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Tethered Sir3p Nucleates Silencing at Telomeres and Internal Loci in *Saccharomyces cerevisiae*

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Rap1p binds to sites embedded within the *Saccharomyces cerevisiae* telomeric $TG_{1–3}$ tract. Previous studies have led to the hypothesis that Rap1p may recruit Sir3p and Sir3p-associating factors to the telomere. To test this, we tethered Sir3p adjacent to the telomere via LexA binding sites in the *rap1-17* mutant that truncates the Rap1p C-terminal 165 amino acids thought to contain sites for Sir3p association. Tethering of LexA-Sir3p adjacent to the telomere is sufficient to restore telomeric silencing, indicating that Sir3p can nucleate silencing at the telomere. Tethering of LexA-Sir3p or the LexA-Sir3p^{N205} gain-of-function protein to a telomeric LexA site hyperrepresses an adjacent *ADE2* gene in wild-type cells. Hence, Sir3p recruitment to the telomere is limiting in telomeric silencing. In addition, LexA-Sir3p^{N205} hyperrepresses telomeric silencing when tethered to a subtelomeric site 3.6 kb from the telomeric tract. This hyperrepression is dependent on the C terminus of Rap1p, suggesting that subtelomeric LexA-Sir3p^{N205} can interact with Rap1p-associated factors at the telomere. We also demonstrate that LexA-Sir3p or LexA-Sir3p^{N205} tethered in *cis* with a short tract of telomeric $TG_{1–3}$ sequences is sufficient to confer silencing at an internal chromosomal position. Internal silencing is enhanced in *rap1-17* strains. We propose that sequestration of silencing factors at the telomere limits the efficiency of internal silencing.

In the yeast *Saccharomyces cerevisiae*, silencing at both telomeres and the silent mating-type loci, *HML* and *HMR*, is initiated and propagated from a discrete set of DNA sequence elements and is mediated by a diverse set of multifunctional and silencing-specific proteins (see references 15, 27, and 40 for reviews). Silent regions are associated with four characteristic phenotypes. First, genes residing within these domains are functionally inaccessible to the basal transcription machinery, resulting in repression under noninducing conditions (2, 18, 27). Second, both *HM* and telomeric loci are present in a closed chromatin state, refractory to both in vivo and in vitro probes (17, 26, 34, 42). Third, origins of DNA replication in telomere-proximal regions are initiated late in S phase (14). *HML* sequences are also replicated late in S phase (38). Fourth, at least some telomeres cluster near the nuclear periphery, suggesting a functional link between perinuclear components and the formation of silent chromatin (35). Telomeric silencing may be a general phenomenon, since variations of this process have been observed in *Schizosaccharomyces pombe* (1), trypanosomes (20a, 39), and *Drosophila melanogaster* (27a, 48). Interestingly, many of these properties are similar to the late-replicating and transcriptionally quiescent heterochromatin identified in other eukaryotes.

Telomeric and *HM* silencing share many *cis*- and *trans*-acting requirements. Mutations in *SIR2*, *SIR3*, *SIR4*, *NAT1*, *ARD1*, and the N termini of histones H3 and H4 reduce or abolish silencing at these loci (1a, 27, 46). Nonetheless, telomeres seem to exhibit a more rudimentary form of silencing than the *HM* loci. This is most apparent when comparing the structure of silencer elements at *HML*, *HMR*, and telomeres. The *HMR-E* silencer contains sites for the binding of three activities: repressor/activator protein 1 (Rap1p); origin recognition complex (ORC), which binds to autonomously replicating sequence elements (3); and autonomously replicating sequence binding factor 1 (Abf1p) (6). ORC and Rap1p binding sites are also present in the *HML-E* silencer. Both *HML* and *HMR* silencers display redundancy in that loss of one binding site is insufficient to eliminate silencing (5, 6, 27). In contrast, the $TG_{1–3}$ tracts of yeast telomeres contain multiple high-affinity binding sites for Rap1p (16, 30) but lack both ORC and Abf1p recognition sequences.

Sir1p plays a key role in the establishment of silencing at the *HM* loci (36). In *sir1* cells, *HML* expression is metastable, with cells switching between transcriptionally repressed and derepressed epigenetic states. Interestingly, this behavior is similar to the *SIR1*-independent transcriptional instability of genes placed adjacent to telomeric $TG_{1–3}$ tracts (1a, 18). The metastable characteristics of telomeric silencing may be due to an inability to recruit Sir1p to telomeres. Indeed, tethering Sir1p to a telomere increases the efficiency of telomeric silencing (10).

The essential sequence-specific DNA-binding protein Rap1p acts at both the *HM* loci and telomeres. Rap1p recognizes a well-defined DNA sequence with high affinity and selectivity, inducing local bending and distortion of the DNA helix (16, 47). Rap1p binds the *HML-E* and *HMR-E* silencers, where it participates in repression of the mating-type genes. This effect is context dependent since Rap1p can also act as an activator at numerous upstream activation sequences (15, 40). At the telomere, Rap1p appears to regulate several functions, including telomere tract size; telomere silencing; and the perinuclear clustering of Sir3p, Sir4p, and Rap1p-telomeric complexes (11, 12, 24–26, 29, 31, 45, 49).

