Note

One-Hybrid Screens at the *Saccharomyces cerevisiae HMR* Locus Identify Novel Transcriptional Silencing Factors

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ABSTRACT

In *Saccharomyces cerevisiae*, genes located at the telomeres and the *HM* loci are subject to transcriptional silencing. Here, we report results of screening a Gal4 DNA-binding domain hybrid library for proteins that cause silencing when targeted to a silencer-defective *HMR* locus.

TRANSCRIPTIONAL silencing in *Saccharomyces cerevisiae* occurs through a specialized chromatin structure at the telomeres and the *HM* loci, *HML* and *HMR* (reviewed in Rusche *et al.* 2003). The *HM* loci consist of mating-type genes that are kept transcriptionally silent by *cis*-acting elements, termed the *E* and *I* silencers (Brand *et al.* 1985; Mahoney *et al.* 1991). The *HMR-E* silencer is composed of *A*, *E*, and *B* sites that are bound by the origin recognition complex, Rap1, and Abf1, respectively (Shore and Nasmyth 1987; Bell *et al.* 1993). These factors, in turn, recruit the *s*ilent *i*nformation *r*egulator proteins, Sir1–4, which leads to silencing of nearby genes (Rusche *et al.* 2003). If two or more silencer elements at *HMR-E* are deleted and replaced by Gal4-binding sites, silencing is lost. Silencing can be restored by expression of Gal4 DNA-binding domain (G_{BD}) hybrids fusing G_{BD} to known silencing proteins (Chien *et al.* 1993; Buck and Shore 1995; Lustig *et al.* 1996; Triolo and Sternglanz 1996); we refer to this as targeted silencing.

A G_{BD} library was screened to identify proteins capable of targeted silencing at *HMR*. Several known silencing proteins and Sir-binding proteins were identified. Novel proteins were also identified and named Esc because they *e*stablish *s*ilent *c*hromatin when targeted to DNA. A few other previously characterized proteins, with no known role in silencing, were also found to give *SIR*-dependent targeted silencing.

Targeted silencing screens: Strains with either two or three silencer elements at *HMR-E* deleted and replaced by binding sites for Gal4 and containing an *hmr::URA3* reporter (designated *Aeb::G* and *aeb::G*) were used for separate screens (Figure 1). The proteins identified in these screens are listed (Table 1) and targeted silencing by some of them is shown (Figure 2).

In the screen with the *Aeb::G* silencer, the known silencing factor Sir1 and Sir-interacting proteins Rad7, Rif1, Ris1, and Ubp10 were identified (Table 1; Hardy *et al.* 1992; Paetkau *et al.* 1994; Zhang and Buchman 1997; Singer *et al.* 1998). Presumably, these gave targeted silencing by binding a Sir protein directly, leading to the recruitment of the Sir protein complex. We also identified Vac8, a component of junctions formed between the nuclear envelope and the vacuole (Pan *et al.* 2000). Perhaps Vac8 brings the derepressed *HMR* locus to the nuclear periphery, where silencing proteins are concentrated (Palladino *et al.* 1993; Gotta *et al.* 1996; Andrulis *et al.* 1998). Two novel proteins with silencing activity were identified and named Esc1 and Esc2. Using two-hybrid assays, we showed that Esc1 interacts with Sir4 (Andrulis *et al.* 2002) and another group showed that Esc2 interacts with Sir2 (Cuperus and Shore 2002); this is probably why they gave targeted silencing. A serine-rich peptide was also found; this peptide is not derived from an open reading frame (ORF). Several proteins predicted to have at least one transmembrane domain (Snc1, Nyv1, Src1, and Gtt3) were also identified (data not shown). We have previously shown that membrane proteins can give targeted silencing, presum-

