α *Aeb::UASg hmr::URA3*	R. Sternglanz
YEA82	*MAT*α *aeB::UASg hmr::URA3*	R. Sternglanz
YEA84	*MAT*α *aeb::UASg hmr::URA3*	R. Sternglanz
PJ69-4a	*MAT***a** *trp1-901 leu2-3,112 ura3-52 his3-200 gal4*Δ *gal80*Δ *LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-LacZ*	James *et al.* (1996)
CTY10-5D	*MAT***a** *ade2-1 trp1-901 leu2-3 his3-200 gal4 gal80 URA3::LexA op-LacZ*	Bartel and Fields (1995)

All GCY strains are isogenic derivatives of W303 (Thomas and Rothstein 1989).

not in GCY213. Two of the *ESC2* clones and two of the *REP1* clones had identical amino-terminal fusion points with Gbd.

Yeast silencing and two-hybrid assays: *HMR*-, telomeric-, and rDNA-silencing assays were performed as described previously (Gottschling *et al.* 1990; Sussel and Shore 1991; Roy and Runge 2000). Tenfold dilutions were used for spotting assays, starting with undiluted overnight cultures in the first spot. Two-hybrid assays in strain CTY10-5D and PJ69-4A were performed as described (Bartel and Fields 1995; James *et al.* 1996).

***In vitro* binding assays and Western blots:** GST and GST-Esc8(524-714)p fusion protein were expressed in *Escherichia coli* strain BL21 and purified essentially as described (Moretti *et al.* 1994), except that 1% Triton X-100 was added to improve solubilization. Typical binding reactions were performed as previously described (Cuperus *et al.* 2000). Input and bound Sir2-9xMyc were detected with the 9E10 monoclonal antibody (hybridoma cell line kindly provided by G. Evan), using the ECL detection system (Amersham, Arlington Heights, IL). Gbd-Esc8 fusion proteins were detected by Western blotting using a mouse monoclonal antibody directed against the Gal4 DNA-binding domain (Santa Cruz Biotechnology), followed by ECL detection.

RESULTS

Search for gene dosage suppressors of *SIR2* class I mutations: To gain insight into the molecular defect(s) of *SIR2* class I mutants we first carried out gene dosage (high-copy) suppressor screens. Two different representative *SIR2* class I mutants were selected on the basis of their distinctive secondary phenotypes (Cuperus *et al.* 2000). The *SIR2-143* mutant is dominant to *SIR2* and the mutant protein can restore silencing when "tethered" to either a telomere or the *HMR* locus (as a Gbd-Sir2-143p fusion) in a cell containing only the mutant *SIR2-143*

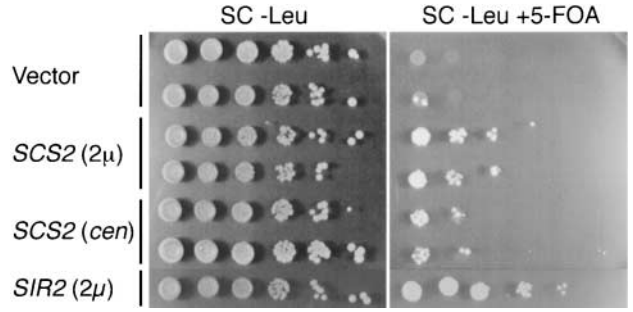

FIGURE 1.—Elevated *SCS2* gene dosage overcomes the dominant effect of the *SIR2-143* mutation on telomeric silencing (TPE). Strain GCY206 contains both *SIR2* and *SIR2-143*, the chromosome VII-L telomeric reporter *adh4::URA3*, and the plasmids indicated on the left. The absence of growth on plates containing the *URA3* counter-selectable drug 5-FOA indicates a loss of *adh4::URA3* silencing. Tenfold serial dilutions of overnight cultures grown in liquid SC-Leu medium were spotted onto SC-Leu (growth) and SC-Leu + 5-FOA (TPE) plates.

allele. By contrast, *sir2-424* is recessive to *SIR2* and the mutant protein is unable to restore silencing when tethered (CUPERUS *et al.* 2000). Strains containing either *SIR2-143* or *sir2-424* in place of wild-type *SIR2*, and a *URA3* reporter gene at the chromosome VII-L telomere (GCY185 and GCY186, respectively), were transformed with a yeast genomic DNA library on a 2μ plasmid. Transformants were screened for 5-FOA resistance (an indication of telomeric silencing on the *URA3* reporter gene) by replica plating. All recovered plasmids contained *SIR2* sequences. We also tested directly whether 2μ plasmids containing *SIR1*, *SIR3*, or *SIR4* could restore telomeric silencing in these *SIR2* class I mutant strains and found that none were able to do so (data not shown). These data suggest that no single gene present in the library that we used is capable of suppressing either *SIR2* mutation when present at elevated dosage.

We therefore designed more elaborate screens aimed at revealing factors that might act together with *SIR2* in telomeric silencing. In one screen we took advantage of the dominant nature of *SIR2-143* (CUPERUS *et al.* 2000). A strain (GCY190) with an *ADE2* reporter gene inserted at the modified chromosome VII-L telomere and containing both *SIR2* and *SIR2-143* alleles forms uniformly white colonies due to the dominant derepressing effect of the *SIR2-143* mutation. We transformed GCY190 with the same 2μ-based genomic library and screened for red-sectored colonies. In addition to the *SIR2* gene itself, *SCS2* was identified as a suppressor in this screen (for details see MATERIALS AND METHODS). This phenotype was confirmed, using a *URA3* marker at the modified chromosome VII-L telomere (GCY206), and is still seen, though to a lesser extent, when *SCS2* is present on a centromeric (*CEN*) plasmid (Figure 1). In fact, *SIR2* overexpression overcomes the dominant negative effect of *SIR2-143* better than *SCS2* overexpression (Figure 1). We presently do not understand how *SCS2* acts to restore silencing, but it is interesting to note that *SCS2* was also identified as a gene dosage suppressor of the telomeric silencing defect seen in a *mec1-21* mutant (CRAVEN and PETES 2001).

Restoration of telomeric silencing in *SIR2* class I mutants by protein tethering: We next turned to a screen in which random yeast genomic fragments fused to DNA encoding the Gal4p Gbd are expressed in the same two *SIR2* class I mutant strains, this time containing Gal4p DNA-binding sites (UASg) immediately adjacent to the telomeric *URA3* reporter gene. The rationale behind this screen is that proteins acting together with Sir2p in telomeric silencing might be able to restore repression if artificially tethered to the telomeric reporter locus. For example, the Rap1p-interacting proteins Sir3p and Sir4p (MORETTI *et al.* 1994) can restore telomeric silencing in certain *rap1* mutant strains when targeted as Gbd hybrid proteins (MARCAND *et al.* 1996).

