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Multiple Interactions in Sir Protein Recruitment by Rap1p at Silencers and Telomeres in Yeast

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Initiation of transcriptional silencing at mating type loci and telomeres in *Saccharomyces cerevisiae* requires the recruitment of a Sir2/3/4 (silent information regulator) protein complex to the chromosome, which occurs at least in part through its association with the silencer- and telomere-binding protein Rap1p. Sir3p and Sir4p are structural components of silent chromatin that can self-associate, interact with each other, and bind to the amino-terminal tails of histones H3 and H4. We have identified a small region of Sir3p between amino acids 455 and 481 that is necessary and sufficient for association with the carboxyl terminus of Rap1p but not required for Sir complex formation or histone binding. *SIR3* mutations that delete this region cause a silencing defect at *HMR* and telomeres. However, this impairment of repression is considerably less than that displayed by Rap1p carboxy-terminal truncations that are defective in Sir3p binding. This difference may be explained by the ability of the Rap1p carboxyl terminus to interact independently with Sir4p, which we demonstrate by in vitro binding and two-hybrid assays. Significantly, the Rap1p-Sir4p two-hybrid interaction does not require Sir3p and is abolished by mutation of the carboxyl terminus of Rap1p. We propose that both Sir3p and Sir4p can directly and independently bind to Rap1p at mating type silencers and telomeres and suggest that Rap1p-mediated recruitment of Sir proteins operates through multiple cooperative interactions, at least some of which are redundant. The physical separation of the Rap1p interaction region of Sir3p from parts of the protein required for Sir complex formation and histone binding raises the possibility that Rap1p can participate directly in the maintenance of silent chromatin through the stabilization of Sir complex-nucleosome interactions.

Related forms of transcriptional silencing in the budding yeast *Saccharomyces cerevisiae* occur at silent mating type loci (*HML* and *HMR*) and immediately adjacent to the TG_{1-3} repeats at telomeres (reviewed in references 15, 21, and 36). Both mating type and telomeric silencing (referred to hereafter as telomere position effect [TPE]) require a common set of *trans*-acting factors that include a complex of silent information regulators (Sir2, Sir3, and Sir4 proteins) and the amino-terminal tails of the core histones H3 and H4. Although the precise molecular mechanisms underlying silencing are not known, genetic and biochemical evidence indicates that it results from the "spreading" of Sir protein complexes from sites of initiation (*HM* silencers or telomeres) to nearby chromatin.

Significantly, both Sir3p and Sir4p can interact directly with the histone H3 and H4 tails in vitro (23), pointing to a possible mechanism for the formation of a closed, or repressive, chromatin structure. Consistent with the idea that Sir2/3/4 protein complexes bring about repression through direct interactions with nucleosomes, silencing is neither promoter nor polymerase specific but instead appears to have a general effect either on chromatin accessibility (19, 34, 54) or on the subsequent action of chromatin-bound factors (51a).

A key question, whose answer is still not clearly understood, is how silencing is initiated only at specific chromosomal sites. None of the Sir proteins appear to recognize DNA directly, and the initiation of silencing thus requires a set of DNA-binding factors to recruit the Sir proteins to their sites of action on the chromosome. At telomeres this is accomplished in part by Rap1p, whose binding sites are contained within the TG_{1-3} repeat tracts that comprise the telomeric DNA (28, 32, 47). Rap1p also binds to three of the four mating type gene silencers, where it collaborates with the origin recognition complex (ORC) and/or Abf1p to initiate silencing (6, 52). Strikingly, none of these three DNA-binding factors is specific to silencers. Rap1p and Abf1p binding sites are found in numerous promoter regions, where they typically act to stimulate transcription (7). Likewise, ORC binding sites are found at all known origins of DNA replication, most of which are not silencers (the only known exception being the *HMR*-E silencer). Therefore, a full understanding of the role of these proteins in the initiation of silencing must also explain why they have quite different functions in other contexts.

An important clue to the molecular mechanism of Rap1p action at telomeres and silencers came from the identification of both Sir3p and Sir4p as Rap1p-interacting proteins in the two-hybrid system (47). Significantly, Rap1 and Sir3p can interact directly in vitro in the absence of other yeast proteins. These observations, together with other studies (11, 32), suggested that the role of Rap1p in the initiation of silencing is to recruit Sir proteins to the chromosome by direct protein-protein interactions with Sir3p and perhaps Sir4p. Strong support for this comes from the observation that direct targeting of Sir proteins to *HMR*, by the use of Gal4p DNA-binding domain (G_{BD})-Sir hybrids, bypasses the requirement for the normal silencer binding sites (10, 40). Similarly, G_{BD}-Sir and LexA-Sir hybrids can restore silencing when targeted to a specific telo-

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mere in cells deleted for the carboxy-terminal Sir interaction domain of Rap1p (10, 37, 40).

To better understand the mechanisms underlying the recruitment of Sir3p and Sir4p to silencers and telomeres, we extended our analysis of the interactions of these two proteins with Rap1p. Using the two-hybrid system and a glutathione *S*-transferase (GST) hybrid pulldown assay, we have identified a Rap1p interaction domain of Sir3p that is both necessary and sufficient for the interaction with the carboxyl terminus of Rap1p. This short region of Sir3p, between amino acids 455 and 481, does not mediate Sir3p self-interaction or the Sir3p interaction with Sir4p, and it does not correspond to a previously characterized histone interaction domain (23). Deletion of this domain debilitates Rap1p-dependent targeted silencing but has much weaker effects on normal *HM* locus and telomeric silencing. In addition, the silencing defect caused by these Sir3p mutations is weaker than that obtained with Rap1p carboxy-terminal deletions, which themselves cause defects in Sir3p binding (47).

We provide evidence that this difference is due to the ability of Rap1p to interact independently with Sir4p. Significantly, overexpression of Sir4p suppresses the *HMR* silencing defect displayed by Sir3p mutants unable to bind Rap1p. These data support a model in which the establishment of silencing at *HM* loci and at telomeres involves the recruitment of the Sir complex to the chromosome via a set of cooperative interactions, involving direct binding of both Sir3p and Sir4p to the carboxyl terminus of Rap1p. Taken together with previous studies of Sir3p and Sir4p binding to histones (23), these new insights into Rap1p-Sir interactions also suggest a mechanism by which Rap1p may participate not only in initiation but also in the propagation and maintenance of silent chromatin.

MATERIALS AND METHODS

Yeast strains and silencing assays. Growth and manipulation of yeast strains were done according to standard procedures (51). The yeast two-hybrid reporter strain CTY10-5D (*MAT***a** *ade2-1 trp1-901 leu2-3,112 his3-200 gal4 gal80 URA3*::*lexAop-lacZ*) was used in all studies involving LexA fusion proteins. This strain, and its derivatives with *HIS3* disruptions of *SIR1*, *SIR2*, *SIR3*, *SIR4*, or *RIF1*, has been described elsewhere (47). All strains used for transcriptional silencing assays are isogenic to strain W303-1B (*MAT*α *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) (59).

The two strains used for targeted silencing with G_{BD}/Rap1p fusions are Leu$^-$ derivatives of two strains (relevant genotypes: $\Delta A\Delta E(UAS_G)_3hmr$::*TRP1 gal4*::*LEU2 sir3*::*URA3* and $\Delta E\Delta B(UAS_G)_3hmr$::*TRP1 gal4*::*LEU2 sir3*::*URA3*) described previously (8). These strains were created as follows. First, the *gal4*::*LEU2* gene was disrupted by gene replacement using a *leu2*::(UAS_G)::*ADE2* construct in which the regulatory sequences of the *ADE2* gene have been replaced with UAS_G, allowing Gal4p-dependent expression of the gene. The disruption was obtained by cotransformation with plasmid pMA210 (38), which expresses Gal4p, followed by selection on plates of synthetic complete (SC) medium lacking Ade and His (SC−Ade−His). Leu$^-$ transformants were identified by replica plating and then grown in liquid yeast extract-peptone-dextrose (YEPD) medium to allow loss of the pMA210 plasmid. The cultures were spread on YEPD plates, and cells with the desired phenotype (Leu$^-$ Ade$^-$ His$^-$) were selected. These cells were then sequentially transformed, first with G_{BD}/Rap1p fusions (8) in the *HIS3* integrating vector pRS303 (53) and then with mutant *sir3* alleles cloned into a low-copy-number *CEN* vector.

The strains used for the analysis of transcriptional silencing at the *HMR* locus and at telomeres are derived from a series of *HMR*::*TRP1* and *HMR*::*ADE2* strains or from a *URA3-Tel VIIL* strain, all of which have been described previously (57, 58). *sir3* disruption derivatives of these strains were obtained by gene replacement using a *sir3*::*HIS3* deletion/disruption construct (*HMR*::*TRP1*, *HMR*::*ADE2*, and *URA3-Tel VIIL* strains) or a *sir3*::*URA3* deletion/disruption (*HMR*::*ADE2*) strain. The *HIS3* disruption removes all Sir3p coding sequences between amino acids 108 and 945. The *URA3* disruption deletes the entire Sir3p open reading frame (978 amino acids) up to position 972. Both gene disruptions cause a complete loss of repression of the reporter gene and can be complemented by a plasmid-borne copy of *SIR3*. For the experiments with mutant alleles of *SIR3*, the cells were transformed with *SIR3* deletions cloned into plasmid pRS415 (*LEU2 CEN*) (53).

