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

Guido Cuperus and David Shore

*Department of Molecular Biology, University of Geneva, Geneva 4, CH-1211 Switzerland*

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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 silent information regulator (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 T13 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; Fritzze 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; Derbishire 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
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/HMR 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 (establishes silent chromatin 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 SWI one (JSWI) protein (Tsukiyama et al. 1999; Gavin et al. 2002), and Ioc3p has been shown directly to be part of an Swi1p-containing nucleosome-remodeling complex (Tsukiyama et al. 1999). We find that deletion of JSWI leads to a relatively severe reduction of HMR silencing and is epistatic to both ESC3 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 ISWI, 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 GLC20-525 (rescued from the suppressor screen), respectively, using primers YOL017-deleta661 (5’ aagtgcagattaattcttccttgaagc3’) and pGBT9-Gbd (5’ gggagtctgactcctg 3’). The PCR product was digested and recombined in pGBT9 (Bartel and Fields 1995) using the unique Sall and BamHI restriction sites. Plasmid GLC525 was recombined in pBTM116 (a LexA fusion vector; Bartel and Fields 1995) using Smal and PstI. ESC8 was fused to glutathione S-transferase (GST) by digesting GLC525 with SmaI and PstI and recombining the fragment in pGEX4-T1 (Pharmacia, Piscataway, NJ) digested with EcoRI and PstI. Gbd-Ioc3p fusions were made by PCR amplification using primer ioc3_5004-27 (5’ aagggcagctgaaaacccaa 3’) for the full-length fusion and primer ioc3_6837-62 (5’ aagttgacgagagagagagaa 3’) for the carboxy-terminal fusion, in combination with primer ioc3_7650-26 (5’ aagttgcaagccagaggggagaa 3’). The PCR products were digested with EcoRI and PstI 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− 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, TRPI, CEN_H2, 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 replicated plated onto 5-FOA-containing plates to identify those in which telomeric silencing had been restored. We isolated 25 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 REPI 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 REPI were both isolated three times in GCY212, but...
not in GGY23. 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; Süssel 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 CUY10-5D 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 Sir2pMyc 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 SIR2-143mutant is dominant to SIR2 and the mutant protein can restore silencing when “tethered” to either a telomere or the HMR locus (as a Gbd-Sir2-143pECL detection system (Amersham, Arlington Heights, IL)).

**TABLE 1**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>W303-1A</td>
<td>MATα ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1</td>
<td>Thomas and Rothstein (1989)</td>
</tr>
<tr>
<td>BUY674</td>
<td>MATα esc2::HIS3</td>
<td>Dhillon and Kamakaka (2000)</td>
</tr>
<tr>
<td>GGY23</td>
<td>MATα RDN1::MET15 ΔAhrm::TRP1 adh4::URA3 Tel (VII-L) sir2 met15 ADE2</td>
<td>Cuperus et al. (2000)</td>
</tr>
<tr>
<td>GGY185</td>
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<td>This study</td>
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<td>This study</td>
</tr>
<tr>
<td>GGY206</td>
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<td>This study</td>
</tr>
<tr>
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<td>MATα adh4::UAGs-URA3 Tel (VII-L) SIR2-143</td>
<td>This study</td>
</tr>
<tr>
<td>GGY213</td>
<td>MATα adh4::UAGs-URA3 Tel (VII-L) sir2-424</td>
<td>This study</td>
</tr>
<tr>
<td>GGY245</td>
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<td>This study</td>
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</tr>
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<td>GGY390</td>
<td>MATα ΔAhrm::TRP1 adh4::URA3 Tel (VII-L) isw1::KanMX</td>
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<tr>
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<tr>
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<tr>
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<td>MATα ΔAhrm::TRP1 adh4::URA3 Tel (VII-L) ioc3::KanMX</td>
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<td>R. Sternaglanz</td>
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<td>MATα Δabr::UAGs hmr::URA3</td>
<td>R. Sternaglanz</td>
</tr>
<tr>
<td>PJ69-4a</td>
<td>MATα trp1-901 leu2-3,112 ura3-2,200 gal4Δ gal80Δ lys2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-LacZ</td>
<td>James et al. (1996)</td>
</tr>
<tr>
<td>CUY10-5D</td>
<td>MATα ade2-1 trp1-901 leu2-3 his3-200 gal4Δ gal80Δ URA3::LexA op-LacZ</td>
<td>Bartel and Fields (1995)</td>
</tr>
</tbody>
</table>