In extensive screens in GCY212 and GCY213 we isolated in-frame fusions between the Gbd and one of three different open reading frames (Figure 2; see MATERIALS AND METHODS for details): the uncharacterized gene *YOL017w* (hereafter named *ESC8*), the *ESC2* gene, and the 2μ plasmid-encoded gene *REP1* (to be described elsewhere; G. CUPERUS and D. SHORE, unpublished results). Since Gbd-Esc2p hybrids were isolated only in GCY212 and not in the GCY213 (*sir2-424*) background, we asked whether this is because they cannot restore silencing in the latter strain. Transformation of both Gbd-Esc2 fusions into GCY213 showed that this is indeed the case: Neither fusion restores silencing in this strain (Figure 2). We also noted that two different Gbd-Esc8p hybrids, Gbd-Esc8(475-714) and Gbd-Esc8(416-714), work poorly or not at all in GCY213 (Figure 2). Finally, we note that expression of Gbd-Esc8(52-714), which lacks only the first 51 amino acids of Esc8p, causes a growth defect (Figure 2). We also directly tested whether Gbd-Sir3p or Gbd Sir4p could suppress the different *sir2* mutations. Interestingly, Sir4p, just like Esc2p, can restore silencing in a *SIR2-143* background but not in a *sir2-424* background (Figure 2).

Since all of the library clones described above contain in-frame fusions with Gbd, it seems likely that both Esc8p and Esc2p must be tethered to the telomere, rather than simply overexpressed, to restore TPE. To test this idea directly, we used the corresponding *SIR2* mutant strains without UASg sites at the telomeric *URA3* reporter gene. As expected, restoration of TPE was no longer observed (data not shown). In addition, targeted silencing initiated by both Gbd-Esc2p and Gbd-Esc8p is completely *SIR2* and *SIR3* dependent (data not shown), suggesting that the normal silencing pathway is not bypassed by either hybrid protein. Moreover, targeting either Gbd-Esc2p or Gbd-Esc8p constructs to a normally nonsilenced locus (*LYS2*) containing a *URA3* reporter gene

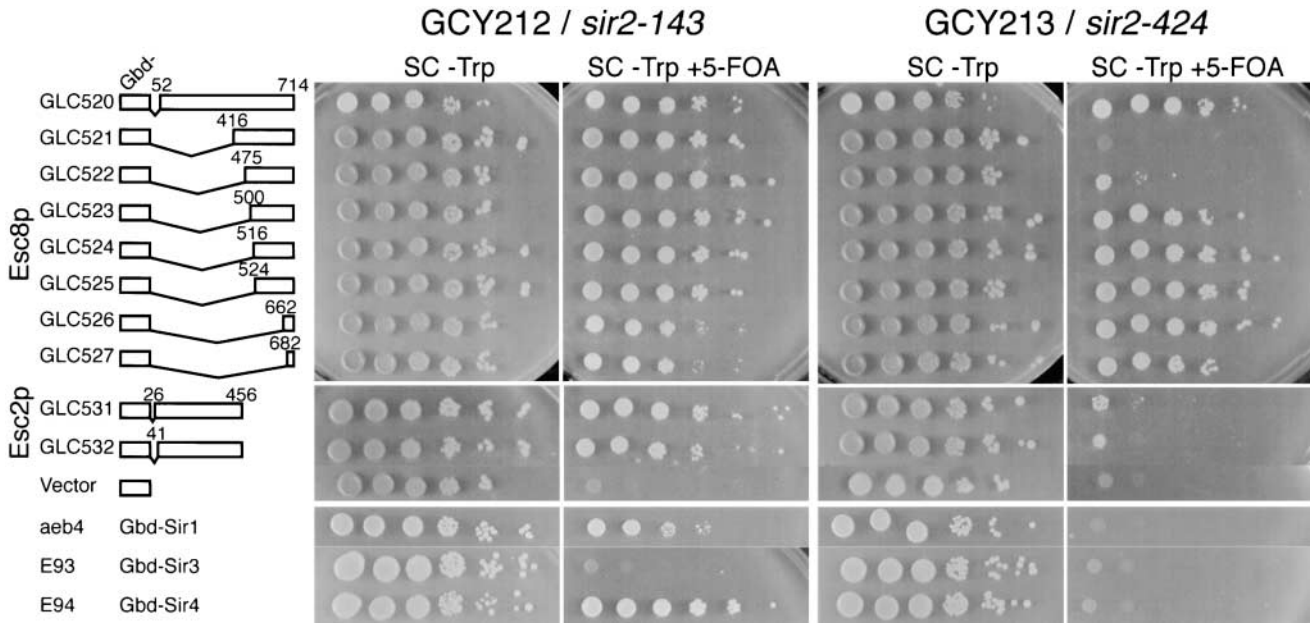

FIGURE 2.—Restoration of telomeric silencing (TPE) by tethering of Gbd-Esc8p and Gbd-Esc2p hybrids in strains GCY212 (*SIR2-143*) and GCY213 (*sir2-424*). The amino acid numbers present in each fusion as well as the plasmid names are shown on the left. Tenfold serial dilutions of overnight cultures grown selectively for the Gbd-containing plasmid (SC-Trp) were spotted onto SC-Trp (growth) and SC-Trp + 5-FOA (TPE) plates. Vector alone and Gbd-Sir3p or Gbd-Sir4p transformants are shown for comparison.

with flanking UASg sites (MARCAND *et al.* 1996) is not sufficient to establish silencing (data not shown). This finding further supports the notion that both Gbd-Esc2p and Gbd-Esc8p must collaborate with other silencing components at telomeres to restore repression in *SIR2* class I mutants.

We were struck by the fact that two identified Gbd-Esc8p fusions (GLC526 and GLC527) encode only 53 and 33 carboxy-terminal amino acids of Esc8p, respectively, yet are still sufficient to at least partially restore TPE in both *SIR2* mutant strains. Furthermore, both of these small hybrids appear to work better in the *sir2-424* background than in the *SIR2-143* strain. To test if this small carboxy-terminal domain is necessary for the action of larger fusions, we deleted the last 53 amino acids in the remaining Gbd-Esc8p constructs (GLC520–525). Significantly, none of these deletion hybrids were able to establish silencing (data not shown). To ask whether the deletions might have destabilized the hybrids, we attempted to measure protein levels for the deletion and parent hybrids by probing Western blots with an antibody against Gbd. Surprisingly, only the two smallest fusion proteins (GLC526 and GLC527) could be detected, and at a very high level (data not shown), making it impossible to determine the relative stability of the carboxy-terminal truncation fusions. Nonetheless, two-hybrid data (see Figure 6) suggest that the truncation fusion proteins are expressed at levels similar to their parental counterparts isolated from the library screen.