Assays for silencing using the *HMR*::*TRP1* reporter were performed by spotting 10-fold serial dilutions of cultures grown in the appropriate synthetic selective medium as described previously (57). Repression was tested in *HMR*::*ADE2* strains by examining colony color after 3 days of growth of the transformants at 30°C, followed by storage of the plates at 4°C for 1 or more days. Determination of the fraction of 5-fluoroorotic acid-resistant (5-FOA^r) and Ura$^+$ cells in *URA3-Tel VIIL* strains was done as follows. Independent colonies were grown in SC−Leu liquid medium overnight, diluted to an appropriate concentration, and then spread on SC−Leu, SC−Leu−Ura, and SC−Leu+5-FOA plates. Colonies were counted after 3 days at 30°C, and results from three or more independent cultures were used to calculate an average value.

Plasmids. The LexA protein and all LexA fusion proteins were expressed from plasmid pBTM116 (2μm origin, *TRP1* pADH1-*lexA*) (2). All LexA/Rap1p hybrids and the LexA/Sir4p(839–1358) and LexA/Sir3p(307–978) constructs have been described elsewhere (47). LexA/Sir1p, LexA/lamin, and LexA/Adh1p were gifts from Rolf Sternglanz (State University of New York at Stony Brook). All the Gal4p activation domain (G_{AD}) hybrids described here were expressed from plasmid pACTII (2μm origin, *LEU2*, pADH1-G_{AD}). The *SIR3* fragment used to create G_{AD}/Sir3p(307–978) was obtained from a LexA/Sir3p(307–978) fusion described previously (47). All the other G_{AD}/Sir3p constructs used here were obtained by two- or three-way ligation of *SIR3* fragments to the pACTII vector.

The construction of some of these constructs required intermediate cloning steps in pUC19 or pIC series plasmids. All the carboxy-terminal deletions of *SIR3* in the pACTII vector have the same amino-terminal junction as G_{AD}/Sir3p(307–978) and were created by cutting the *SIR3* sequence with a suitable restriction enzyme followed by repair of the end with the Klenow fragment or T4 DNA polymerase and ligation to a flushed site in the polylinker of the vector. All amino-terminal G_{AD}/Sir3p fusions other than G_{AD}/Sir3p(307–978) were created by using a restriction sites in the *SIR3* open reading frame or by creating a site at a suitable position by PCR. More detailed information on these constructs is available upon request.

G_{AD}/Sir4p(839–1358) was obtained by ligating a *Bam*HI-*Sal*I fragment of *SIR4* to the pACTII vector cut with *Bam*HI and *Xho*I. G_{AD}/Sir4p(839–1275) was obtained by removing *SIR4* sequences beyond the *Eco*RV site. The internal deletions of *SIR3* were created either by joining the flushed ends of DNA fragments cut at suitable restriction sites or by PCR cloning. All deletions cause the removal of *SIR3* sequences without causing the insertion of amino acids that are not normally present in the protein. Allele Δ440–502 was created by joining an amino-terminal fragment cut with *Eco*RI followed by Klenow repair of the end to a carboxy-terminal fragment cut with *Hin*dIII and repaired with Klenow. Mutant Δ482–502 was created in the same manner by joining a *Bsr*FI end repaired with Klenow to the same blunt *Hin*dIII end as above. All remaining *SIR3* deletions were constructed using PCR by creating restriction sites at new positions and subsequently removing sequences between the novel site and an existing site in *SIR3*.

Alleles Δ440–480 and Δ440–454 were constructed by creating an *Eco*RI site at positions 481 and 455, respectively, followed by removal of *SIR3* sequences between this novel site and the *Eco*RI site at amino acid position 439. Allele Δ456–479 was constructed by creating a *Bsr*FI site at amino acid position 455 followed by ligation to the *Bsr*FI site at position 480. Mutant Δ333–357 was constructed by creating a novel *Eag*I site at position 333, and alleles Δ358–437 and Δ398–437 were constructed by creating new *Eco*RI sites at positions 357 and 397, respectively. Fragments from these deletion constructs of *SIR3* were cloned in the pACTII vector for two-hybrid studies, in a pT7-Sir3p construct for in vitro studies, and in the pRS415 and pRS425 plasmids to test for transcriptional silencing in vivo. The GST/Rap1p and pT7-Sir3p(1–978) constructs used in this work have been described previously. pT7-Sir4p(839–1358) and pT7-Sir4p(839–1275) are derived from the corresponding G_{AD}/Sir4p constructs. Constructs used for the overexpression of *SIR1* and *SIR4* were created by cloning a gene fragment in the 2μm *URA3* vector pRS426 and in the *CEN URA3* vector pRS316, respectively.

Transcriptional activation assays. Transcriptional activation assays using LexA hybrids or LexA and G_{AD} hybrid combinations were performed as previously described (47).

In vitro protein-binding assays. Protein expression and purification and all in vitro binding studies were performed as described previously (47).

RESULTS

Amino acids 455 to 481 of Sir3p are sufficient to mediate a two-hybrid interaction with Rap1p but not Sir3p or Sir4p. We showed previously that the carboxy-terminal two-thirds of Sir3p (amino acids 307 to 978) can interact with Rap1p, Sir4p, and itself in two-hybrid assays (47). Here we have extended this analysis to ask whether a defined region of Sir3p that is specifically involved in binding to Rap1p could be identified. Beginning with a construct expressing G_{AD}/Sir3p(307–978), two sets of G_{AD}/Sir3p hybrids with progressive carboxy- or amino-terminal deletions of Sir3p were created and tested for the ability to interact with a LexA/Rap1p(679–827) hybrid. As shown in the top panel of Fig. 1, G_{AD}/Sir3p fusions with amino-terminal endpoints at positions 307, 356, 439, and 455 (and continuing to the carboxyl terminus at position 978) do not significantly differ in the strength of their interaction with LexA/Rap1p(679–827). However, two shorter fusions, G_{AD}/Sir3p (481–978) and G_{AD}/Sir3p(503–978), are completely defective in this interaction. The failure of these latter two G_{AD}/Sir3p constructs to give a signal in the two-hybrid assay is not due to lack of expression of a functional protein, since both fusion proteins interact normally with LexA/Sir3p(307–978) (data not shown). From these results, we place the amino-terminal endpoint of the minimal region of Sir3p required for the two-hybrid interaction with Rap1p between amino acids 455 and 481.

The analysis of a series of carboxy-terminal deletions gives a more complex picture than the amino-terminal set. Deletion of Sir3p sequences between amino acids 978 and 910 does not significantly affect the strength of the two-hybrid interaction with Rap1p, whereas two larger deletions (truncations at positions 798 and 762) completely abolish the two-hybrid signal (Fig. 1). Again, loss of the Rap1p interaction with these two latter constructs is not due to lack of expression of functional G_{AD}/Sir3p hybrids, since both of these fusions can interact with LexA/Sir4p(839–1358) as strongly as G_{AD}/Sir3p(307–978) (data not shown). Surprisingly, with Sir3p deletions beyond amino acid 762, the two-hybrid signal with LexA/Rap1p is restored, weakly in the case of G_{AD}/Sir3p(307–734) and G_{AD}/Sir3p(307–685), but to high levels with truncations at positions 598, 503, and 481 of Sir3p. The interaction is abolished again with further deletions to endpoints at amino acids 455 and 439. Taken together, these data suggest that the carboxy-terminal limit of the minimal Sir3p region required for the interaction with Rap1p may lie between amino acids 481 and 455. Although we do not know why the carboxy-terminal truncations ending between residues 685 and 798 of Sir3p weaken or abolish interactions with Rap1p in the two-hybrid assay, we note that this region of Sir3p has been linked to its binding to core histone N-terminal tail sequences (23). Perhaps, in the Sir3p truncations in question, histone interaction regions become exposed in such a way as to titrate out the two-hybrid interaction with Rap1p.

To rule out the possibility that the amino-terminal 307 amino acids of Sir3p that were not included in this analysis also contain sequences able to mediate an interaction with Rap1p, we tested a G_{AD}/Sir3p(1–503) and a G_{AD}/Sir3p(1–439) fusion. Only the first of these two constructs is able to give a signal in a two-hybrid assay with LexA/Rap1p(679–827) (data not shown), suggesting that the first 439 amino acids of Sir3p do not contain regions that are able to interact with Rap1p. This result, as well as all data shown in Fig. 1, were replicated with LexA/Rap1p(635–827) and LexA/Rap1p(653–827) hybrids.