All GGY strains are isogenic derivatives of W303 (Thomas and Rothstein 1989).
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 Sir4p (TPE). Strain GCY206 contains both Sir4p, just like Esc2p, can 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-Esc8p(52-714), which lacks only the first 51 amino acids of Esc8p, causes a growth defect (Figure 2).2000). A strain (GCY190) with an inserted at the modified chromosome VII-L telomere (GCV185 and GCV186, 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 (GCV190) 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 GCV190 with the same 2μ-based genomic library and screened for red-sector 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 mcm1Δ 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-Esc8p(475-714) and Gbd-Esc8p(416-714), work poorly or not at all in GCY213 (Figure 2). Finally, we note that expression of Gbd-Esc8p(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 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.

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.

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. Andurulis 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 (in vitro complex 3 protein) was identified biochemically as a polypeptide that copurifies with Isw1p, a member of the SWI/SNF2 family of ATPases (Tsukiyama et al. 1999) and has been implicated in nucleosome spacing and displacement activities (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
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 esc8Δ strain shows no silencing defect at hmrΔ::TRP1 (a weakened silenced 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 esc8Δct strain containing the same set of reporters (data not shown). However, both esc8Δ and esc8Δct mutations do weaken silencing of a highly sensitive hmrΔ::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 hmrΔ::ADE2 reporter, but no obvious effect on TPE. Similarly, ioc3Δ 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 iow3Δ strain, indicating the ISW1 is epistatic to both ESC8 and IOC3 (Figure 5A). We also examined the effect of an esc2Δ mutation (kindly provided by R. Kamakaka), either alone or in combination with esc8Δ and ioc3Δ, 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 hmrΔ::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, esc8Δ, esc8Δct, esc2Δ, and ioc3Δ all lead to a significant (and roughly equivalent) further destabilization of hmrΔ::ADE2.
ADE2 silencing (Figure 5B). Interestingly, the two double-mutant combinations tested (escΔ ioc3Δ and escΔ ioc3Δ) 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 isw1Δ strain is much more defective in this HMR silencing assay than ioc3Δ, esc8Δ, esc2Δ, or esc2Δ single-mutant strains (Figure 5B) and even shows a derepression phenotype that is more severe than that of the ioc3Δ esc8Δ esc2Δ 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 GLC527) yield no transformants in combination with GAD-Sir2p in the two-hybrid strain P[69-4A] (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-Esc8p (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).

To verify the two-hybrid interaction between Sir2p and Esc8p, a GST pull-down assay was performed using GST-Esc8p (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
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-Sir2p mutant hybrids of both the class I and class II types paired against either Gbd-Esc2p or Gbd-Esc8p. 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 esc2Δ and esc8Δ (or esc8Δct) mutants do not support this idea. We also examined two other Sir2p-related rDNA functions in esc8Δ 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 esc8Δ mutation (data not shown).

**TABLE 2**

<table>
<thead>
<tr>
<th>Class I mutants</th>
<th>Esc2p</th>
<th>Esc8p</th>
<th>Class II mutants</th>
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</tr>
</thead>
<tbody>
<tr>
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<td>++</td>
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<td>Sir2-199p</td>
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</tr>
</tbody>
</table>

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

**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-Esc8p(524-714) 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 (9E10) against the Myc tag. (A) Positions of molecular weight standards are indicated on the left and protein identity on the right.

**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; Susse 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/HMR 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/HMR 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 SCS2 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, YOLO17w(E8C8) 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 Sir2p 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” (establishes silent chromatin) genes, including NET1/ESC5. Their screen employed a number of different mutated silencer elements, but did not include a mutation in a transacting 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 ISWI, 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 ISWI 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 ISWI homologs to examine their role in Gbd-Esc8p-tethered telomeric silencing. (As mentioned above, ISWI itself is not required for tethered silencing at telomere VII-L by Gbd-Esc8p). Included in this analysis were ISW2 (the closest homolog of ISWI) as well as CHD1, RAD5, RAD16, RIS1, and SWRI. 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 mRNAs 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 PGD1 (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 rap1-1 mutations (Susse 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 (Bucr 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 TBPl 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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