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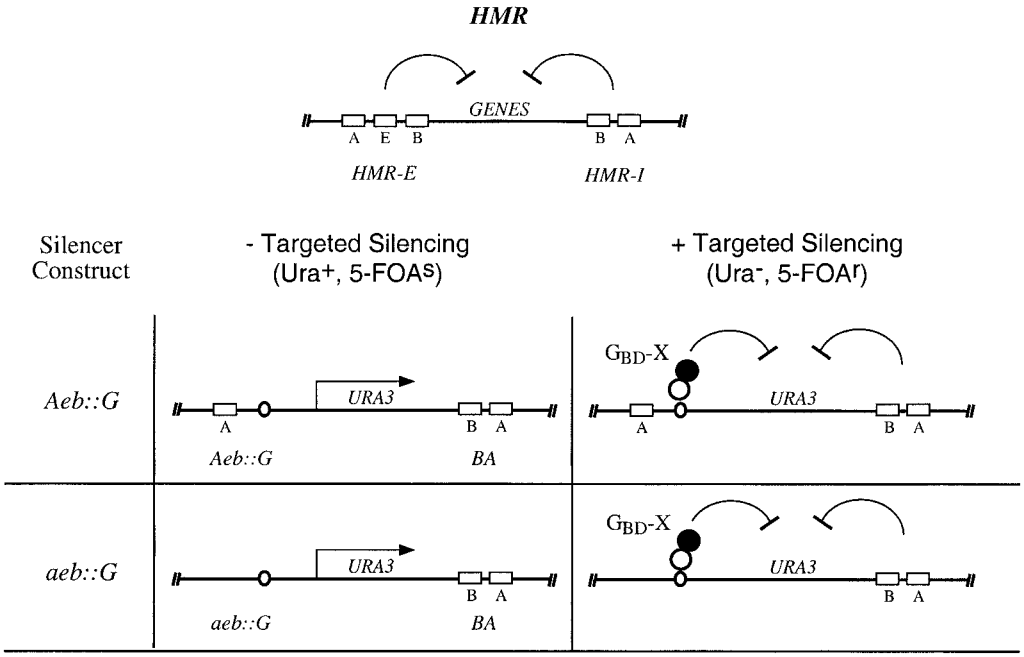

FIGURE 1.—Schematics of the strains used for the targeted silencing screens. The *Aeb::G* strain (YEA76) and the *aeb::G* strain (YEA78) both have a *URA3* reporter gene at *HMR*. The phenotype of the strains without and with targeted silencing is indicated. G_{BD}-X represents a hybrid protein from the library that gives targeted silencing.

ably because they bring the *HMR* locus to the nuclear periphery where there is a higher concentration of Sir proteins (ANDRULIS *et al.* 1998).

In the screen with the *aeb::G* silencer, many proteins were found (Table 1). Sir1, Rif1, Esc2, and Ris1, identified in the first screen, were isolated again. The Sir1-binding protein, Orc1, and the Sir2 homolog, Hst1, were identified. The Sir2-interacting Net1 protein was also found, as were Hir1, a repressor of histone gene transcription (SHERWOOD *et al.* 1993), and Rpb4, an RNA polymerase II subunit. A previously uncharacterized protein was also identified and named Esc4. The serine-rich peptide found in the first screen was identified twice in this screen. Finally, a G_{BD} hybrid to a 17-amino-acid peptide with the sequence IFLRLVKRPWP GQNFAP gave silencing. This peptide, like the serine-rich peptide, is not derived from an ORF. It is possible that these peptides may mimic a binding site for a silencing protein.

A targeted silencing screen also was undertaken with a strain that had both *HMR* silencers deleted and replaced with G_{BD}-binding sites. Two proteins, Esc1 and Rif2, a Rap1-interacting factor (WOTTON and SHORE 1997), were identified (data not shown).