Gbd-Esc2p and Gbd-Esc8p can also restore silencing at *HMR*: Since *SIR2-143* and *sir2-424* mutants are also defective in *HMR* silencing, which requires many of the same factors involved in TPE (APARICIO *et al.* 1991), we tested the effect of tethering both Gbd-Esc8p and Gbd-Esc2p to *HMR*. In the first set of experiments we targeted the hybrids in the wild-type *SIR2* strain YEA80, which contains an *HMR*-E silencer whose Rap1p and Abf1p binding sites (E and B sites) are deleted and replaced by three copies of UASg. In addition, the *a1* gene at this modified *HMR* locus is replaced by *URA3*. The silencer mutation abolishes repression of *URA3* and renders this strain completely 5-FOA sensitive. Most of the Gbd-Esc8p and Gbd-Esc2p constructs can restore silencing at this mutated *HMR* locus when targeted (Figure 3, top). Surprisingly, however, the two smallest Gbd-Esc8p fusions (GLC526 and GLC527), as well as the largest one (GLC520), are nearly inactive in this assay, in marked contrast to their function in the TPE assay in *SIR2* mutant backgrounds (Figure 2). We also examined targeted silencing at the same *hmr::URA3* reporter locus in strains carrying the *SIR2-143* or *sir2-424* mutations. The same overall pattern was observed (Figure 3, middle and bottom), except for the fact that Gbd-Esc8(475-714) and Gbd-Esc8(416-714) failed to restore silencing at *HMR* in the *sir2-424* background, just as they failed to restore TPE in this particular *SIR2* mutant (see Figure 2). Finally, we repeated these tests in strain YEA82, in which the UASg sites replace a deletion of the A and E elements (ORC- and Rap1p-binding sites) at the silencer, and found essentially the same results (data not shown). Taken together, these data strongly support the idea that the function of Gbd-Esc8p and Gbd-Esc2p

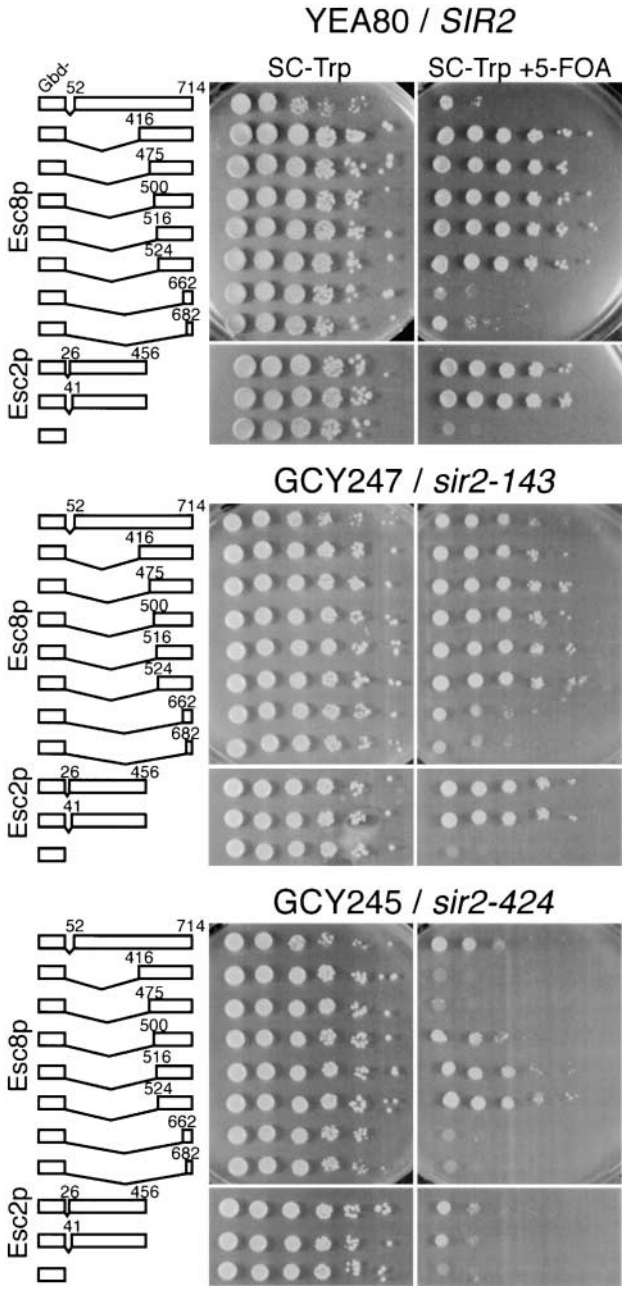

Figure 3.—Gbd-Esc8p- and Gbd-Esc2p-targeted silencing at *HMR* in a *SIR2*$^{+}$ (YEA80), *SIR2-143* (GCY247), or *sir2-424* (GCY245) strain background. Constructs used are indicated on the left and plasmid numbers are the same as in Figure 2. Tenfold serial dilutions from overnight cultures were spotted onto SC-Trp (growth) and SC-Trp + 5-FOA (*HMR* silencing) plates.

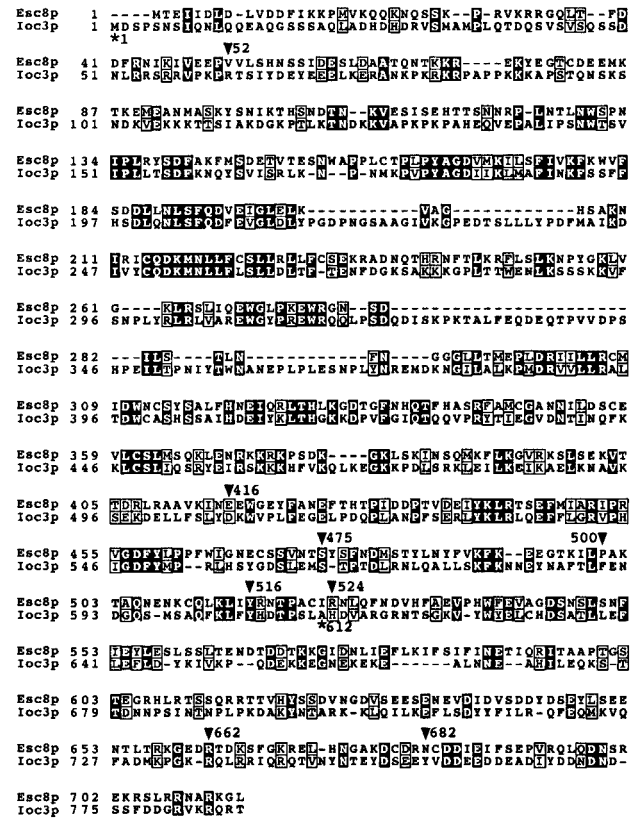

Figure 4.—The Isw1p-associated protein Ioc3 is highly related to Esc8p. Alignment was performed using the ClustalW algorithm and the output was formatted with BOXSHADE. Identities are highlighted in black and boxed residues indicate similarities (conservative substitutions). The amino-terminal endpoints of the eight Gbd-Esc8p hybrids identified in the screen are indicated above the Esc8p sequence by solid arrowheads, and the end points of the two Gbd-Ioc3p hybrids that were constructed and tested are marked below with asterisks.

hybrids is similar both at a telomere and at *HMR* and that the particular *SIR2* mutants tested lead to similar defects at the two loci.