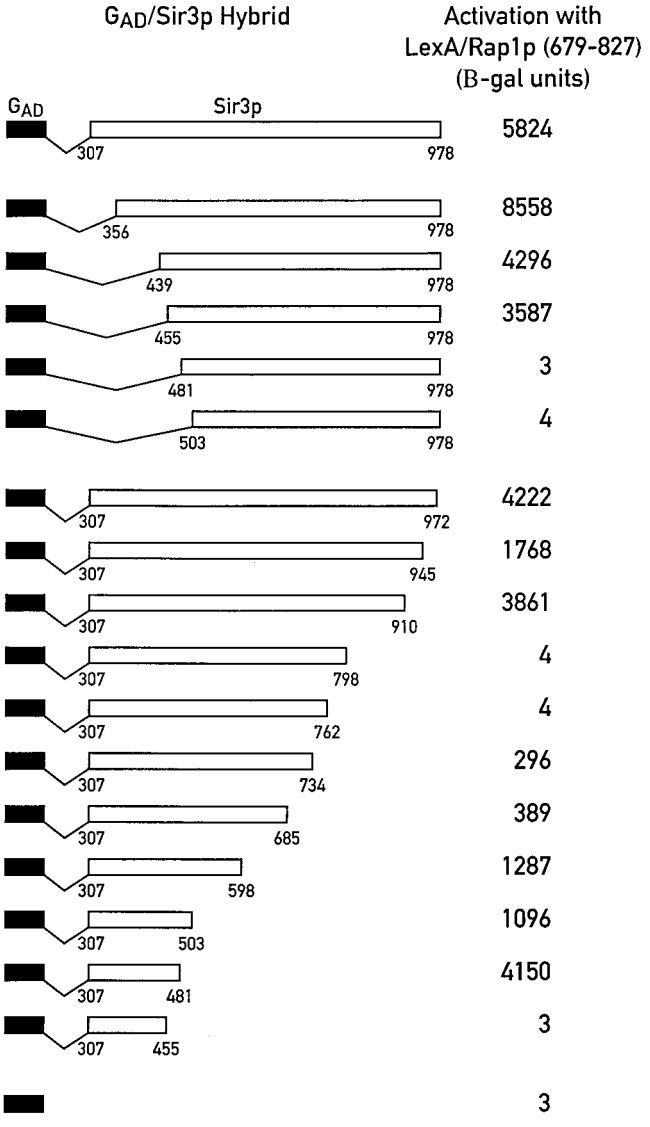

FIG. 1. Interaction of a series of G_{AD}/Sir3p truncations with LexA/Rap1p(679–827) in the two-hybrid system. G_{AD}/Sir3p fusions with progressive amino- and carboxy-terminal deletions of *SIR3* sequences were assayed for their interaction with LexA/Rap1p(679–827) using the two-hybrid reporter strain CTY10-5D. Transcriptional activation, measured as units of β-galactosidase (B-gal) activity, was normalized to a value of 10,000 U for LexA/Gal4p(768–881) [(LexA/G_{AD})], which was included as a control in all experiments (not shown in the figure).

We have shown previously that LexA/Rap1p carboxy-terminal fusions interact genetically with the endogenous *SIR2*, *SIR3*, *SIR4*, and *RIF1* genes. Specifically, mutations in any one of these four genes enhance a cryptic activation potential of certain LexA/Rap1p hybrids. In addition, Rif1p and Sir3p appear to compete for binding to the carboxyl terminus of Rap1p in the two-hybrid assay (47). To ask if the analysis reported above was influenced by competition between the G_{AD}/Sir3p fusions and endogenous Sir2, Sir3, Sir4, or Rif1 protein, we repeated these experiments in a set of reporter strains contain-

TABLE 1. Short stretch of 27 amino acids of Sir3p is sufficient to mediate a two-hybrid interaction with Rap1p but not Sir3p or Sir4p

LexA hybrid	β-Galactosidase (U)	
	G_{AD}/Sir3p(455–481)	G_{AD}
LexA/Rap1p(679–827)	80	3
LexA/Sir3p(307–978)	3	3
LexA/Sir4p(839–1358)	4	5

ing null mutations in each of the corresponding genes. Only minor quantitative differences between mutant and wild-type reporters were observed (data not shown), arguing against the possibility of significant perturbation by any of the endogenous factors tested.

The data described above suggest that amino acids 455 to 481 of Sir3p may contain all of the sequences required for a two-hybrid interaction with the carboxyl terminus of Rap1p. To test this hypothesis directly and to determine whether this small Sir3p fragment also mediates homodimerization or association with Sir4p, we constructed a G_{AD}/Sir3p(455–481) fusion and tested it with LexA/Rap1p(679–827), LexA/Sir3p (307–978), and LexA/Sir4p(839–1358). As shown in Table 1, G_{AD}/Sir3p(455–481) displays a significant association with LexA/Rap1p(679–827), demonstrating that a fragment of only 27 amino acids of Sir3p is sufficient to mediate a two-hybrid interaction with the carboxyl terminus of Rap1p. In contrast, this small Sir3p fragment does not give a signal with either LexA/Sir3p(307–978) or LexA/Sir4p(839–1358), suggesting that amino acids 455 to 481 of Sir3p are not sufficient to mediate a Sir3p self-interaction or binding to Sir4p.

Deletion of amino acids 456 to 479 of Sir3p specifically abolishes the two-hybrid interaction with Rap1p. To test the hypothesis that the region between amino acids 455 and 481 in Sir3p is necessary for the association with Rap1p but not required for either Sir3p self-interaction or binding to Sir4p, we constructed a series of G_{AD}/Sir3p(307–978) fusions with small deletions spanning the region between amino acid positions 333 and 502. As shown in Table 2, the deletion of 24 amino acids of Sir3p between positions 456 and 479 completely abolishes the interaction with LexA/Rap1p(679–827), as do two larger deletions encompassing the same region (Δ440–480 and Δ440–502). None of these three mutations significantly impair the interaction with LexA/Sir4p(839–1358) or LexA/Sir3p(307–978) hybrids (Table 2), demonstrating the specificity of their effect on the interaction with Rap1p.

To determine whether Sir3p sequences adjacent to the short region between amino acids 456 and 479 play a role in this interaction, constructs with deletions amino-terminal to position 456 (Δ440–454, Δ398–437, Δ358–437, and Δ333–357) and one deletion carboxy-terminal of position 479 (Δ482–502) were created. As shown in Table 2, none of these constructs show any defect in the interaction with LexA/Rap1p(679–827), LexA/Sir4p(839–1358), or LexA/Sir3p(307–978). The results of this analysis did not change when the interactions were tested in two-hybrid reporter strains carrying mutations in either *SIR2*, *SIR3*, *SIR4*, or *RIF1* (data not shown). In summary, these data demonstrate that amino acids 456 to 479 of Sir3p are necessary for the interaction with the carboxyl terminus of Rap1p in the two-hybrid system but do not play any detectable role in binding of Sir3p to itself or Sir4p.

Amino acids 455 to 481 of Sir3p are required for binding to carboxyl terminus of Rap1p in vitro. Using a GST pulldown assay, we showed previously that Sir3p can bind to the carboxyl terminus of Rap1p (47). The same approach was used to test whether Sir3p sequences required for Rap1p interaction in the two-hybrid assay are also necessary for binding in vitro (see Materials and Methods for details). Three ^{35}S-labeled Sir3p mutant proteins were analyzed, two of which (Δ456–479 and Δ440–480) contain deletions that abolish the Rap1p-Sir3p two-hybrid interaction. The third mutant (Δ440–454) has a deletion of sequences that do not appear to play a role in the Rap1p interaction, as judged by the two-hybrid assay.

As shown in Fig. 2, wild-type Sir3p and Sir3p(Δ440–454) are both able to bind to the GST/Rap1p(562–827) fusion. In contrast, Sir3p(Δ456–479) and Sir3p(Δ440–480) show a strong impairment in binding to GST/Rap1p. These data confirm the results of the two-hybrid analysis of the interaction between Rap1p and Sir3p and indicate that amino acids 456 to 479 of Sir3p are required for strong binding to the carboxyl terminus of Rap1p in vitro. Previous studies have shown that this region of Sir3p does not appear to be required for in vitro binding to the amino termini of histones H3 and H4 (23). Taken together, the results described above identify amino acids 455 to 481 of Sir3p as a Rap1p interaction domain of this protein.

Targeted silencing by G_{BD}/Rap1p hybrids requires Rap1p interaction domain of Sir3p. If the Rap1p interaction domain of Sir3p identified above plays any role in repression of transcription in vivo, its deletion should cause an impairment of silencing. In addition, mutations of Sir3p that do not affect the interaction with Rap1p, Sir4p, or Sir3p should not cause a silencing defect unless other functions required for silencing were affected. To test these ideas, we first used a targeted silencing assay (8) in which G_{BD}/Rap1p hybrids are tethered to mutated *HMR*-E silencer elements containing Gal4p binding sites (UAS_G). In this assay, the restoration of silencing is critically dependent upon a small carboxy-terminal domain of Rap1p(667–827) with which Sir3p interacts.