***SIR*-dependent targeted silencing:** As mentioned, many of the proteins identified in these screens bind to Sir proteins. To test the *SIR* dependence of targeted silencing by the G_{BD} hybrids, each of the hybrid proteins was introduced into a targeted silencing reporter strain deleted for the *SIR2*, *SIR3*, or *SIR4* gene. None of the hybrid proteins gave targeted silencing when tested in these *sir* mutant strains (data not shown). Thus, it is very likely that the silencing observed was due to the endogenous silencing machinery. It is not clear why

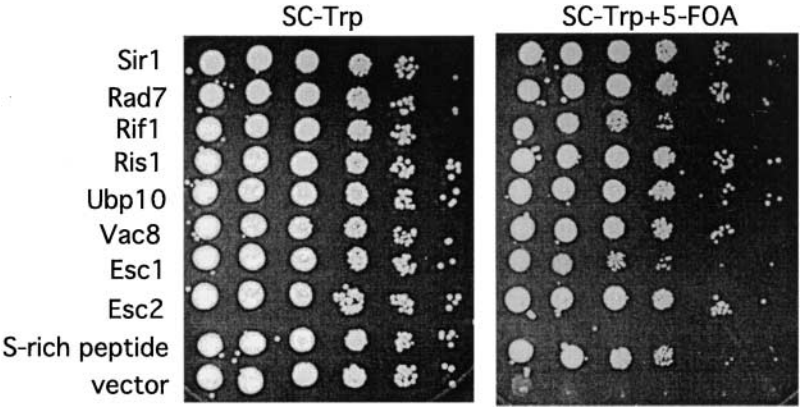

FIGURE 2.—Targeted silencing by all the proteins identified with the *Aeb::G* strain (listed in Table 1). Serial dilutions of cultures plated on SC-Trp medium to indicate the number of cells plated, and on SC-Trp+5-FOA medium to measure silencing of the *URA3* reporter gene, are shown. Good growth on the latter medium indicates good silencing.

TABLE 1

Proteins isolated in the targeted silencing screens

Protein	Fused at/total residues[a]	Comments	Reference
		Aeb::G screen results	
Sir1	163/678	Silencing protein	Stone *et al.* (1991)
Rad7	94/566	Binds Sir3	Paetkau *et al.* (1994)
Rif1	1589/1917	Binds Rap1	Hardy *et al.* (1992)
Rif1	1742/1917	Binds Rap1	
Ris1	1398/1601	Previously called Dis1; binds Sir4	Zhang and Buchman (1997)
Ubp10	78/791	Also called Dot4; binds Sir4	Singer *et al.* (1998)
Vac8	1/578	Vacuolar protein	
Esc1	1395/1658	Binds Sir4	Andrulis *et al.* (2002)
Esc2	115/456	Binds Sir2; SUMO-like domain	Cuperus and Shore (2002)
S-rich peptide[b]	18/50		
		aeb::G screen results	
Sir1	163/678	Silencing protein	Stone *et al.* (1991)
Orc1	37/914	Binds Sir1	Triolo and Sternglanz (1996)
Rap1	65/827	Binds Sir3 and Sir4	Moretti *et al.* (1994); Moretti and Shore (2001)
Rap1	637/827	Binds Sir3 and Sir4	
Rif1	1742/1917	Binds Rap1	Hardy *et al.* (1992)
Ris1	398/1601	Binds Sir4	Zhang *et al.* (2002)
Ris1	467/1601	Binds Sir4	
Hst1	45/503	Sir2 homolog	Brachmann *et al.* (1995); Derbyshire *et al.* (1996)
Rpb4	87/221	RNA polII subunit	
Hir1	475/840	Histone gene repressor	Sherwood *et al.* (1993)
Esc2	115/456	Binds Sir2	Cuperus and Shore (2002)
Esc4	−1/1070	Has six BRCT motifs	
Net1	566/1189	Binds Sir2	Cuperus and Shore (2002)
S-rich peptide[b]	18/50		
S-rich peptide[b]	22/50		
Peptide[c]		IFLRLVKRPWPGQNFAP	