We note that Gbd-Esc2p was initially identified by E. Andrulis and R. Sternglanz (personal communication; see http://www.proteome.com/databases/YPD/reports/ESC2.html) in a "one-hybrid" library screen in strain YEA80. The *ESC2* gene was subsequently isolated in a high-copy suppressor screen of *SIR1* mutants defective in *HMR* silencing (Dhillon and Kamakaka 2000). However, neither of these screens identified *ESC8*.

The Esc8p homolog Ioc3p forms part of a nucleosome-remodeling complex: To better understand the cellular function of *ESC8*, we performed a BLAST search using the predicted Esc8 protein sequence (Altshul *et al.* 1990). The best match is a *S. cerevisiae* protein, Ioc3p, which has 26% identity and 42% similarity to Esc8p over a region of 570 amino acids (Figure 4). Interestingly, Ioc3p (*i*mitation SWI *o*ne *c*omplex 3 protein) was identified biochemically as a polypeptide that copurifies with Isw1p, a member of the SWI2/SNF2 family of ATPases (Tsukiyama *et al.* 1999). The Isw1p complex (containing Ioc3p) has nucleosome spacing and displacement activities *in vitro* (Tsukiyama *et al.* 1999) and has been implicated in nucleosome displacement and transcriptional repression at specific genes *in vivo* (Kent *et al.* 2001). More recently, in a large-scale TAP-tag purification project, affinity purification of tagged Isw1p led to the identification of both Ioc3p and Esc8p as Isw1p-interacting proteins (Gavin *et al.* 2002).

The similarity between Esc8p and Ioc3p and their

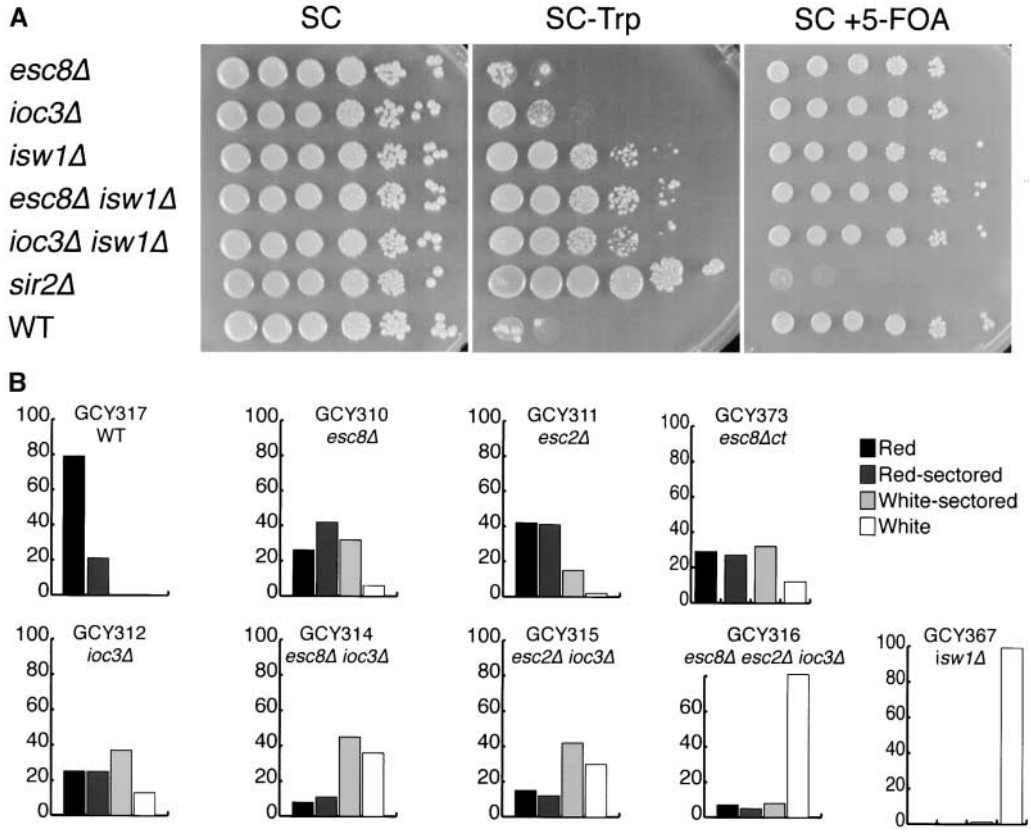

FIGURE 5.—(A) Silencing phenotypes of *esc8*Δ, *ioc3*Δ, and *isw1*Δ strains. Relevant genotypes are indicated on the left. Tenfold serial dilutions of overnight cultures were spotted onto SC (growth), SC-Trp (*HMR* silencing), and SC + 5-FOA (TPE at telomere VII-L) plates. (B) Silencing of Δ*Ahmr::ADE2* is affected by *esc2*Δ, *esc8*Δ, *esc8*Δ*ct*, *ioc3*Δ, and *isw1*Δ. The colony color phenotype of individual colonies was determined by eye and assigned to one of four different categories: solid red (silenced), red with white sectors (mostly silenced), white with red sectors (mostly derepressed), and solid white (no silencing). The data are presented as percentage of each colony type for the single-, double-, or triple-mutant genotype indicated and are based upon the scoring of >300 individual colonies for each strain.

possible presence in a common complex prompted us to ask whether Ioc3p might also restore silencing in any of the tethering assays described above. To test this idea we constructed two Gbd-Ioc3 fusion proteins: (i) a full-length fusion (amino acids 1–787) and (ii) an amino-terminal truncated form, Gbd-Ioc3(612-787), corresponding to the Gbd-Esc8(534-714) fusion (GLC525; see Figure 4). However, neither Gbd-Ioc3p fusion restored silencing in these assays (data not shown).

Involvement of *ESC8*, *ESC2*, *IOC3*, and *ISW1* in silencing: To investigate the possible role of native Esc8p in silencing, we deleted its predicted open reading frame in the chromosome. Surprisingly, an *esc8*Δ strain shows no silencing defect at *hmr*ΔA*::TRP1* (a weakened silencer reporter locus where the ORC binding site at the *HMR*-E silencer is deleted and the *a1* gene replaced by *TRP1*), the telomere VII-L *URA3* reporter, or the rDNA repeats (Figure 5A and data not shown). Since we had shown that the 53 carboxy-terminal amino acids of Esc8p are necessary and (often) sufficient to restore silencing in tethering assays (see Figure 2), we decided to generate a truncation allele of endogenous *ESC8* that would specifically remove only this part of the protein, by introducing a stop codon after amino acid 681. Again, no silencing defect is observed in an *esc8*Δ*ct* strain containing the same set of reporters (data not shown). However, both *esc8*Δ and *esc8*Δ*ct* mutations do weaken silencing of a highly sensitive *hmr*ΔA*::ADE2* reporter (see below).