Two reporter strains with different mutant *HMR*-E silencers were used in these experiments. In the first strain, the *HMR*-E silencer is deleted for the ORC binding site (A) and the Rap1p-binding site (E), and the two elements are replaced by three UAS_G sites [$\Delta A\Delta E(UAS_G)_3$]. In the second strain, the

TABLE 2. Deletion of as few as 24 amino acids of Sir3p abolishes the interaction with Rap1p but not Sir3p or Sir4p in the two-hybrid system

G_{AD}/Sir3p hybrid	β-Galactosidase (U)		
	LexA/Rap1p (679–827)	LexA/Sir4p (839–1358)	LexA/Sir3p (307–978)
G_{AD}/Sir3p(307–978)	5,824	3,238	3,597
G_{AD}/Sir3p(Δ456–479)	3	4,297	1,384
G_{AD}/Sir3p(Δ440–480)	4	3,354	1,201
G_{AD}/Sir3p(Δ440–502)	4	3,331	1,704
G_{AD}/Sir3p(Δ440–454)	5,337	3,555	3,292
G_{AD}/Sir3p(Δ398–437)	5,709	2,944	3,136
G_{AD}/Sir3p(Δ358–437)	6,587	2,501	3,333
G_{AD}/Sir3p(Δ333–357)	6,445	2,845	3,378
G_{AD}/Sir3p(Δ482–502)	7,052	2,329	2,694
G_{AD}	3	5	2

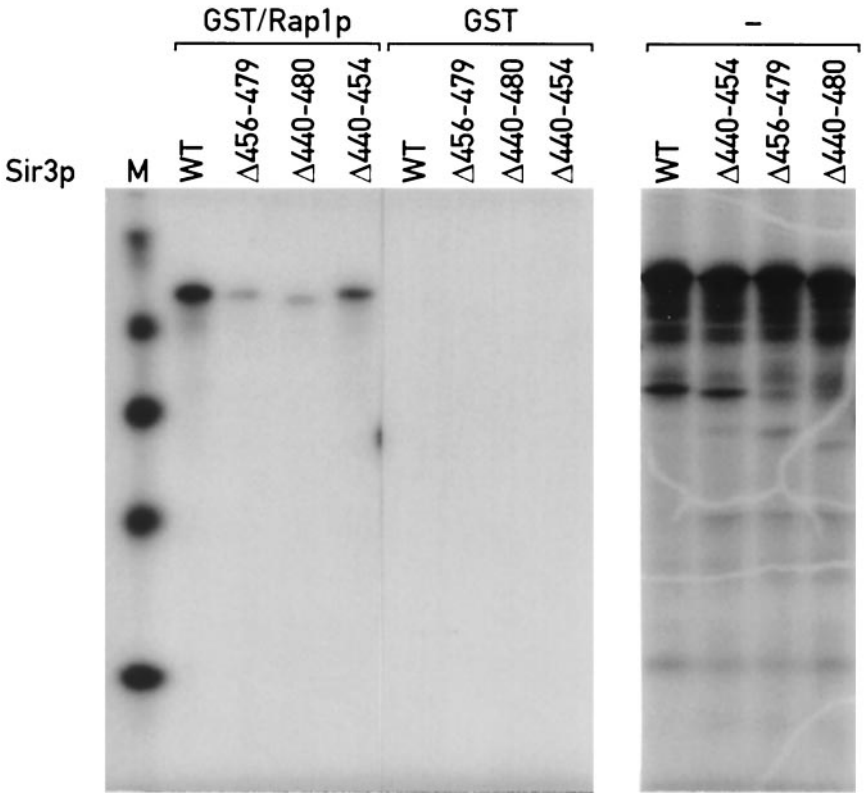

FIG. 2. Sir3p mutants with a deletion of amino acids 456 to 479 are defective in binding to GST/Rap1p(562–827) in vitro. See Materials and Methods for details of the binding assay. Right, in vitro-produced labeled wild-type (WT) and mutant proteins (from a separate gel) before addition to GST/Rap1p(562–827) or GST-agarose beads. Left, material bound to the GST/Rap1p beads in each lane [Sir3p(1–978), Sir3p (Δ456–479), Sir3p(440–480), and Sir3p(440–454)] has the same mobility as the primary high-molecular-weight translation product in the right panel (note that the order of lanes in the two gels is not the same). Lane M, size standards.

Rap1p-binding site and the Abf1p-binding site (B) are removed and replaced by the same UAS_G trimer [$\Delta E\Delta B(UAS_G)_3$]. In both strains, the *MAT***a***1* gene at *HMR* is replaced by *TRP1*, and silencing is measured by the ability of the strains to grow on medium lacking tryptophan (57). To target the Rap1p carboxyl terminus to these altered silencers, a construct expressing a G_{BD}/Rap1p (653–827) fusion protein is integrated at the *HIS3* locus. To test for the *SIR3* dependence of targeted silencing, the chromosomal copy of *SIR3* is disrupted in these strains and either the wild-type or a series of mutant *SIR3* alleles (the same mutations tested in the two-hybrid analysis introduced into the full-length *SIR3* gene) are expressed from a low-copy-number centromere-containing (*CEN*) vector.

The top panel of Fig. 3 shows results obtained with the $\Delta A\Delta E(UAS_G)_3$::*TRP1* strain. As expected, the *TRP1* gene is efficiently repressed (as judged by an approximately 100-fold decrease in the ability to form colonies on medium lacking tryptophan) in the presence of wild-type *SIR3* on a plasmid (top row) and is completely derepressed (full growth on −Trp medium) in cells transformed with the control vector alone (bottom row). Significantly, four *SIR3* alleles with a deletion of the Rap1p interaction domain (Δ456–479, Δ440–480, Δ440–502, and Δ356–502) display an almost complete loss of repression (Fig. 3, rows 2 to 5). In contrast, Sir3p deletions that did not cause a defect in the two-hybrid interaction with Rap1p (Δ440–454, Δ398–437, Δ358–437, and Δ333–357) support silencing in this assay as well as wild-type Sir3p (Fig. 3, rows 6 to 9).

The results obtained with the same set of *SIR3* alleles in the $\Delta E\Delta B(UAS_G)_3$::*TRP1* strain (bottom panel of Fig. 3) are qualitatively similar. In this strain, however, targeted silencing in the *SIR3* wild-type background appears much stronger, and the derepressing effect of the Rap1p interaction domain deletions (rows 2 to 5) is correspondingly weaker than in the $\Delta A\Delta E(UAS_G)_3$ silencer strain. Nonetheless, we again observe little or no silencing defect for the *SIR3* alleles with deletions outside of the Rap1p interaction domain (rows 6 to 10). This difference between the two silencers suggests that the ORC plays a more important role than Abf1p in recruitment of the silencing complex to the chromosome in cooperation with Rap1p (60) (see Discussion). In both strains these results were replicated using G_{BD}/Rap1p hybrids expressing either a longer or a shorter fragment of the carboxyl terminus of Rap1p (data not shown), suggesting that the different effect of the *SIR3* mutations in the two strains is not the result of a peculiar feature of the G_{BD}/Rap1p(653–827) hybrid.

The direct targeting of either Sir3p or Sir4p to the *HMR* locus in cells lacking a functional *HMR*-E silencer is also sufficient to restore repression (40). In order to determine whether the defect displayed by the *SIR3* alleles tested above was specific to Rap1p-dependent silencing, we analyzed G_{BD}-Sir3p