Screens were performed as follows: Strains YEA76 (*Aeb::G*) or YEA78 (*aeb::G*) were transformed with a G_{BD} yeast genomic library constructed in a derivative of the vector pGBT9c (Bartel *et al.* 1996). Trp^+ transformants were selected and then replica plated to 5-fluoroorotic acid (5-FOA)-containing medium. 5-FOA^R colonies were selected and assessed for plasmid dependence. Plasmid DNA was isolated from appropriate candidates and retransformed into the same yeast strain. Positive candidates were sequenced. Rif1 (fused at residue 1589) was identified six times, Ubp10 two times, Esc2 eight times, and the rest of the proteins one time.

[a] The number on the left indicates the amino acid residue in the protein at its junction point with G_{BD}. The number on the right indicates the total number of amino acids in the protein. Each hybrid included all amino acids from the junction point to the C terminus of the protein.

[b] This peptide is encoded by the noncoding strand of the *RSE1* gene, ORF *YML049c*. The sequence of this peptide beginning with residue 18 of a hypothetical (and most likely not naturally expressed) ORF is DICNISISSSISSSSSSLLSSPPPFSCLLTLDS.

[c] This peptide, whose sequence is shown, is not encoded by an ORF. The DNA coding for it is from near the 3′ end of the *RGM1* gene.

Hir1 or Rpb4 gave *SIR*-dependent targeted silencing. Perhaps Hir1 binds appropriately modified histones and thus seeds the formation of the Sir2–4 complex. At first sight, *SIR*-dependent targeted silencing by Hst1 does not seem surprising because Hst1 is a fairly close homolog of Sir2 and overexpression of Hst1 can partially suppress the silencing defect of a *sir2* mutant at *HMR* (Brachmann *et al.* 1995). However, a novel form of silencing mediated by the SUM1-1 protein requires Hst1 but is independent of Sir2, Sir3, and Sir4 (Sutton *et al.* 2001).

Characterization of Esc2: We decided to focus our attention on one of the three previously uncharacterized proteins identified in these screens, Esc2. Esc1 has already been described (Andrulis *et al.* 2002), and Esc4 will be described elsewhere. Targeted silencing by Esc2 was most efficient in the presence of one remaining *HMR-E* silencer element, but also was seen when all three were absent (Figure 3A). Silencing by Esc2 required the Gal4-binding sites and endogenous Sir2, Sir3, and Sir4 proteins, but was only partially dependent on Sir1 (Figure 3A). The C-terminal region of Esc2 protein shows a significant similarity to the small ubiquitin-like