Given the possible connection between Esc8p and Isw1p and the high degree of similarity between Esc8p and Ioc3p, we also asked whether *ISW1* or *IOC3* have any role in silencing. As shown in Figure 5A, a complete ORF deletion of *IOC3* results in a weak derepression of the *hmr*ΔA*::TRP1* reporter, but no obvious effect on TPE. Similarly, *ioc3*Δ has no effect on rDNA silencing (data not shown). Interestingly, an *ISW1* deletion has a strong *HMR* silencing defect but does not affect TPE (Figure 5A) or rDNA silencing (data not shown). Significantly, both *esc8 isw1* and *ioc3 isw1* double mutants display an *HMR* silencing defect indistinguishable from that observed in a *isw1*Δ strain, indicating the *ISW1* is epistatic to both *ESC8* and *IOC3* (Figure 5A). We also examined the effect of an *esc2*Δ mutation (kindly provided by R. Kamakaka), either alone or in combination with *esc8*Δ and *ioc3*Δ, but observed no further silencing defect other than the minor *HMR* silencing defect and the reduction of TPE described previously (DHILLON and KAMAKAKA 2000; data not shown).

To better understand the role of Esc8p, Ioc3p, and Isw1p in *HMR* silencing we took advantage of the very sensitive *hmr*Δ*A::ADE2* reporter (SUSSEL *et al.* 1993). In this otherwise wild-type strain, the *ADE2* gene is silenced, as indicated by the uniformly red color of most (~80%) colonies. However, derepression of *ADE2* is observed in some cells within a fraction (~20%) of colonies, as indicated by white sectors (clones of cells) within an otherwise red colony (Figure 5B). Using this assay, *esc8*Δ, *esc8*Δ*ct*, *esc2*Δ, and *ioc3*Δ all lead to a significant (and roughly equivalent) further destabilization of *hmr*Δ*A::*

ADE2 silencing (Figure 5B). Interestingly, the two double-mutant combinations tested (*esc8*Δ *ioc3*Δ and *esc2*Δ *ioc3*Δ) displayed additive effects, and the triple mutant appeared to be largely derepressed (Figure 5B). These data suggest that *ESC8*, *ESC2*, and *IOC3* act in nonoverlapping pathways in *HMR* silencing, but do not address the question of whether the effects of these genes are direct or through the regulation of other genes involved in silencing. Again, we found that an *isw1*Δ strain is much more defective in this *HMR* silencing assay than *ioc3*Δ, *esc8*Δ, *esc8*Δ*ct*, or *esc2*Δ single-mutant strains (Figure 5B) and even shows a derepression phenotype that is more severe than that of the *ioc3*Δ *esc8*Δ *esc2*Δ triple mutant. Taken together, these data implicate Isw1p, either directly or indirectly, in *HMR* silencing and suggest that the Isw1p-interacting proteins Esc8 and Ioc3 contribute only in part to this function (see DISCUSSION).

Esc8p and Esc2p both interact with Sir2p: To restore TPE in strain GCY212 (*SIR2-143*), it is sufficient to tether Sir2-143p to the telomere by fusing it to the Gbd (CUPERUS *et al.* 2000). It is therefore possible that Gbd-Esc8p and Gbd-Esc2p work in the GCY212 strain by themselves, recruiting the mutant Sir2-143 protein to the telomere. In this regard, it is interesting to note that Gbd-Sir4p, which is known to interact with Sir2p, also restores TPE in GCY212 but not GCY213 (Figure 2). To test whether either Esc2p or Esc8p interacts with Sir2p we took advantage of the Gbd fusion proteins rescued from the suppressor screen and performed a two-hybrid assay against full-length *SIR2* fused to the Gal4p activation domain (GAD). As predicted by the recruitment model, Gbd-Esc2p does interact with GAD-Sir2p in this assay (Figure 6). In addition, several Gbd-Esc8p hybrids also interact strongly with GAD-Sir2p. However, an examination of the full set of Gbd-Esc8p hybrids in this assay revealed some interesting features (Figure 6). For example, two similar carboxy-terminal Gbd-Esc8p hybrids (GLC524 and GLC525, with amino-terminal endpoints at 516 and 524, respectively) show a stronger interaction with GAD-Sir2p than do several larger Gbd-Esc8p constructs. Perhaps more peculiar, however, was the finding that GLC520 (the nearly full-length Gbd-Esc8p hybrid) and the two smallest carboxy-terminal hybrids (GLC526 and GCL527) yield no transformants in combination with GAD-Sir2p in the two-hybrid strain PJ69-4A (and therefore could not be tested). This apparent toxic effect was specific to these particular combinations of Gbd and GAD fusions. Finally, deleting the 53 carboxy-terminal amino acids of the Gbd-Esc8p hybrids abolishes their interaction with Sir2p [and also the toxicity of the Gbd-Esc8(52-714) hybrid in combination with GAD-Sir2p]. These results indicate that the extreme carboxy terminus of Esc8p is required for Sir2p binding and, in combination with the targeted silencing experiments with the carboxy-terminal truncations, strongly suggest that this interaction plays an important role in Gbd-Esc8p-tethered silencing. Taken together, these observations indicate that Gbd-Esc8p and Gbd-Esc2p probably restore silencing in GCY212 by recruiting *SIR2-143*, but that Esc8p must have at least one additional function, since recruitment of Sir2-424p to the telomere in strain GCY213 is not sufficient to restore TPE (CUPERUS *et al.* 2000).