The carboxyl-terminal 160 amino acids of Rap1p are both

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necessary and sufficient for *HMR* silencing in a modified nonredundant silencer and are required for telomeric silencing (Fig. 1) (8, 19, 26). This region encompasses two mutationally defined regions, the *rap1*^{s} and C-terminal tail domains, important for telomere silencing and telomere size control (29, 45).

Several genetic studies have suggested that Rap1p mediates its silencing function(s) through interaction with Sir3p and Sir4p. First, null alleles of *SIR3* and *SIR4* lack the perinuclear clustering of Rap1p-telomeric complexes (35). Conversely, loss of the Rap1p C-terminal tail domain eliminates Sir3p peripheral localization (11). Second, several gain-of-function alleles of *SIR3* (*SIR3*$^{L\text{-}31}$ and *SIR3*^{N205}) and Sir3p overexpression restore telomeric silencing to *rap1* silencing-defective alleles with partial *rap1* allele specificity (28, 29). This genetic evidence for Rap1p-Sir3p interaction has been corroborated by two-hybrid, biochemical, and immunological studies, which demonstrate the capability of both Sir3p and Sir4p to associate with Rap1p (11, 33).

Several findings implicate Sir3p as a major component in the formation of silent chromatin. First, Sir3p overexpression increases spreading of telomeric silencing into the chromosome (37). Second, *SIR3* suppressors that restore the loss of *HML* silencing conferred by a mutation within the histone H4 N terminus have been identified (21, 22). Indeed, Sir3p can physically interact with the N termini of both histones H3 and H4 in vitro (20). Third, while subtelomeric regions of wild-type cells are inaccessible to exogenously introduced *dam* methylase, *sir3* mutations obliterate this protection (17). Fourth, nucleosomes both at the silent mating-type loci and adjacent to telomeres are hypoacetylated in wild-type cells but not in *sir2* and *sir3* mutant cells (7). Fifth, Sir3p can self-associate, as well as interact with both Sir4p and Rap1p (33). Therefore, transcriptional repression may result in the assembly of Rap1p-Sir3p-Sir4p complexes.

These genetic and biochemical data have led to the hypothesis that the primary role of Rap1p in telomeric silencing is to recruit Sir3p and Sir3p-associating factors to the telomere. To test this hypothesis and to further explore the mechanisms underlying the role of Sir3p in telomeric silencing, we have analyzed the function of Sir3p and the Sir3p^{N205} gain-of-function protein when tethered to telomeric, subtelomeric, and internal loci. We demonstrate that tethering LexA-Sir3p to a telomeric LexA site suppresses the effects of *rap1* C-terminal truncation alleles and facilitates telomeric silencing in wild-type cells. These data argue that a major role of Rap1p in telomeric silencing is to recruit Sir3p to the telomere and that Sir3p recruitment is limiting for silencing even in wild-type *RAP1* cells. We also demonstrate that Sir3p^{N205} tethered to a subtelomeric site facilitates telomeric silencing in wild-type *RAP1* strains, suggesting that elements of the silencing machinery can act at a distance. Remarkably, in a strain lacking the C-terminal 165 amino acids of Rap1p, silencing can be initiated from the subtelomerically tethered site, rather than from the telomere. These data support a model in which subtelomeric and telomeric regions can functionally interact in silencing. We further define a minimal silencer in which Sir3p tethered to an internal site, in conjunction with Rap1p binding, is sufficient to confer silencing.

MATERIALS AND METHODS

Plasmid construction. The LexA-Sir3p and LexA-Sir3p^{N205} fusions were constructed by cloning the *Sal*I-*Pst*I fragment of either *SIR3* from pAR16/*SIR3* or *SIR3*^{N205} from pAR16/*SIR3*^{N205} (7, 28), which both contain an artificial *Sal*I site 7 bp in front of the *SIR3* start codon, into pBTM116 (a gift of P. Bartel and S. Fields). A *Pst*I fragment containing the downstream sequence of *SIR3* was then cloned into the unique *Pst*I site to generate pBTM116/*SIR3* and pBTM116/*SIR3*^{N205} plasmids. This construction places Sir3p and Sir3p^{N205} in frame with the LexA protein.

pVIIL-*URA3*-LexAS2-TEL and pVIIL-*URA3*-LexAS3-TEL were constructed by cloning a 42-bp oligonucleotide (13), which contains two binding sites for LexA, into the unique *Bam*HI site of pVIIL-*URA3*-TEL (18). Sequencing of the resulting plasmids, and all other plasmids bearing the LexA-binding sites, revealed that plasmids with apparently multiple 42-bp oligonucleotides, as judged by Southern hybridization, contained only one to three unrearranged sites for a LexA dimer, indicating a strong selection against multiple LexA binding sites on the plasmid in *Escherichia coli*. S2 and S3 refer to the presence of two and three intact LexA binding sites, respectively. The number of intact sites was determined on the basis of the consensus sequence for LexA binding (13) and by the ability of LexAS1-1, which deviates from the consensus at 2 of 12 residues, to function efficiently in vivo.

pVIIL-*URA3*-*ADE2*-LexAS1-1-TEL, pVIIL-*URA3*-*ADE2*-LexAS1-2-TEL, pVIIL-*URA3*-*ADE2*-LexAS3-TEL, and pVIIL-*URA3*-LexAS1-*ADE2*-TEL were constructed by cloning the same 42-bp oligonucleotide into one of the two *Bam*HI sites upstream and downstream of the *ADE2*-containing *Bam*HI fragment by ligation into pVIIL-*URA3*-*ADE2*-TEL (18) after partial digestion with *Bam*HI. The recovered plasmids were subjected to Southern analysis and sequenced. S1-1 (ATGTACATATAACCAC) and S1-2 (CTGTATATAAAACG) refer to constructs containing single LexA binding sites of different sequences. The subtelomeric site is identical to S1-2. S3 refers to the presence of three LexA binding sites.