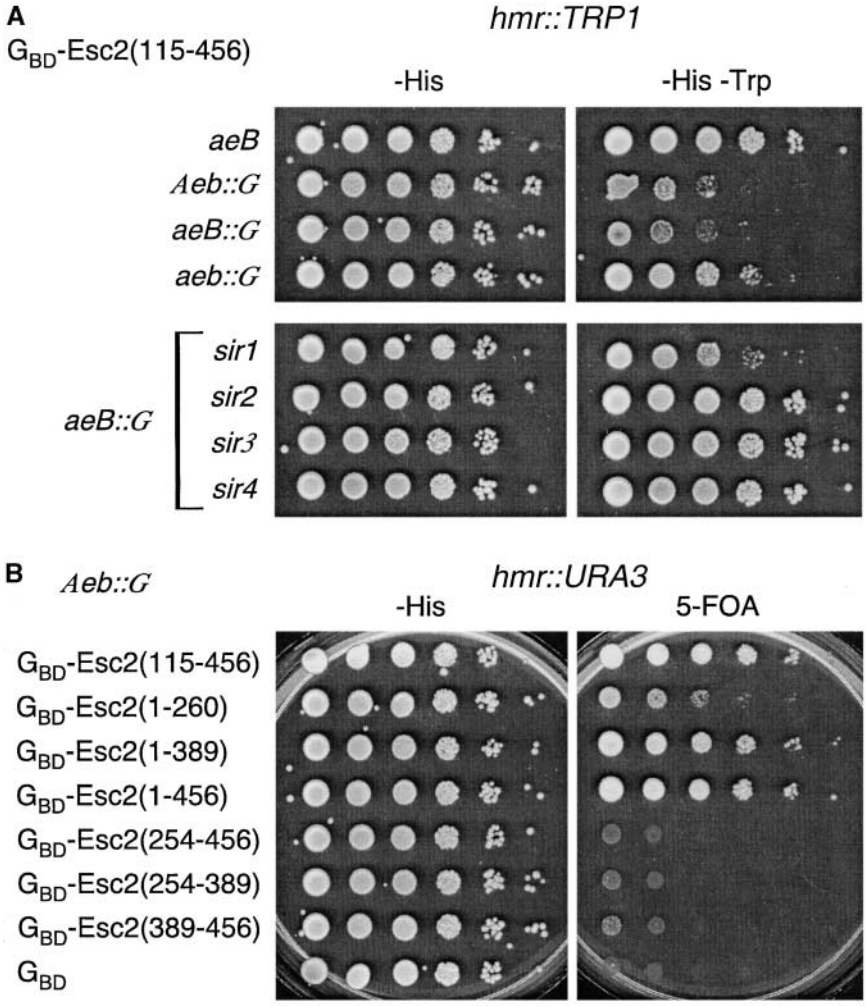

FIGURE 3.—Silencing by Esc2. (A) Silencing by G_{BD}-Esc2 (115–456). The various strains all have a *TRP1* reporter gene at *HMR*. To measure silencing in these strains, the originally isolated *ESC2* fragment, encoding amino acids 115–456 (Table 1 and Figure 2), was cloned in frame with G_{BD} into the vector pTT63, which has a *HIS3* selectable marker. The strains used were YSB1 (*aeB*, with no Gal4-binding site), YSB35 (*Aeb::G*), YSB2 (*aeB::G*), YSB41 (*aeb::G*), and the *sir1*, *sir2*, *sir3*, and *sir4* derivatives of YSB2, RS1172, RS1042, RS1061, and RS1067, respectively. Serial dilutions were plated on −His medium to indicate the number of cells plated and on −His-Trp medium to measure silencing. Lack of growth on the latter medium indicates good silencing. (B) Delineation of the silencing domain of Esc2. Various Esc2 fragments were amplified by PCR and cloned into pTT63. The reporter strain is YEA76 (*Aeb::G*), and, as in Figure 2, silencing is indicated by growth on 5-FOA medium.

protein, SUMO (58% identical or similar residues for amino acids 393–452 of Esc2). To address the role of the Esc2 C-terminal SUMO-like domain in silencing, we constructed several G_{BD} hybrids containing Esc2 fragments and tested them for targeted silencing. Removal of the Esc2 C-terminal SUMO-like domain did not affect the ability of Esc2 to give targeted silencing (Figure 3B). Also, this domain alone was not sufficient for silencing the reporter gene. These deletion studies suggest that the targeted silencing domain of Esc2 does not include the SUMO domain and lies between amino acid residues 115 and 260 (Figure 3B). While this article was in preparation, two other groups identified Esc2 by use of genetic screens for silencing proteins (DHILLON and KAMAKAKA 2000; CUPERUS and SHORE 2002).

Future directions: These targeted silencing screens allowed us to identify proteins implicated in the formation of silent chromatin. The screens have not been saturated, as we have identified only one clone for most of the proteins. It is likely that other factors that can establish silencing at *HMR* remain to be identified. The silencing peptides discovered here may provide insight into critical domains to which silencing proteins bind. This one-hybrid silencing approach can be applied to identify factors that are locus specific by performing targeted silencing screens at derepressed *HML* or ribosomal DNA loci with Gal4-binding sites. Future studies using this system should enhance our understanding of the general mechanisms of transcriptional silencing.

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