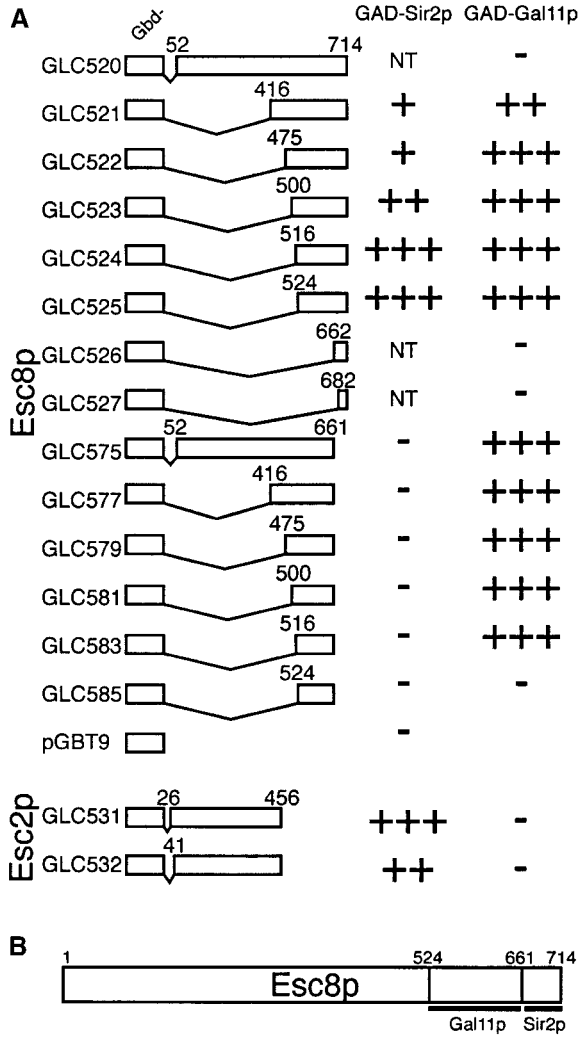

FIGURE 6.—(A) Two-hybrid analysis of Gbd-Esc8p and Gbd-Esc2p with either GAD-Sir2p or GAD-Gal11p in strain PJ69-4A. The different Gbd constructs used are shown on the left with the plasmid number and relevant amino acid end points indicated. −, no growth on SC-His plates after 5 days; +, growth on SC-His and on SC-Ade plates; ++, growth on SC-His + 2 mM aminotriazole (an inhibitor of the *HIS3* gene product) and on SC-Ade plates; +++, growth on SC-His + 10 mM aminotriazole and on SC-Ade plates; NT, no transformants. (B) Schematic representation of Esc8p showing the domains involved in Gal11p and Sir2p interactions. Relevant amino acid numbers are indicated.

To verify the two-hybrid interaction between Sir2p and Esc8p, a GST pull-down assay was performed using GST-Esc8(524-714)p and yeast whole-cell extracts made from strains expressing 9xMyc-tagged Sir2p (see MATERIALS AND METHODS). As shown in Figure 7, Sir2p

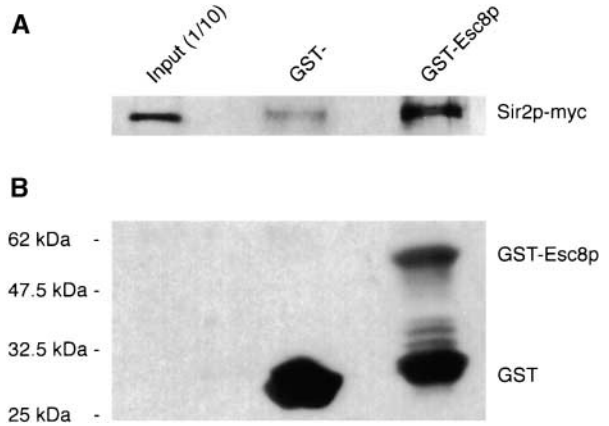

FIGURE 7.—Esc8p interacts with Sir2p *in vitro*. (A) Binding of 9xMyc epitope-tagged Sir2 protein (Sir2p-myc) from yeast whole-cell extract to *E. coli*-produced GST or GST-Esc8(524-714)p bound to glutathione agarose beads. Sir2p from one-tenth of the yeast extract input, or the eluates from the respective glutathione agarose beads, was detected by Western blotting using a monoclonal antibody (9E10) against the Myc tag. (B) The same input and eluate samples as in A were probed by Western blotting, using a polyclonal antibody against GST. The positions of molecular weight standards are indicated on the left and protein identity on the right.

specifically binds to GST-Esc8p in this assay, providing independent confirmation of the significance of the two-hybrid results.

A priori, it is possible that the loss of the Esc2p or Esc8p interaction (or both) with Sir2p might at least partially explain the silencing defect in *SIR2* class I mutants (CUPERUS *et al.* 2000). Alternatively, one could imagine that the Esc2p-Sir2p or Esc8p-Sir2p interactions play a specific role in rDNA silencing (or some other Sir2p function) that can be coopted at telomeres or *HM* loci by tethering. In an attempt to distinguish between these different possibilities, we performed two-hybrid assays with GAD-Sir2 mutant hybrids of both the class I and class II types paired against either Gbd-Esc8p or Gbd-Esc2p. Somewhat surprisingly, we found that several *SIR2* class II mutants (defective in rDNA silencing) fail to interact with both Esc2p and Esc8p, whereas all of the *SIR2* class I mutants (defective in telomeric/*HMR* silencing) retain both interactions (Table 2). These results were confirmed in the CTY10-5D reporter strain, with *ESC2* and *ESC8* cloned in frame with LexA (data not shown). Although the simplest interpretation of these data might be that Esc2p and Esc8p work in a common complex implicated in rDNA silencing, the phenotypes of *esc2*Δ and *esc8*Δ (or *esc8*Δ*ct*) mutants do not support this idea. We also examined two other Sir2p-related rDNA functions in *esc8*Δ mutants: inhibition of rDNA recombination (GOTTLIEB and ESPOSITO 1989; FRITZE *et al.* 1997) and involvement in a meiotic checkpoint (SAN-SEGUNDO and ROEDER 1999). However, neither rDNA recombination nor sporulation kinetics are affected by the *esc8*Δ mutation (data not shown).

TABLE 2

Two-hybrid analysis of GAD-Sir2p/Gbd-Esc2p or Gbd-Esc8p interactions

Class I mutants	Esc2p	Esc8p	Class II mutants	Esc2p	Esc8p
Sir2-12p	+++	+++	Sir2-81p	+++	+++
Sir2-143p	+++	+++	Sir2-82p	+++	+++
Sir2-146p	+++	+++	Sir2-85p	+++	+++
Sir2-213p	+++	+++	Sir2-86p	+++	+++
Sir2-424p	+++	+++	Sir2-88p	−	−
Sir2-512p	+++	+++	Sir2-89p	+++	+++
			Sir2-93p	−	−
			Sir2-131p	−	−
Controls			Sir2-167p	−	−
Sir2p	+++	+++	Sir2-199p	−	−
Vector	−	−	Sir2-87p	−	−

−, no growth on SC-His plates after 5 days; +++, growth on SC-His + 10 mM aminotriazole and on SC-Ade plates.