To construct the integrating plasmid p*URA3*-LexAS3-TEL, the *Sal*I-*Eco*RI fragment containing the *URA3*-LexAS3-TEL sequence from the plasmid pVIIL-*URA3*-LexAS3-TEL was cloned into pRS303 (41). For construction of p*URA3*-LexAS3, p*URA3*-LexAS3-TEL was digested with *Eco*RI and *Bam*HI to replace the 81-bp TG_{1-3} tracts with an *Eco*RI-*Bam*HI linker. The 42-bp oligonucleotide containing the LexA-binding sites was then cloned into the unique *Bam*HI site. The resulting plasmids have three functional LexA binding sites. All plasmids were transformed into *E. coli* HB101, JM105, JM109, or TOP10F′ (Invitrogen).

Yeast strain constructions and media. The strains used in this study are listed in Table 1. All strains were derived from W303. Yeast gene deletions were constructed by one-step gene disruption (23). The *sir3*::*ADE2*, *sir2*::*ura3*, and *sir4*::*ura3* deletion strains were constructed as described elsewhere (10, 28). The latter two alleles were disrupted by a mutated version of the *URA3* gene (28). All transplacements and disruptions were confirmed by Southern hybridization.

To construct the CLY series of strains containing the *URA3*-LexAS-marked VIIL telomeres, YDS/D130 was transformed with linearized pVIIL-*URA3*-LexAS-TEL derivatives as described previously (18). To construct VIIL-*URA3*-LexAS1-*ADE2*-TEL, the plasmid pVIIL-*URA3*-LexAS1-*ADE2*-TEL was digested with *Sal*I and *Not*I, and the resulting fragment was transformed into YDS/D130. To construct the VIIL-*URA3*-*ADE2*-LexAS-TEL derivatives, pVIIL-*URA3*-*ADE2*-LexAS1-1-TEL, pVIIL-*URA3*-*ADE2*-LexAS1-2-TEL, and pVIIL-*URA3*-*ADE2*-LexAS3-TEL were digested with *Sal*I and *Not*I and transformed into YDS/D130 cells. The telomeric and subtelomeric *ADE2*-marked telomeres gave rise to the CZY series of strains. The presence of the marked telomeres in these cells was confirmed by Southern analysis and mitotic stability assays. The various *rap1* alleles were introduced into these strains by plasmid shuffles as described elsewhere (28, 29). CLY4 (W303α/*URA3*-LexAS3) and CLY5 (W303α/*URA3*-LexAS3-GT) were constructed by integrating *Nhe*I-digested p*URA3*-LexAS3 or p*URA3*-LexAS3-TEL at the *HIS3* locus in W303α. Strains carrying a single copy of the insert were identified by Southern analysis. To construct *rap1-17* derivatives containing these internal silencers, CLY4 and CLY5 were crossed to the *rap1-17* strain AJL441-3d to generate the strains AJL 479 and AJL 474, respectively.

To construct strains deleted for *SIR1*, a *sir1*::*ura3* disruption strain (containing a nonfunctional *URA3* gene) was constructed in W303 (28) and crossed to VIIL-*URA3*-LexAS1-*ADE2*-TEL, to form diploids AJL 481 and AJL 484, or to VIIL-*URA3*-LexAS3-TEL, to form diploid AJL 490. Spore segregants containing the *sir1* disruption, a *rap1*::*LEU2* disruption, pD130, and the marked telomere were identified by genetic and Southern analyses. These segregants were subjected to a plasmid shuffle to replace pD130 with the indicated plasmid carrying the wild-type or mutant allele of *RAP1*.

All strains were transformed with pBTM116, pBTM116-*SIR3*, or pBTM116-*SIR3*^{N205}, encoding LexA, LexA-Sir3p, and LexA-Sir3p^{N205}, respectively. The presence of the plasmid was confirmed by Southern analysis, and the presence of the fusion protein was confirmed in a subset of cases by Western analysis (immunoanalysis) with antibodies directed against the LexA protein (kindly provided by Roger Brent) in an enhanced chemiluminescence assay (Amersham).

Cell growth and transformations were carried out by standard methods (23). YPD, synthetic complete medium, synthetic complete omission medium, and 5-fluoroorotic acid (FOA)-containing medium were prepared as described previously (23, 25).