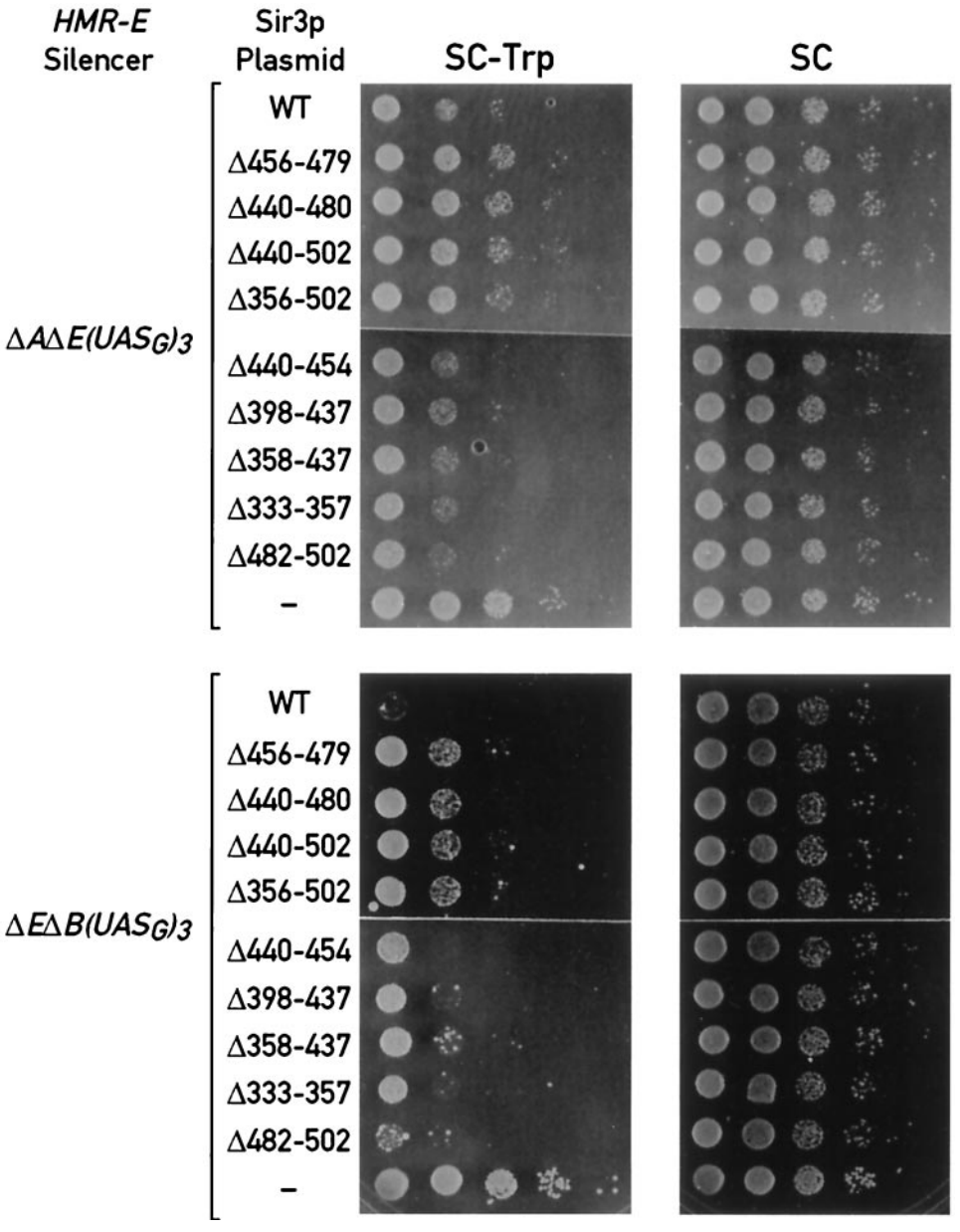

FIG. 3. Targeted silencing of an *hmr*::*TRP1* reporter by a G_{BD}/Rap1p(653–827) hybrid with different *HMR*-E silencer deletions and a series of *SIR3* alleles. Silencing is measured by comparing the ability of cells to grow in the absence (SC−Trp) and presence (SC) of tryptophan. Each row consists of spots representing 5-μl aliquots from a set of 10-fold serial dilutions of an overnight liquid culture. Photographs were taken after 2 to 3 days of growth at 30°C.

(1–978)- and G_{BD}-Sir4p(1–1358)-dependent repression in the presence of the same *SIR3* mutants tested above. None of the *SIR3* alleles displays a noticeable silencing defect with either G_{BD}/Sir3p or G_{BD}/Sir4p (data not shown). These data demonstrate that the defect caused by the *SIR3* mutations is bypassed when either Sir3p or Sir4p is targeted to the silencer by a Rap1p-independent mechanism. Therefore, these small *SIR3* deletions do not cause a general impairment of repression and are thus more likely to specifically affect a step in which Sir3p is recruited by Rap1p to the silencer.

Deletion of Rap1p interaction domain of Sir3p impairs silencing at *HMR* locus when the silencer ORC binding site is deleted. We next tested the effect of *SIR3* mutations on silencing initiated by wild-type silencers at *HMR* as well as two mutated but fully functional silencers in which the redundant ORC (A element) or Abf1p (B element) binding sites at *HMR*-E were deleted (5, 26, 57). As shown in the top and bottom panels of Fig. 4, both wild-type and mutant alleles of *SIR3* are able to restore full silencing in both *HMR*::*TRP1* and *hmr*Δ*B*::*TRP1* cells. Only control cells lacking a functional copy of *SIR3* are fully derepressed (last row of each panel in Fig. 4). In contrast, Sir3p mutants with a deletion of the Rap1p interaction domain (Δ456–479, Δ440–480, Δ440–502, and Δ356–502) display a partial silencing defect in *hmr*Δ*A*::*TRP1* cells (middle panels of Fig. 4). In these mutants, the ability to grow on medium lacking tryptophan is increased about 10^3-fold

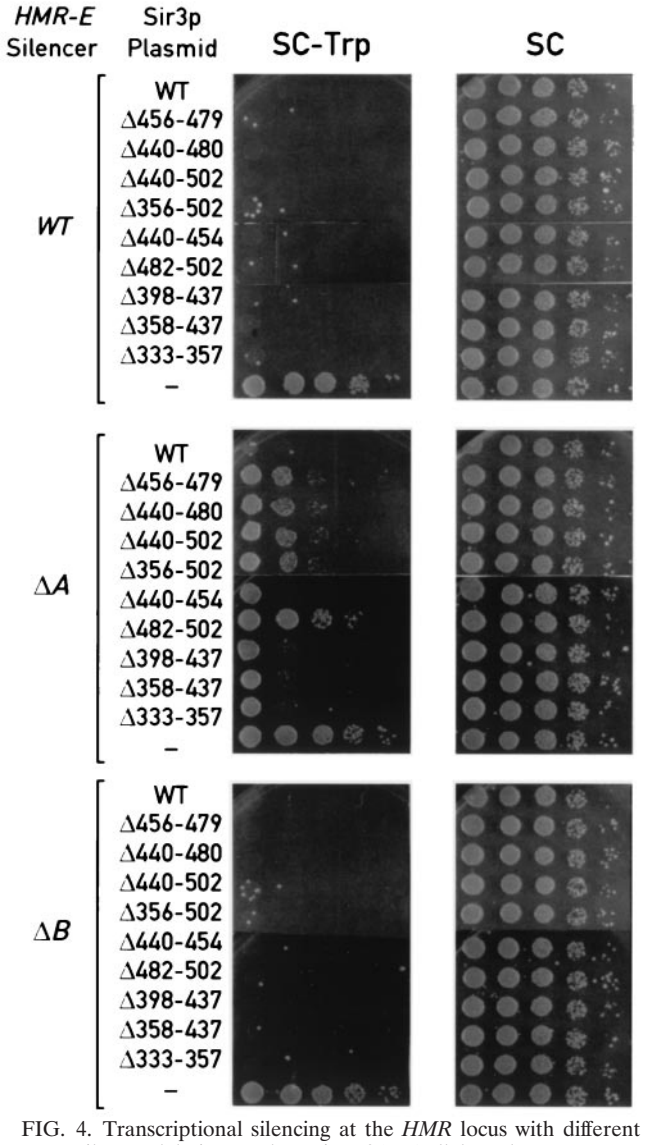

FIG. 4. Transcriptional silencing at the *HMR* locus with different *HMR*-E silencer deletions and a series of *SIR3* alleles. The assays were performed as described for Fig. 3.

compared with cells expressing wild-type Sir3p (first rows of the panels), but is reduced 10^{-2}- to 10^{-3}-fold compared with cells transformed with the *CEN* plasmid control (last rows of the panels). Significantly, deletion of Sir3p sequences amino-terminal of the Rap1 interaction domain (Δ440–454, Δ398–437, Δ358–437, and Δ333–357) does not cause any impairment of repression in *HMRΔA*::*TRP1* cells.

The fact that Sir3p mutants defective in the Rap1p interaction have a partial silencing defect in *hmrΔA*::*TRP1* cells but no impairment in *HMR*::*TRP1* or *hmrΔB*::*TRP1* cells differs from results obtained with Rap1p mutants defective in Sir3p interaction, in which there is a complete silencing defect in both *hmrΔA*::*TRP1* and *hmrΔB*::*TRP1* cells but no loss of repression in *HMR*::*TRP1* cells (47). To test the possibility that these Sir3p mutants have a weak silencing defect that our assay was not sensitive enough to detect, we tested one of them (Δ456–479) in strains in which the *ADE2* reporter is integrated at *HMR* in cells containing wild-type or mutant *HMR*-E silencer (Δ*A* and Δ*B*) (58). The *ADE2* gene has been shown to be efficiently repressed at *HMR* and allows very sensitive detection of weak silencing defects by a simple nonselective colony color assay. Although two different *SIR3* deletion-insertion mutations (removing amino acids 108 to 945 or nearly the entire open reading frame [ORF], amino acids 1 to 972) caused strong derepression of all three *ADE2* reporters, the Sir3p (Δ456–479) deletion showed a strong silencing defect only in the *hmrΔA*::*ADE2* strain (data not shown), consistent with the results obtained in the *TRP1* reporter strains. Taken together, these data suggest that loss of Sir3p binding to the carboxyl terminus of Rap1p causes a partial silencing defect at *HMR*, but only when the *HMR*-E silencer lacks a functional ORC binding site.

Deletion of Rap1p interaction domain of Sir3p impairs telomeric silencing. At telomeres, multiple Rap1p binding sites are found within the terminal TG_{1-3} repeats (16, 33), where the Rap1 protein is involved in regulation of both telomere structure and telomeric silencing (8, 27, 28, 32, 41, 47). We showed previously that mutations of the Rap1p carboxyl terminus that impair Sir3p binding also impair telomeric repression (47). If the Sir3p mutants described above were unable to bind to native Rap1p in vivo, one would predict that they should cause a telomeric silencing defect.

We tested this idea using a standard telomeric silencing assay in which the *URA3* gene is placed immediately adjacent to a telomere created at the *ADH4* locus (20). In these cells, the telomeric *URA3* reporter gene is subject to a variegated form of silencing that results in the repression of the gene in ≈50% of the cells in a culture, which can be quantified by measuring the ability of the cells to grow in the presence of 5-FOA, which kills cells expressing *URA3*. We created a *sir3*Δ derivative of this strain and transformed the cells with plasmids carrying wild-type *SIR3*, two alleles encoding a deletion of the Rap1p interaction domain [Sir3p(Δ456–479) and Sir3p(Δ440–480)], and one allele with a mutation that does not cause an impairment of Rap1p interaction [Sir3p(Δ333–357)].