Esc8p interacts with Gal11p, a component of the RNA pol II holoenzyme: Following the analysis of the Sir2p-Esc8p interaction, we performed a two-hybrid screen using Gbd-Esc8p(524-714) (GLC525) as bait, in an attempt to identify other Esc8p-interacting factors that might provide an additional clue as to the molecular function of this protein. This screen identified a clone encoding a carboxy-terminal fragment of Gal11p (amino acids 902–1081), a subunit of the RNA Pol II mediator complex. This result might seem puzzling given the role of Esc8p in silencing and the more common description of Gal11p as an activator of gene transcription (KOLESKE and YOUNG 1995). However, a repression function for Gal11p has also been uncovered (FASSLER and WINSTON 1989; YU and FASSLER 1993; SUSSEL *et al.* 1995; HAN *et al.* 2001; see DISCUSSION). Nonetheless, when tethered upstream of a promoter as a LexA fusion, Gal11p (and in particular a carboxy-terminal region encompassing amino acids 865–911) can function as a strong activator (HIMMELFARB *et al.* 1990). Although the *GAL11* fragment isolated in our two-hybrid screen does not comprise this entire domain, we found that it did function as a transcriptional activator in the two-hybrid reporter strain CTY10-5D (data not shown). To map more precisely the region of Esc8p sufficient for Gal11p interaction, we tested all of our Gbd-Esc8p clones as well as deletions derived from them (Figure 6). Interestingly, the carboxy terminus of Esc8p, necessary for the Sir2p interaction, is not required for Gal11p binding in this assay. Instead, a region between amino acids 524 and 561 appears to be necessary and sufficient for the Esc8p interaction with Gal11p.

DISCUSSION

In a previous study we identified a class of *SIR2* mutants specifically defective in telomeric/*HM* silencing

and described evidence for the existence of unidentified Sir2p-interacting factor(s) required at these loci (Cuperus *et al.* 2000). Here we have presented results of different suppressor screens designed to identify factors involved with Sir2p in telomeric/*HM* silencing. Because a standard gene dosage ("high-copy plasmid") suppressor screen failed to uncover genes (other than *SIR2* itself) that would restore repression in *SIR2* class I mutant strains, we turned to two other types of screens: dosage suppression of the dominant-negative *SIR2-143* mutation and suppression via protein tethering at a telomeric reporter.

The first screen yielded the *SCS2* gene, which encodes a conserved integral membrane protein of the endoplasmic reticulum. Although the precise molecular function of Scs2p is still obscure, the protein clearly plays a role in lipid metabolism through the activation (either direct or indirect) of *INO1* expression (Kagiwada *et al.* 1998). We presently do not understand the mechanism of either the dominant negative effect of *SIR2-143* or its suppression by *SCS2*. It is interesting to note, however, that *SCS2* was also identified as a high-copy suppressor of the telomeric silencing defect of a *mec1-21* mutant (Craven and Petes 2001). Because it seems unlikely that the TPE defects of *SIR2* class I mutants and the *mec1-21* mutation are related, we imagine that the effect of elevated *SCS2* gene dosage on silencing is indirect, perhaps through increased expression of limiting silencing factor(s). Interestingly, *YOL017w(ESC8)* was also identified in the *mec1-21* suppressor screen (referred to as pMOS7), although its effect was much weaker than that of *SCS2* (Craven and Petes 2001).

Esc8p and Esc2p: Sir2-interacting proteins that rescue class I mutations when tethered to a telomere or *HMR* silencer: Through a protein tethering strategy we were able to identify Esc8p, Esc2p, and Rep1p as *SIR2* class I mutant suppressors. Significantly, both Esc8p and Esc2p proteins were subsequently shown to interact with Sir2p in two-hybrid assays, and the Esc8p-Sir2p interaction was confirmed biochemically. The simplest interpretation of these data would be that both proteins function in the tethering assay by directly recruiting Sir2p (and thus indirectly Sir3p and Sir4p) to the reporter locus. This explanation fits well for the case of Gbd-Esc2p and its effect in the *SIR2-143* strain, where we know that tethering of the mutated Sir2 protein itself will restore repression (Cuperus *et al.* 2000). It is also consistent with the observation (Figure 2) that Gbd-Sir4p, another Sir2p-interacting protein (Moazed *et al.* 1997; Cuperus *et al.* 2000), can restore silencing in the *SIR2-143* strain. However, this Sir2p recruitment mechanism does not explain the unique action of Gbd-Esc8p in the *sir2-424* background, where, in tethering of the mutant Sir2-424 protein itself or of Gbd-Sir4p, both fail to restore repression (Cuperus *et al.* 2000; Figure 2). Interestingly, Gbd-Rep1p does not seem to interact with Sir2p and is therefore believed to restore silencing by a different mechanism (G. Cuperus and D. Shore, unpublished results).

We thus hypothesize that Esc8p has a silencing function that goes beyond a simple ability to interact physically with Sir2p. Remarkably, this function would appear to be carried out by the same small (~50 amino acid) carboxy-terminal region of Esc8p necessary (and probably sufficient) for its Sir2p interaction, since the two smallest Gbd-Esc8p hybrids (GLC526 and GLC527) can restore TPE in a *sir2-424* background. Gal11p seems unlikely to be involved directly in this function, since it does not interact with this extreme carboxy-terminal region of Esc8p. Despite the specific effect of tethered Esc8p in *SIR2* class I mutant strains, it is clear that the native Esc8 protein plays either a minimal or highly redundant role in silencing (see below). One possible explanation for this conundrum is that the interaction between the C terminus of Esc8p and Sir2-424p at an *HMR* silencer or a telomere is sufficient to activate an otherwise nonfunctional mutant Sir2 protein. This might be a fortuitous interaction or one which in the context of the native wild-type proteins serves only a minor or redundant function in silencing. Consistent with this model for a direct allosteric effect of Esc8p on the Sir2-424 mutant protein, we find that the Isw1 protein, with which Esc8p is associated, is not required for Gbd-Esc8p action in telomeric silencing (data not shown).

We were surprised to find that certain *SIR2* class II mutant proteins, but not a single class I mutant, failed to interact with Esc8p in a two-hybrid assay (Table 2). It seems clear that the loss of the Sir2p-Esc8p interaction in these class II mutants would not explain their rDNA silencing defect, since none of the *ESC8* mutations we tested had any effect on rDNA silencing. However, these particular *SIR2* class II mutants might be (partially) defective in an additional interaction, which, in combination with the loss of Esc8p binding, might result in a complete breakdown of rDNA silencing. In any event, we have no additional evidence that either Esc2p or Esc8p plays a role in rDNA silencing. But, even if they do, data presented here and by Dhillon and Kamakaka (2000) clearly implicate these proteins in silencing at both *HMR* and telomeric sites.

It is worth pointing out that the tethering suppression screen described here is conceptually similar to a screen carried out first by E. Andrulis and R. Sternglanz (personal communication) that led to the identification of *ESC2* and many other "ESC" (*e*stablishes *s*ilent *c*hromatin) genes, including *NET1(ESC5)*. Their screen employed a number of different mutated silencer elements, but did not include a mutation in a *trans*-acting factor, as was the case here. This difference between the two screens is apparently significant. To begin with, the screen described here identified only one gene (*ESC2*) isolated in the original Andrulis and Sternglanz screens. In addition, we tested six other clones identified in the original ESC screens and found that only one (a Gbd-

Sir1 hybrid) restored repression in the *SIR2-143* strain, while none worked in *sir2-424* (Figure 1 and data not shown). These observations underscore some unique feature of our screen and the special property of the Gbd-Esc8p hybrid to restore repression in the *sir2-424* strain.