Assays for transcriptional repression. The expression of the *URA3*-marked VIIL telomere was monitored by determining the median fraction of cells that were capable of growth on FOA-containing medium, which allows the growth of Ura3^{-}, but not Ura3^{+}, cells (4, 18, 26). Each trial consisted of a fluctuation assay using at least seven independent colonies. Statistical significance between the distribution of FOA^{r} frequencies generated by the various alleles or between

TABLE 1. Yeast strains

Strain	Genotype
W303a[a]	*MAT***a** *leu2-3,112 HIS3 ade2-1 trp1 ura3-1 SIR3*
W303α[a]	*MAT*α *leu2-3,112 his3 ade2-1 trp1 ura3-1 SIR3*
YDS/D130	*MAT*α *rap1*::*LEU2 leu2-3,112 trp1 his3 ade2-1 ura3-1* pD130/RAP1
CLY1/*RAP1*[b]	*MAT*α *rap1*::*LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 SIR3* VIIL::*URA3* pRS313/*RAP1*
CLY1/*rap1-21*[b]	*MAT*α *rap1*::*LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 SIR3* VIIL::*URA3* pRS313/*rap1-21*
CLY1/*rap1-22*[b]	*MAT*α *rap1*::*LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 SIR3* VIIL::*URA3* pRS313/*rap1-22*
CLY1/*rap1-17*[b]	*MAT*α *rap1*::*LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 SIR3* VIIL::*URA3* pRS313/*rap1-17*
CLY2/*RAP1*	*MAT*α *rap1*::*LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 SIR3* VIIL::*URA3* LexAS2 pRS313/*RAP1*
CLY2/*rap1-21*	*MAT*α *rap1*::*LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 SIR3* VIIL::*URA3* LexAS2 pRS313/*rap1-21*
CLY2/*rap1-22*	*MAT*α *rap1*::*LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 SIR3* VIIL::*URA3* LexAS2 pRS313/*rap1-22*
CLY2/*rap1-17*	*MAT*α *rap1*::*LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 SIR3* VIIL::*URA3* LexAS2 pRS313/*rap1-17*
CLY3/*RAP1*	*MAT*α *rap1*::*LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 SIR3* VIIL::*URA3* LexAS3 pRS313/*RAP1*
CLY3/*rap1-21*	*MAT*α *rap1*::*LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 SIR3* VIIL::*URA3* LexAS3 pRS313/*rap1-21*
CLY3/*rap1-22*	*MAT*α *rap1*::*LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 SIR3* VIIL::*URA3* LexAS3 pRS313/*rap1-22*
CLY3/*rap1-17*	*MAT*α *rap1*::*LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 SIR3* VIIL::*URA3* LexAS3 pRS313/*rap1-17*
CLY3/*rap1-17*$^{\Delta sir1}$	*MAT*α *rap1*::*LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 SIR3 sir1*::*ura3* VIIL::*URA3* LexAS3 pRS313/*rap1-17*
CLY3/*rap1-17*$^{\Delta sir2}$	*MAT*α *rap1*::*LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 SIR3 sir2*::*ura3* VIIL::*URA3* LexAS3 pRS313/*rap1-17*
CLY3/*rap1-17*$^{\Delta sir4}$	*MAT*α *rap1*::*LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 SIR3 sir4*::*ura3* VIIL::*URA3* LexAS3 pRS313/*rap1-17*
CZY1/RAP1	*MAT*α *rap1*::*LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 SIR3* VIIL::*URA3*/*ADE2* pRS313/*RAP1*
CZY2/RAP1	*MAT*α *rap1*::*LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 SIR3* VIIL::*URA3*/*ADE2* LexAS1-1 pRS313/*RAP1*
CZY3/RAP1	*MAT*α *rap1*::*LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 SIR3* VIIL::*URA3*/*ADE2* LexAS1-2 pRS313/*RAP1*
CZY4/RAP1	*MAT*α *rap1*::*LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 SIR3* VIIL::*URA3*/*ADE2* LexAS3 pRS313/*RAP1*
CZY4/rap1-17	*MAT*α *rap1*::*LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 SIR3* VIIL::*URA3*/*ADE2* LexAS3 pRS313/*rap1-17*
CZY5/RAP1	*MAT*α *rap1*::*LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 SIR3* VIIL::*URA3*/LexAS1/*ADE2* pRS313/*RAP1*
CZY5/rap1-17	*MAT*α *rap1*::*LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 SIR3* VIIL::*URA3*/LexAS1/*ADE2* pRS313/*rap1-17*
W303aΔsir1	*MAT*α *leu2-3,112 HIS3 ade2-1 trp1 ura3-1 SIR3 sir1*::*ura3*
AJL 481-3a, 16b/RAP1	*MAT*α *rap1*::*LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 SIR3 sir1*::*ura3* VIIL::*URA3*/LexAS1/*ADE2* pRS313/*RAP1*
AJL 481-3a, 16b/rap1-17	*MAT*α *rap1*::*LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 SIR3 sir1*::*ura3* VIIL::*URA3*/LexAS1/*ADE2* pRS313/*rap1-17*
CLY4	*MAT*α *RAP1 leu2-3,112 his3 ade2-1 trp1 ura3-1 SIR3 HIS3*::*URA3* LexAS3::*HIS3*
CLY5	*MAT*α *RAP1 leu2-3,112 his3 ade2-1 trp1 ura3-1 SIR3 HIS3*::*URA3* LexAS3/TG1-3::*HIS3*
AJL 441-3d	*MAT***a** *rap1-17 leu2-3,112 his3 ade2-1 trp1 ura3-1 SIR3* VIIL::*ura3*/*ADE2*
AJL 474	CLY5 × AJL 441-3d
AJL 479	CLY4 × AJL 441-3d
AJL 493	CLY5 × WaΔsir1
AJL 495	AJL 474-1a × WaΔsir1

[a] From the work of Kurtz and Shore (24a).
[b] From the work of Liu et al. (29).

median values of multiple experiments was determined by the rank sum test (43). When internal silencing was assayed, only FOA^r colonies capable of reversing to the Ura$^+$ phenotype were scored as silencing events. A subset or all colonies from a FOA plate were plated directly onto uracil omission medium. When a subset was used, the data were extrapolated to the whole population in estimating the frequency.