As shown in Table 3, growth in medium containing 5-FOA is impaired in cells expressing Sir3p(Δ456–479) and Sir3p(Δ440–480) compared with cells expressing wild-type Sir3p. About 45% of the cells expressing wild-type Sir3p are able to grow in the presence of 5-FOA. On the other hand, only 27 and 26% of the total cell population are able to grow in the same medium for cells expressing Sir3p(Δ456–479) and Sir3p(Δ440–480), respectively, resulting in a decrease of about 40 to 43% compared with the wild type. In contrast, the control mutant Sir3p(Δ333–357) gives wild-type levels of growth in 5-FOA of about 50%, demonstrating the specificity of the deletion of the Rap1p interaction domain in telomeric repression. No significant differences were measured between wild-type and mutant alleles of *SIR3* in growth in the absence of uracil.

These data indicate that, as was the case for native silencing at *HMR*, loss of a single direct contact between Rap1p and Sir3p causes a relatively weak impairment of telomeric silencing compared to the effect of deleting the carboxy-terminal Sir3p-interacting domain of Rap1p (28, 47). One possible explanation for these results is that the Sir3p mutations that we created cause only a partial loss of Rap1p-Sir3p binding in vivo and thus do not significantly compromise the ability of Rap1p

TABLE 3. *SIR3* alleles with a deletion of the Rap1p interaction domain have a defect in telomeric silencing[a]

Sir3p construct	Growth (%) on medium:	
	Containing 5-FOA	Lacking uracil
Wild type	45.3	68.5
Sir3p(Δ456–479)	27.2	69.9
Sir3p(Δ440–480)	25.6	66.0
Sir3p(Δ333–357)	49.9	75.6

[a] A Δ*sir3* reporter strain containing the *URA3* gene immediately adjacent to a telomere created at the *ADH4* locus on chromosome VII-L was used in these experiments. The percentage of 5-FOA^r and Ura$^+$ cells was calculated by averaging the values obtained from three or more independent *SIR3* transformants. These values reflect the number of cells carrying the plasmid-borne copy of *SIR3* that are able to grow on the selective plates.

to recruit Sir3p to the chromosome. An alternative, though not mutually exclusive, possibility is that the carboxyl terminus of Rap1p possesses additional mechanisms to recruit a functional Sir complex. For example, Orc1p has been shown to play an important role in transcriptional silencing (3, 14, 44) and has extensive regions of similarity with Sir3p over the full length of the protein (4). We thus used the two-hybrid system to ask whether the Rap1p carboxyl terminus might also be able to interact with Orc1p, which might in turn recruit the Sir2/3/4 complex through its ability to interact with Sir1p (60). However, we found that a G_{AD}/Orc1p(5–914) hybrid, capable of interacting with LexA/Sir1p, failed to interact with either LexA/Rap1p(635–827) or LexA/Rap1p(679–827) (data not shown). This result suggests that Orc1p may not interact with the carboxyl terminus of Rap1p and may be unable to substitute for Sir3p in Sir complex recruitment. Another possible explanation of the relatively weak silencing effect caused by a Rap1p/Sir3p interaction defect is that Rap1p interacts directly with Sir4p, which can itself associate with Sir3p. This possibility is addressed below.

Sir4p binds to Rap1p in vitro and interacts with Rap1p carboxyl terminus in vivo in the absence of endogenous Sir or Rif1 proteins. We had previously shown that the carboxyl terminus of Sir4p can interact with Rap1p in a two-hybrid assay, but were unable to determine whether or not this interaction is direct (47). To address this question, we first used a GST pulldown assay. The same GST/Rap1p(562–827) fusion and binding conditions used in the analysis of the Rap1p-Sir3p interaction were used with two different ^{35}S-labeled fragments of Sir4p [Sir4p(737–1358) and Sir4p(737–840)]. As shown in Fig. 5, the Sir4p(737–1358) fragment can interact with GST/Rap1p(562–827), whereas the shorter Sir4p(737–840) fragment cannot bind specifically to Rap1p even when larger amounts of the ^{35}S-labeled protein are used. These data indicate that functionally important parts of Sir4p and Rap1p can interact directly in vitro or at least without the assistance of other yeast proteins.

To determine whether Rap1p and Sir4p might associate directly in vivo, we extended our previous two-hybrid analysis in which an interaction between G_{AD}/Sir4p(1205–1358) and LexA/Rap1p(635–827) but not shorter Rap1p fusions (amino-terminal endpoints at positions 647, 653, 655, 667, 679, and 691) was detected (47). Reasoning that a G_{AD}/Sir4p construct expressing a larger fragment of the carboxyl terminus of Sir4p might be able to interact more strongly with Rap1p, we constructed and tested a G_{AD}/Sir4p(839–1358) fusion with the same series of LexA/Rap1p constructs. As shown in Fig. 6 (first and third columns), LexA/Rap1p fusions with amino-terminal endpoints between positions 647 and 679 can weakly but specifically interact with G_{AD}/Sir4p(839–1358). These five LexA/Rap1p fusions are also able to interact with G_{AD}/Sir3p(307–978) and G_{AD}/Rif1p(1614–1916) in two-hybrid assays (47). In contrast, LexA/Rap1p(691–827) does not show a significant difference in β-galactosidase signal with G_{AD}/Sir4p(839–1358) compared to the G_{AD}-alone control. This last LexA/Rap1p fusion is unable to interact with either G_{AD}/Sir3p or G_{AD}/Rif1p but expresses a protein of the expected size and in amounts comparable to those produced by the other LexA/Rap1p constructs (data not shown). As a control for the Rap1p-Sir4p interaction, we tested G_{AD}/Sir4p(839–1358) with a LexA/Adh1p fusion. The last two rows of Fig. 6 show that G_{AD}/Sir4p(839–1358) does not interact significantly above background with either LexA/Lamin or LexA/Adh1p, suggesting that the Rap1p-Sir4p interaction is specific.

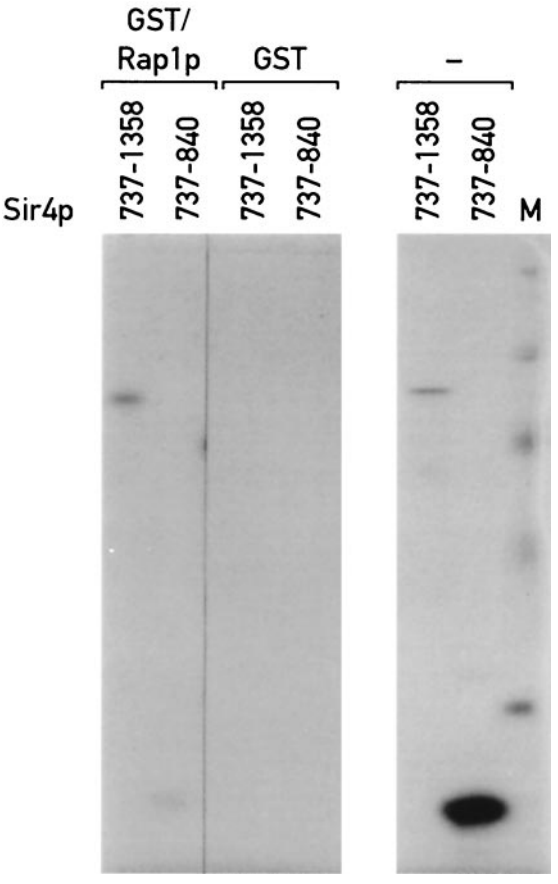

FIG. 5. Sir4p carboxyl terminus binds to GST/Rap1p(562–827) in vitro. See Materials and Methods for details of the binding assay. Right, in vitro-produced labeled carboxy-terminal fragments of Sir4p (839–1358) and Sir4p(839–1275) (from a separate gel) before addition to GST/Rap1p(562–827) or GST-agarose beads. Left, material bound to the GST/Rap1p beads in the Sir4p(839–1358) lane has the same mobility as the higher-molecular-weight translation product in the right panel. Lane M, size standards.

Based on the results shown above, the possibility remains that the in vivo Sir4p-Rap1p interaction is indirect, mediated,

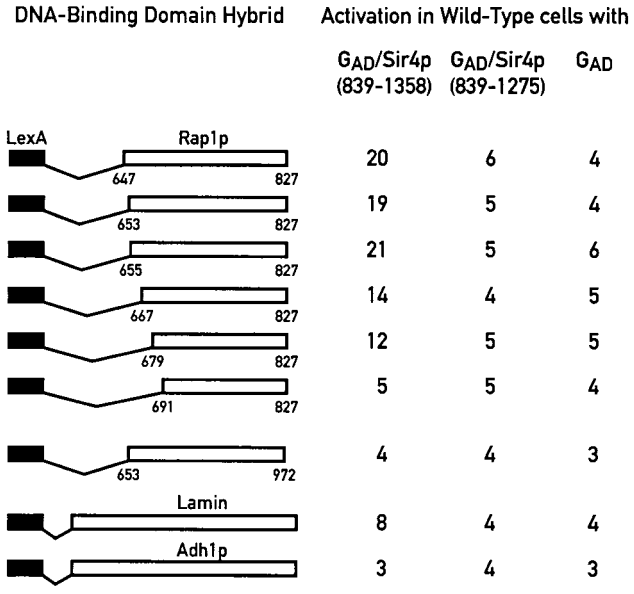

FIG. 6. Interaction of G_{AD}/Sir4p(839–1358), G_{AD}/Sir4p(839–1275), and G_{AD} with a series of LexA/Rap1p hybrids using the two-hybrid system. LexA/Rap1p hybrids with different amino-terminal Rap1p fusion endpoints and either a wild-type Rap1p carboxyl terminus (647–827, 653–827, 655–827, 667–827, 679–827, and 691–827) or a linker insertion mutation at amino acid position 825 (653–825*) were assayed as before. The LexA/Lamin and LexA/Adh1p hybrids were included as negative controls.

for example, by an interaction between these two proteins and Sir3p (47) or another silencing protein(s). To determine whether the Rap1p-Sir4p two-hybrid interaction requires the function of the endogenous Sir proteins or Rif1p, we tested all of the interactions reported above in strains containing mutations in *SIR1*, *SIR2*, *SIR3*, *SIR4*, or *RIF1*. None of the interactions are abolished in the mutant strains (data not shown), supporting the idea that Rap1p and Sir4p interact directly in vivo and that this interaction does not require a functional Sir2/3/4 complex.