A role for chromatin remodeling complexes in silencing? The homology between Esc8p and Ioc3p (26% identity and 42% similarity observed over a 570-amino-acid region; Figure 4) is compelling and immediately suggested a connection between the Gbd-Esc8p silencing function and chromatin remodeling. Consistent with this notion, deletion of either *ESC8* or *IOC3* weakens *HMR* silencing, albeit to a small extent. However, the effects of these two mutations are at least partially additive, suggesting that their functions are nonoverlapping with respect to silencing. Furthermore, deletion of the SWI/SNF homolog gene *ISW1*, whose product interacts biochemically with both Esc8p (Yol017p) and Ioc3p (Tsukiyama *et al.* 1999; Gavin *et al.* 2002), results in a rather strong reduction of silencing at the *HMR* locus. At present, our genetic data and the available biochemical evidence suggest that both Esc8p and Ioc3p act in association with Isw1p in a silencing pathway(s). However, it is still unclear whether Esc8p and Ioc3p function in the same complex with Isw1p or in two separate Isw1p complexes. In any event, our epistasis data indicate that Isw1p has a silencing function in addition to or distinct from that provided by the combined action of the interacting proteins Esc8 and Ioc3. It is interesting to note that ISWI in the fruit fly Drosophila has been found in three distinct chromatin remodeling complexes (NURF, ACF, and CHRAC) implicated by some biochemical experiments in gene activation (Varga-Weisz and Becker 1998) but also in repression functions (Deuring *et al.* 2000). Similarly, although the SWI2/SNF2 complex and other SWI/SNF-like ATPases in yeast have been implicated in gene activation, both *ISW1* and its closest homolog in yeast, *ISW2*, have been linked to transcriptional repression (Goldmark *et al.* 2000; Kent *et al.* 2001).

Given the possible redundancy of SWI/SNF complex function in silencing, we also deleted several *ISW1* homologs to examine their role in Gbd-Esc8p-tethered telomeric silencing. (As mentioned above, *ISW1* itself is not required for tethered silencing at telomere VII-L by Gbd-Esc8p). Included in this analysis were *ISW2* (the closest homolog of *ISW1*) as well as *CHD1 RAD5*, *RAD16*, *RIS1*, and *SWR1*. None of these genes were required for Esc8p-mediated targeted silencing, arguing either that they are not Esc8p partners or that their activity is not required together with Esc8p in the tethering assay. *MOT1* and most members of the RSC complex could not be tested in this way since they are essential genes.

At present, then, our genetic data, together with the biochemical results from other groups (Tsukiyama *et al.* 1999; Gavin *et al.* 2002), are consistent with a model in which an Isw1-containing nucleosome remodeling complex (or complexes) contributes to silencing at the *HMR* locus. We do not know yet whether this effect is direct or indirect (for example, on the expression of genes with a specific silencing function at *HM* loci) or whether it requires Isw1p ATPase activity. Interesting possibilities are that Isw1p nucleosome remodeling activities (perhaps redundant with those of other SWI/SNF-like complexes) are required for efficient silencer-binding protein interactions at the silencers, for effective SIR protein recruitment, or for generating a nucleosome spacing arrangement that promotes SIR complex-histone interactions. In regard to the last possibility, it is worth noting that nucleosomes appear to be highly ordered at *HM* silent chromatin, compared to the same regions in a (*sir*$^{-}$) active conformation (Weiss and Simpson 1998; Ravindra *et al.* 1999). Finally, we think it is interesting to note that *ESC8* mRNA levels are cell-cycle regulated, with a peak at mid-G1 phase and a strong reduction during M phase (Spellman *et al.* 1998). This could be consistent with a role in the initiation or inheritance of silencing (supported by the data in Figure 5B), a process that shows a still-unspecified S-phase requirement (Kirchmaier and Rine 2001; Li *et al.* 2001) that might be related to a nucleosome remodeling step that normally acts on newly replicated DNA.

Possible implications of the Esc8p-Gal11p interaction: The identification of *GAL11* as an Esc8p-interacting protein was somewhat surprising since Gal11p is a subunit of the RNA Pol II mediator complex, which has generally been associated with gene activation, rather than with repression or silencing (Kim *et al.* 1994; Koleske and Young 1995). However, several genetic studies have in fact implicated Gal11p in repression (reviewed in Carlson 1997) and other reports indicate that *GAL11* mutants, as well as mutants in an associated mediator component *PDG1(MED3/HRS1)*, are defective in telomeric silencing (Suzuki and Nishizawa 1994; Piruat *et al.* 1997). Curiously, a *GAL11* mutation actually improves silencing at a weakened *HMR* locus in strains carrying *rap1*^{s} mutations (Sussel *et al.* 1995). This opposite effect at *HMR* might be explained, however, by the relief of TPE and the consequent release of limiting amounts of Sir proteins to act at other sites (Buck and Shore 1995; Marcand *et al.* 1996). Although the precise role of Gal11p in transcriptional regulation is unclear, biochemical studies have placed the protein within the Rgr1p subcomplex of the mediator, in a "module" containing Pgd1p, Med2p, and Sin4p (Li *et al.* 1995; reviewed in Malik and Roeder 2000; Myers and Kornberg 2000; Liu *et al.* 2001).

How might an Esc8p-Gal11p interaction contribute to silencing? The following speculative model is based upon the phenotype of *GAL11* mutants and an analogy to an emerging connection between the Tup1 repressor and mediator (Papamichos-Chronakis *et al.* 2000). We suggest that tethered Gbd-Esc8p can bind to mediator

at nearby promoter regions to transmit a negative signal to Pol II, the nature of which is unclear. This signal may be reinforced by the action of the deacetylase Sir2p, as well as the combined action of the Sir3 and Sir4 proteins, both of which are believed to assemble onto chromatin through interactions with hypoacetylated histone H3 and H4 amino-terminal tails. This hypothetical model for Esc8p action finds a striking parallel with the Tup1p repressor, which appears to be able to contact mediator (Papamichos-Chronakis *et al.* 2000), interact directly with histone tails (Edmondson *et al.* 1996), and recruit a specific deacetylase complex to the site of repression (Watson *et al.* 2000). Recent results from Sekinger and Gross (2001) lead to the surprising conclusion that SIR-mediated silencing excludes neither DNA-bound activators nor TBP and Pol II themselves from the repressed chromatin. The action of Esc8p or related factors might help to explain how silent chromatin could apparently trap or block the transcription machinery at a silent promoter. Additional experiments will obviously be required to test these ideas.

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