The repression of the *ADE2*-marked VIIL telomere was monitored by measuring the median frequency of red colonies or sectors obtained in a fluctuation assay (18, 26). To assay the expression of *ADE2* on plates containing FOA, the concentration of adenine in these plates was reduced to 25% of normal concentrations.

To measure the frequency of switching from the *URA3* repressed state to the induced state, identical fluctuation tests were performed, except that cells were initially grown on FOA-containing tryptophan-omission medium and subsequently plated to tryptophan-omission medium, FOA-containing tryptophan-omission medium, or tryptophan- and uracil-omission medium. Statistical significance was determined as described above.

RESULTS

Tethering of Sir3p to the telomere restores silencing in silencing-defective *rap1* alleles. The C-terminal 165 amino acids of Rap1p were previously shown to be essential for telomeric silencing (26) (Fig. 1). A mutation that truncates this region of Rap1p (the *rap1-17* allele) confers a complete loss of telomeric silencing. Site-directed mutagenesis has further identified a C-terminal tail domain, comprising the terminal 28 amino acids of Rap1p, which is required for telomeric silencing. A nonsense mutation (*rap1-21*) that truncates the terminal 28 amino acids and a missense mutation (*rap1-22*; H810Y) within this domain eliminate silencing (29). Interestingly, loss of the C-terminal tail domain leads to the delocalization of Sir3p from the nuclear periphery, suggesting a role for Rap1p in the recruitment of Sir3p into a multimeric complex (11).

We reasoned that if the C-terminal tail domain functions in telomeric silencing by recruiting Sir3p, then tethering Sir3p adjacent to a marked telomere in *cis* might restore silencing in these *rap1* alleles. To this end, we constructed a LexA-Sir3p fusion protein, containing the entire open reading frame of *SIR3* in frame with LexA, and introduced plasmids encoding either LexA or the LexA-Sir3p fusion protein into the *rap1* silencing-defective strains. Each of these strains contains a *URA3*-marked telomere on the left arm of chromosome VII (VIIL), in the presence of zero, two (LexAS2), or three (LexAS3) LexA binding sites adjacent to the telomeric tract (Fig. 2, top). The presence of the fusion proteins in these strains was confirmed by Western analysis (data not shown). Transcriptional silencing of the subtelomeric *URA3* gene was assayed by measuring the ability of cells to grow on FOA, which allows the growth of Ura3$^-$, but not Ura3$^+$, cells (4).

As expected, the introduction of LexA alone had no effect on telomeric silencing in either the presence or the absence of a LexA binding site (Table 2; Fig. 3). Similarly, in the absence of a LexA binding site, production of LexA-Sir3p did not restore silencing in any of the *rap1* alleles. In contrast, in the presence of LexA binding sites, production of LexA-Sir3p restored silencing in the *rap1-22* missense allele to FOA^r values just 2-fold (for three LexA binding sites) to 40-fold (for two

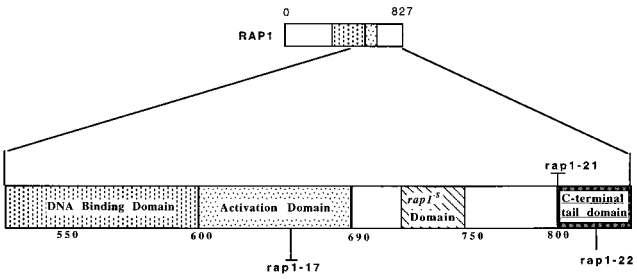

FIG. 1. RAP1 domain structure. The DNA binding, activation, *rap1*^{s}, and C-terminal tail domains are shown in the expansion (see references 15 and 40). The positions of the *rap1-17* and *rap1-21* nonsense mutations and the *rap1-22* (H810Y) missense mutation are also shown.

LexA binding sites) lower than wild type. Similarly, in strains lacking the C-terminal tail domain (*rap1-21*), which abrogates silencing, production of LexA-Sir3p restored silencing to levels only 4-fold (for two LexA binding sites) to 20-fold (for three LexA binding sites) lower than wild type. Production of the LexA-Sir3p fusion protein in strains lacking the entire 165-amino-acid C terminus of Rap1p (*rap1-17*) led to restoration of silencing, with FOA^r values only 20-fold lower than wild type. Although the exact FOA^r values vary nonsystematically with the number of LexA binding sites within a 20-fold range, this variation is small relative to the $\approx 10^3$- to 10^5-fold increase in silencing at the telomere over cells expressing only LexA or lacking the LexA binding site. Since the LexA protein contains a dimerization domain, these data indicate that four to six molecules of Sir3p bound to the LexA site are sufficient to rescue silencing in *rap1* silencing-defective alleles.