Silencing-defective mutations in Rap1p or Sir4p abolish the two-hybrid interaction between the two proteins. Truncation of Sir4p at position 1237 in the *sir4-42* mutant causes a silencing defect at *HM* loci and telomeres (25). To determine whether this phenotype might result from an inability of the truncated protein to interact with Rap1p, we created a G_{AD}/Sir4p(839–1275) fusion and tested it for the ability to interact with the carboxyl terminus of Rap1p. As shown in the middle column of Fig. 6, deletion of the last 83 amino acids of Sir4p completely abolishes the two-hybrid interaction with all LexA/Rap1p fusions. This result demonstrates that the interaction of G_{AD}/Sir4p with LexA/Rap1p is dependent on Sir4p sequences of the G_{AD} hybrid and suggests that a short carboxy-terminal domain of Sir4p required for silencing may also be necessary for its physical association with Rap1p in vivo.

To ask whether the converse is also true, we analyzed a silencing-defective Rap1p mutation for its effect on the two-hybrid interaction with Sir4p. As shown in Fig. 6, the incorporation of a small linker insertion mutation at position 825 of Rap1p [Rap1p(825*)], which results in the addition of five amino acids at the carboxyl terminus of the protein, completely abolishes the ability of the LexA/Rap1p(653–825*) fusion to interact with G_{AD}/Sir4p(839–1358). Since LexA/Rap1p(653–825*) is much less severely impaired in its interactions with both Sir3p and Rif1p compared to the wild type (1.6- and 3.8-fold decreases, respectively [47]), the complete absence of an interaction with G_{AD}/Sir4p(839–1358) is unlikely to be due to decreased expression or stability of the LexA/Rap1p fusion protein. Taken together, these data are consistent with the idea that a direct Rap1p-Sir4p interaction plays an important role in silencing.

Increased gene dosage of *SIR4* but not *SIR1* improves silencing in *sir3* mutants. The gene dosage of both *SIR1* and *SIR4* can have profound effects on *HMR* silencing. For example, elevated gene dosage of *SIR1* can suppress various silencing mutations (29, 55, 57), whereas increased *SIR4* dosage can improve or disrupt silencing depending on the genetic background and the number of added copies of the gene (39, 42, 50, 57, 58). One possible mechanism for an improvement in silencing with increased *SIR1* or *SIR4* dosage is increased availability of these proteins at the silencers, which might act to strengthen the formation of a complex that nucleates heterochromatin assembly.

We reasoned that the decreased silencing seen at *hmrΔA*::*TRP1* in *SIR3* mutants defective in Rap1p binding might be explained by the combined loss of two independent interactions, one between the Sir2/3/4p complex and Rap1p and the other between the Sir2/3/4 complex and Sir1p/ORC (60). The loss of the first interaction would be the result of the *SIR3* mutation, whereas the loss of the second would be explained by the deletion of the ORC binding site (the silencer A element). We therefore asked whether increased *SIR1* or *SIR4* dosage might improve silencing under these mutant conditions. As shown in Fig. 7, one or two extra copies of *SIR4* can strongly suppress the silencing defect exhibited by *SIR3* mutants that are defective in binding to Rap1p. The partial derepression of *hmrΔA*::*TRP1* in the presence of Sir3p(Δ456–479) or Sir3p (Δ440–480) is completely reversed by the addition of one extra copy of *SIR4* (Fig. 7, rows 2 and 3 of top panels). In contrast, much higher gene dosage of *SIR1* (present on a 2μm plasmid) has no effect under these conditions (rows 2 and 3 of bottom panels). These results are consistent with the hypothesis that Sir4p interacts with the carboxyl terminus of Rap1p and contributes to the recruitment of the Sir complex to the *HMR*-E silencer. In addition, the data suggest that Sir1p may act at the *HMR*-E silencer only when a strong ORC binding site is present (10, 60).

DISCUSSION

Specific Rap1p interaction domain of Sir3p. We have identified a minimal Rap1p interaction domain of Sir3p between amino acids 455 and 481 that, by several criteria, appears to play an important and specific role in the initiation of silencing. First, this short region is sufficient to mediate a two-hybrid interaction with the carboxyl terminus of Rap1p yet is incapable of interacting with either Sir3p or Sir4p. Second, deletion of amino acids 456 to 479 in Sir3p completely abolishes its association with Rap1p in the two-hybrid assay but does not affect its Sir3p or Sir4p interactions. Third, deletion of the 456 to 479 region impairs Sir3p binding to the carboxyl terminus of

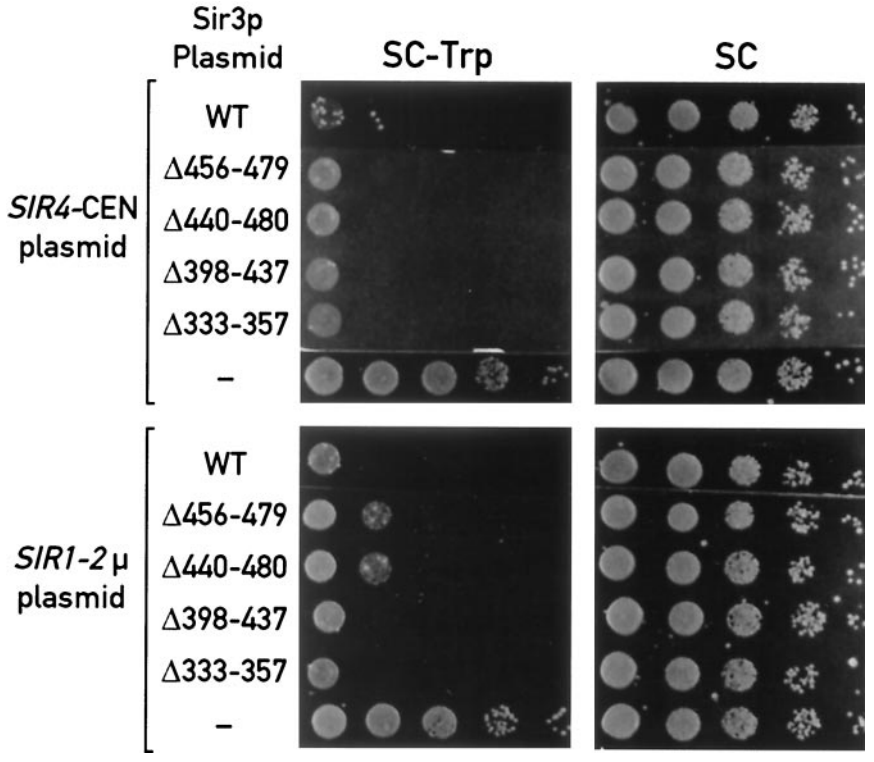

FIG. 7. Extra dosage of *SIR4* suppresses the silencing defect caused by mutation of the ARS consensus sequence at the *HMR*-E silencer in combination with the deletion of the Rap1p interaction domain of Sir3p. Cells expressing different *SIR3* alleles were transformed with a low-copy-number *CEN* plasmid containing the *SIR4* gene (top panels) or a high-copy-number 2μm plasmid containing *SIR1* (bottom panels). The assays were performed as described for Fig. 3.

Rap1p in vitro. Fourth, deletion of this small region of Sir3p causes a complete loss of silencing at *HMR* when the initiation of repression is dependent on targeting of the carboxyl terminus of Rap1p, but not when it is initiated by either Sir3p or Sir4p targeting.

It is worth noting that the Rap1p interaction domain of Sir3p identified here maps well upstream on the linear protein sequence of regions required for self-association, Sir4p binding, or histone binding. A Sir3p interaction with the amino-terminal tails of histones H4 and H3 requires regions between amino acids 623 and 762 and 799 and 910 of Sir3p and is unaffected by deletion of the Rap1p interaction domain defined here (23). Similarly, we have also mapped Sir3p sequences required for self-association and Sir4p binding to two separate regions, both of which are carboxy-terminal to the Rap1p interaction domain (unpublished results), and within a broad region defined by Park and colleagues (49). The picture that emerges from these studies is that Sir3p may be able to interact simultaneously with several different proteins (Rap1p, Sir4p, and histones H3 and H4). The possible significance of this observation is discussed below.