These results suggest that a major function of the Rap1p C terminus in telomeric silencing is to recruit Sir3p and Sir3p-associating factors to the telomere and that recruitment of Sir3p is sufficient to nucleate telomeric silencing. Interestingly, Sir3p retains function even when separated in *cis* from the telomeric TG_{1-3} sequences, indicating that the presumed Rap1p requirement for telomeric binding can be physically separated from its role in Sir3p recruitment.

We have shown elsewhere that the *SIR3*^{N205} gain-of-function suppressor (a dominant missense mutation that replaces the aspartic acid at amino acid 205 with an asparagine) restores silencing in cells carrying the *rap1-21* allele but not in strains carrying the more extreme *rap1-17* allele (28). The characteristics of this suppressor suggest that it is likely to act through facilitated interactions with downstream factors (28). We transformed the various *rap1* strains containing marked VIIL telomeres with the LexA-Sir3^{N205} fusion protein and assayed the levels of FOA^r cells (Table 2; Fig. 4). In the absence of the binding site, LexA-Sir3p^{N205} did not significantly affect FOA^r levels in any of the *rap1* silencing-defective alleles, including *rap1-22* and *rap1-21*, indicating that this fusion protein has lost the ability to suppress these *rap1* alleles. However, in strains

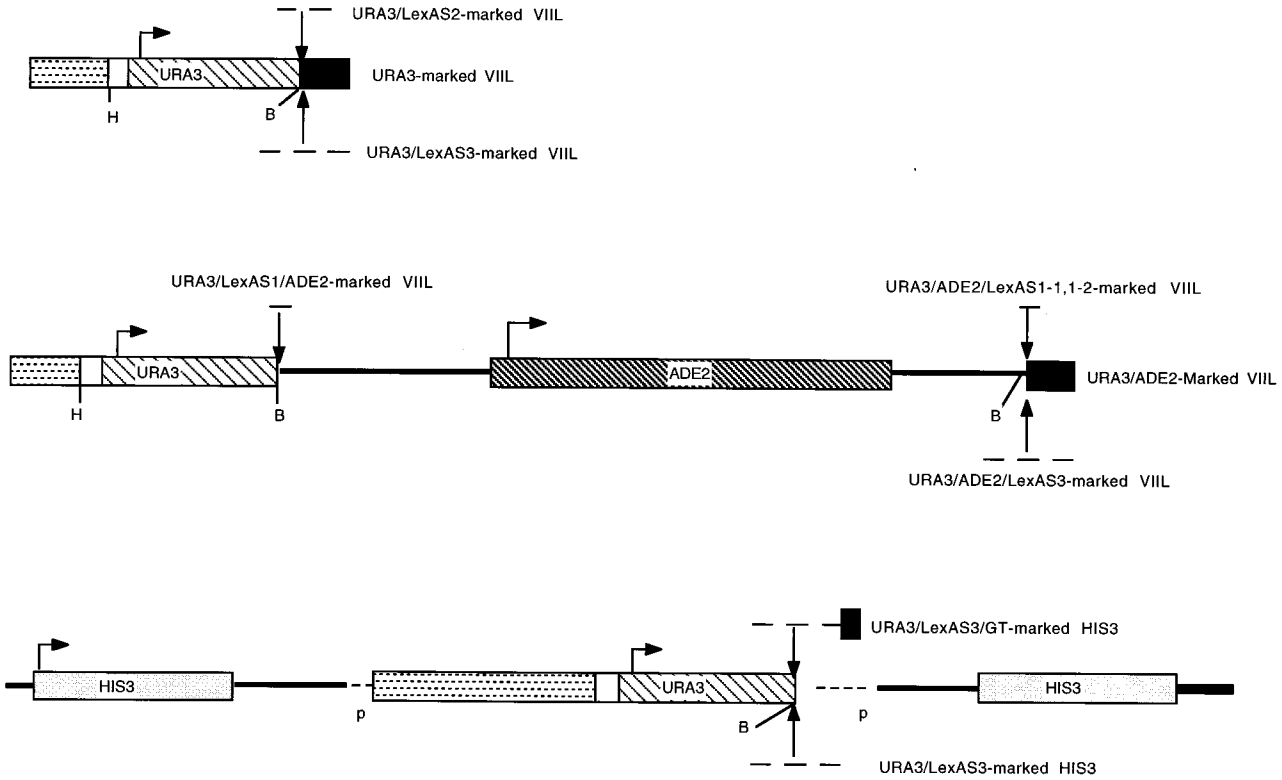

FIG. 2. Marked telomeres used in this study. Shown here are the marked termini of the left arm of chromosome VII (VIIL) and the internal silencers at the *HIS3* locus used in this study. In the case of the internal silencers, plasmid sequences (p) present in pRS303 flanking the *URA3* gene have been omitted for simplicity. Directional arrows denote the sites of transcriptional initiation. The positions of the LexA sites (and adjacent TG_{1-3} when relevant) are denoted by vertical arrows with the number of corresponding dashes indicating the number of sites. The designation of each telomere is adjacent to the inserted LexA site. LexAS1, LexAS2, and LexAS3 refer to the presence of one, two, or three LexA binding sites. The site in *URA3*/LexAS1/*ADE2* is identical in sequence to the site in the *URA3*/*ADE2*/LexAS1-2 construct. Dashed box, *ADH4* sequences from the left arm of chromosome VII; white box, genomic sequences upstream from the *URA3* gene; light striped box, the *URA3* gene; dark striped box, the *ADE2* gene; stippled box, the *HIS3* gene; black box, TG_{1-3} telomeric or internal sequences; horizontal lines, genomic sequence surrounding either the *ADE2* gene in the *URA3*/*ADE2*-marked telomeres or the *HIS3* gene in the internal silencers. Restriction sites: H, *Hin*dIII site; B, *Bam*HI site.

TABLE 2. Tethering of Sir3p adjacent to TG_{1-3} tracts overcomes the requirement for the Rap1p C terminus in telomeric silencing

Allele	No. of sites[a]	LexA			LexA-Sir3p			LexA-Sir3p^{N205}		
		Median FOA^r % (fold increase)[b]	Range[c]	No. of trials	Median FOA^r % (fold increase)[b]	Range[c]	No. of trials	Median FOA^r % (fold increase)[b]	Range[c]	No. of trials
RAP1	−	37 (1)	12–63	3	0.063 (0.0017)	0–0.8	3	45 (1.2)	28–95	3
	+++	36 (1)	12–90	3	25 (0.7)	14–39	2	85 (2.4)	59–176	3
	++	36 (1)	9–53	1	NT			94 (2.6)	52–120	1
rap1-22	−	0.0025 (1)	0.0005–0.009	2	<0.0003 (<0.12)		1	0.0041 (1.6)	0.0007–0.012	2
	+++	0.003 (1)	0.0003–0.012	2	18 (6,000)	13–25	1	44 (15,000)	13–119	2
	++	NT			1.0 (330)	0.07–1.4	1	NT		
rap1-21	−	<0.00015		2	<0.00015		1	<0.0004		2
	+++	<0.00006 (1)[d]		1	2.0 (>33,000)	0–5	2	32 (>530,000)	9–65	2
	++	NT			9.6 (>160,000)	9–12	1	NT		
rap1-17	−	<0.0001		1	<0.0003		1	<0.0006		1
	+++	<0.0005 (1)		3	1.7 (>3,400)	0.6–4	2	41 (>82,000)	17–95	3
	++	0.0003 (1)	0–0.003	2	NT			55 (180,000)	46–96	1

[a] −, +++, and ++ refer to the presence of zero, three, and two telomeric LexA binding sites, respectively.
[b] Median values are presented as the percentage of cells containing the *URA3*-marked VIIL telomere (with or without LexA binding sites) capable of growth on FOA medium, together with the fold increase over the LexA control in parentheses. Each trial consists of a fluctuation analysis using at least seven independent colonies. NT, not tested.
[c] The range of values is derived from the pooled set of data from the number of trials listed.
[d] A second trial gave a finite value of 3.7×10^{-5}.

that contained either two or three telomeric LexA binding sites, the introduction of LexA-Sir3p^{N205} completely restored wild-type levels of silencing in the *rap1-22*, *rap1-21*, and *rap1-17* alleles, the latter truncating the region thought to be necessary for Sir3p association (Fig. 4; Table 2) (33). Western analysis demonstrated that this effect is not due to an increased abundance of LexA-Sir3p^{N205} (data not shown). We note that LexA-Sir3p tethering restores the silencing defects of these alleles without altering their effects on telomere size (data not shown).

Restoration of silencing by tethering of LexA-Sir3p or LexA-Sir3p^{N205} is dependent on Sir2p and Sir4p but is independent of Sir1p (Table 3). This is identical to the behavior previously reported for telomeric silencing (1). These data indicate that tethered silencing is operating through (or sharing elements with) the normal pathway for telomeric silencing.

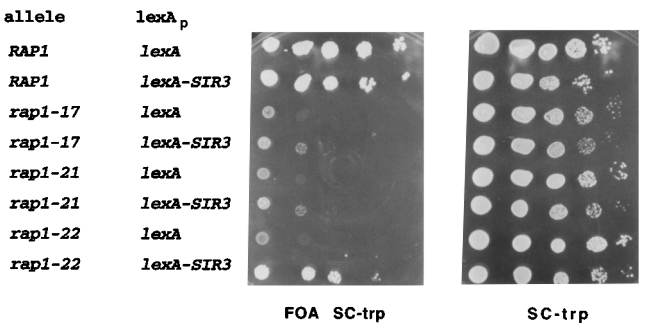

FIG. 3. Tethering of LexA-Sir3p restores silencing in *rap1* silencing-defective strains. *SIR3* strains containing either the *rap1-17*, *rap1-21*, *rap1-22*, or *RAP1* allele and the *URA3*-LexAS3-marked VIIL telomere were transformed with pBTM116, encoding LexA, or pBTM116/*SIR3*, encoding the LexA-Sir3p fusion protein. Tenfold serial dilutions of cells were plated onto either tryptophan omission medium (SC-trp) to select for the *TRP1*-containing pBTM116 and pBTM116/*SIR3* or tryptophan omission medium containing FOA (FOA SC-trp). Cells were grown at 30°C. We note that spot assays yield only a qualitative indication of the restoration of silencing. See Table 2 for frequencies determined by fluctuation analysis.