Rap1p carboxy-terminal silencing domain can interact directly and independently with both Sir3p and Sir4p. Previous studies showed that Rap1p and Sir4p coimmunoprecipitate from yeast extracts (11, 24, 56) and interact in the two-hybrid system (47). However, none of these studies were able to address the question of whether Rap1p and Sir4p interact directly and, if so, whether their association is important for silencing. Here we present the first demonstration that Rap1p and Sir4p can bind to each other directly in vitro in the absence of other yeast proteins and that their in vivo interaction in a two-hybrid assay is independent of *SIR* function. These findings suggest that the Rap1p-Sir4p association seen in large chromatin complexes is due, at least in part, to direct interactions that contribute to the stability of these complexes. As discussed in detail below, genetic data reported here indicate that direct binding between Rap1p and Sir4p is important in vivo for silencing.

On the basis of the results presented here, we propose that Sir3p and Sir4p interact directly and independently with partially overlapping regions of the carboxyl terminus of Rap1p. Although both Sir interactions require an intact Rap1p carboxyl terminus, the amino-terminal boundaries of the Rap1p sequences required for Sir3p and Sir4p association differ. A relatively small carboxy-terminal fragment (amino acids 679 to 827) interacts strongly with Sir3p but only weakly with Sir4p, which requires sequences upstream of position 679 for stronger Rap1p binding. This increase is specific to Sir4p and thus unlikely to be a trivial consequence of increased protein stability, because these larger Rap1p hybrids actually interact less well with Sir3p (47). The importance of Rap1p sequences upstream of 679 for silencing is underscored by the observation that G_{BD}/Rap1p hybrids containing these additional sequences initiate repression much more efficiently than does G_{BD}/Rap1 (679–827) (8). It is worth pointing out that more amino-terminal sequences of Rap1p might also contribute to Sir protein

binding, particularly for the case of Sir4p. However, LexA/Rap1p hybrids containing such sequences, which include the Rap1p DNA-binding domain, do not work in the two-hybrid system.

Given the above considerations, we favor the idea that Sir3p and Sir4p simultaneously contact the Rap1p carboxyl terminus in vivo. Although our experiments do not address this question directly, the fact that Sir3p and Sir4p can interact with each other (47) through regions not required for their interactions with Rap1p (our unpublished data) suggests that these two Sir proteins form a unique ternary complex with Rap1p that is stabilized by interactions among all three proteins. An alternative possibility that we cannot rule out at present is that Rap1p-Sir3p and Rap1p-Sir4p interactions are mutually exclusive, so that individual Rap1p molecules interact with one or the other Sir protein in forming a Rap1/Sir complex.

Cooperativity in Sir complex recruitment by Rap1p. The apparent ability of Rap1p to interact independently with both Sir3p and Sir4p and the ability of these two proteins to interact with themselves and each other (9, 47) suggest a cooperative and redundant mechanism for recruitment of the Sir complex by Rap1p. This notion is strongly supported by the genetic studies described here. Thus, although a small region of Sir3p (455–481) is required (by both in vitro and in vivo criteria) for an interaction with Rap1p, *SIR3* mutants lacking this specific domain display only a weak silencing defect even when the silencer being tested is totally dependent on Rap1p. Significantly, however, when such mutants are tested in a targeted silencing system (where Sir4p recruitment by G_{BD}/Rap1p may be poor), they are severely silencing defective. The simplest interpretation of these results is that the Rap1p-Sir3p interaction that we have characterized is important but not essential for Rap1p's action at either *HMR* or a telomere. The same appears to hold for the Rap1p-Sir4p interaction, which is specifically abolished by a linker insertion mutation very near the carboxyl terminus of Rap1p with relatively little effect on TPE (47). In contrast, the loss of a Rap1p interaction with both Sir3p and Sir4p (due to Rap1p truncations at amino acids positions 716, 703, and 695) causes a complete TPE defect (28, 47).

The idea that Sir3p and Sir4p cooperate in recruitment to silencers and telomeres by Rap1p is further supported by the observation that increased *SIR4* gene dosage significantly improves silencing in cells carrying a deletion of the Rap1p-interacting domain of Sir3p. Finally, the idea that Sir3p can interact independently with Rap1p in vivo is strongly supported by coimmunoprecipitation studies using an antigen-tagged version of Sir3p (24), which showed that mutation of *SIR4* reduces but does not abolish Rap1p binding to Sir3p.

In addition to the Rap1p-Sir protein interactions described here, a large number of other protein-protein interactions have been implicated in either the establishment or maintenance of silencing at either *HM* mating type loci or telomeres (e.g., ORC-Sir1p, Yku70p-Sir4p, Sir1p-Sir4p, Sir3p-Sir3p, Sir4p-Sir4p, Sir4p-Sir2p, Sir3p-H3, Sir3p-H4, Sir4p-H3, and Sir4p-H4) (9, 11, 24, 31, 45–47, 60). Additional protein-protein interactions (e.g., Rap1-Rif1p, Rap1p-Rif2p, and Sif2p-Sir4p) appear to downregulate silencing at telomeres (12, 22, 28, 61). At present it is unclear why the recruitment and assembly of Sir proteins at *HM* loci and telomeres involve such a complex network of interactions. One possibility is that this complexity is necessitated by the tight regulation of Sir2/3/4-mediated silencing, which is particularly stable at *HM* mating type loci, less so at telomeres, and excluded from most other chromosomal sites (62). This "hierarchy" of silencing (1) has been linked to the different complexity of the silencers themselves, and the present work lends further support to this idea. For example, we found that mutation of the Rap1p interaction domain of Sir3p has a more severe effect at an *HMR*-E silencer lacking the ORC binding site (A element) than at a silencer lacking the Abf1 site (B element). These data indicate that the different silencer elements make independent and quantitatively different contributions to the strength of the silencer. This conclusion is supported by evidence that the silencer A element recruits Sir1p and, indirectly, Sir4p through the ORC (60), whereas Abf1p appears to act by recruiting Sir3p (P. Moretti and D. Shore, unpublished data).

Structural role for Rap1p in silent chromatin? Initial molecular models suggested that silencer- or telomere-binding proteins (e.g., Rap1p, ORC, Abf1p, and Yku70/80) initiate silencing by recruiting a Sir2/3/4 complex to the chromosome, but do not participate directly in the subsequent "spreading" of this complex along adjacent nucleosomal DNA (21, 35, 36). However, several studies using chromatin immunoprecipitation (ChIP) have now shown that Rap1p and Yku80p are bound at distances of 2 to 4 kb from a telomere end, together with the Sir2/3/4 proteins (13, 24, 43, 56). These results are surprising in light of the fact that the $TG_{1–3}$ repeat tracts (which constitute the telomeric Rap1p DNA-binding sites) extend only about 300 to 400 bp from the chromosome end and that Yku protein is generally thought to interact with the end of the $TG_{1–3}$ repeat tract, based on its in vitro preference for DNA ends or duplex/single-strand junctions. The ChIP results have thus been interpreted to mean that the telomere repeat tract folds back on more internal nucleosomal regions through Sir-Sir interactions between these two domains (13, 17, 56), thus associating repeat tract-bound Rap1p indirectly with distal heterochromatin.

Our results suggest an additional explanation for this finding. Specifically, we propose that Rap1p might contribute directly to the stability of silent chromatin through simultaneous interactions with the Sir2/3/4 complex and (nonspecific) DNA sites. According to this model, Rap1p spreads together with the Sir2/3/4 complex by virtue of its ability to bind cooperatively to Sir3p and Sir4p and to nonspecific DNA sites. The finding that Sir3p may be able to simultaneously contact both Rap1p and histone tails raises the intriguing possibility that the Sir complex can promote the stable coassociation of Rap1p and nucleosomes on silent regions. This model might help to better explain two puzzling observations regarding telomeric heterochromatin. First, coimmunoprecipitation experiments with antibodies against Sir3p show that interactions with Rap1p and histone H4 are surprisingly interdependent, so that Sir3p-Rap1p binding appears to be lost in strains containing H4 amino-terminal tail mutations (23). These data are difficult to reconcile with a simple version of the telomere "fold-back" model but are consistent with the proposal that the bulk of Rap1p-Sir3p interactions at telomeres actually occurs within the silent chromatin itself through cooperative interactions between histones and Sir3/4 proteins on the one hand and

DNA-bound Rap1p and Sir3/4 on the other. Second, indirect immunofluorescence experiments have revealed a significant loss of punctate Rap1p staining in strains carrying either *SIR3* or *SIR4* mutations (48), which cannot be explained by a loss of telomere clustering (18). This result would be predicted if one assumes again that a large pool of telomere-associated Rap1p is not bound directly to the TG_{1-3} repeat tracts but is instead held there by nonspecific binding and cooperative interactions with the Sir complex (17). It may prove difficult, however, to distinguish experimentally between different models to explain the spreading of Rap1p in telomeric heterochromatin. Furthermore, none of these models are mutually exclusive. Perhaps a good test of the idea that Rap1p is a structural component of the silent chromatin would be to determine if Rap1p also spreads together with the Sir complex at *HM* silent mating type loci, where a telomere fold-back model would not apply.

While our manuscript was under revision, a whole-genome analysis of both Rap1p and Sir protein binding in vivo was published (30). Data presented in that paper indicate considerable spreading of Rap1p (together with Sir2p, Sir3p, and Sir4p in most cases) to sequences outside of those bounded by the E and I silencers at both *HML* and *HMR*. As pointed out above, this observation is consistent with our model but difficult to accommodate in the context of a fold-back-type model for Rap1p spreading outside of its high-affinity binding sites.

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