We note that LexA-Sir3p activity also depends on the presence of wild-type Sir3p (data not shown). This characteristic has been previously reported for a G_{AD} (Gal4 activation domain)-Sir3p fusion protein and is thought to be the consequence of disruption of Sir3p N-terminal function (10, 44b). In addition, LexA-Sir3p displays a dominant negative behavior in some contexts (see below). This interference with normal silencing may lead to an underestimate of the effect of LexA-Sir3p tethering. In contrast, LexA-Sir3p^{N205} is able to overcome this defect and functions as Sir3p in the presence or absence of wild-type Sir3p.

Tethering of Sir3p or Sir3p^{N205} at the telomere stabilizes the repressed state in wild-type cells. Tethering of LexA-Sir3p^{N205} also increases the efficiency of silencing in a wild-type background. In wild-type cells that lack the LexA binding site and contain LexA-Sir3p^{N205}, wild-type levels of silencing were observed (45% FOA^r cells; Table 2). In cells containing the telomeric LexA binding site, LexA-Sir3p^{N205} conferred a highly repressed state, with 85 to 94% of cells displaying FOA resistance. Qualitatively, LexA-Sir3p displayed a similar pat-

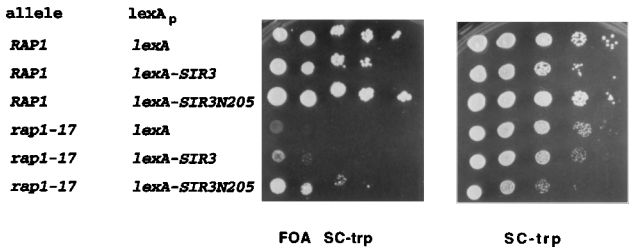

FIG. 4. Tethering of LexA-Sir3p^{N205} leads to higher levels of telomeric silencing in *rap1-17* and wild-type cells. *SIR3* strains containing either the *rap1-17* or *RAP1* allele and the *URA3*-LexAS3-marked VIIL telomere were transformed with pBTM116, encoding LexA; pBTM116/*SIR3*, encoding LexA-Sir3p; or pBTM116/*SIR3*^{N205}, encoding LexA-Sir3p^{N205}. Tenfold serial dilutions of cells were plated onto either tryptophan omission medium (SC-trp) to select for the *TRP1*-containing pBTM116 plasmids or tryptophan omission medium containing FOA (FOA SC-trp). Cells were grown at 30°C.

TABLE 3. Tethered silencing is *SIR2* and *SIR4* dependent

Genotype	LexA			LexA-Sir3p			LexA-Sir3p^{N205}		
	Median FOA^r % (fold increase)[a]	Range	No. of trials	Median FOA^r % (fold increase)[a]	Range	No. of trials	Median FOA^r % (fold increase)[a]	Range	No. of trials
SIR1 SIR2 SIR4	<0.0005 (1)[b]			1.7 (>3,400)[b]			41 (>82,000)[b]		
sir1 SIR2 SIR4	0.002 (1)[b]	0–0.07	2	0.7 (350)	0.02–0.2	2	34 (17,000)	28–52	1
SIR1 sir2 SIR4	<0.0002		3	<0.0003		2	<0.0003		3
SIR1 SIR2 sir4	<0.0002		2	<0.0002		2	<0.0003		3

[a] Median values are presented as described in Table 2 together with the fold increase over the LexA control in parentheses. Values were determined with *rap1-17* cells carrying the *URA3*-LexAS3-marked VIIL telomere.
[b] Values are from Table 2.

tern. In the absence of a telomeric LexA binding site, the LexA-Sir3p fusion protein decreased silencing efficiency, displaying a dominant negative effect. However, wild-type levels of silencing were regained by introduction of the telomeric LexA binding sites in strains containing LexA-Sir3p, suggesting a competition between the negative and positive effects of LexA-Sir3p on silencing. For both LexA-Sir3p and LexA-Sir3p^{N205}, silencing is fully dependent on Sir2p and Sir4p but is independent of Sir1p (data not shown).

Silencing at the telomere is restricted to the repression of basal transcription (2). The Ppr1p transactivator is induced under conditions of uracil starvation and activates *URA3* expression (38a). Induction of *PPR1* under conditions of uracil starvation or by *GAL1-10*-mediated overexpression leads to an almost quantitative switching of the *URA3*-marked VIIL locus from the repressed (FOA^r) to the activated (Ura$^+$) state (2). To test the stability of the hyperrepressed state observed after tethering of LexA-Sir3p^{N205}, we measured the ability of silenced FOA^r cells to be induced under conditions of uracil starvation. As expected, in cells transformed with LexA, 65 to 80% of FOA^r cells switched to the Ura$^+$ phenotype after 3 days of growth. In contrast, only 0.5% of cells containing LexA-Sir3p^{N205} switched to the Ura$^+$ phenotype, even after extended growth. Similarly, of cells carrying the LexA-Sir3p protein, only 16% switched to the derepressed state under the same conditions. Thus, tethering of either Sir3p or Sir3p^{N205} stabilizes the transcriptionally repressed state, even under conditions of *URA3* activation.

Tethering of Sir3p at an *ADE2*-marked telomere hyperrepresses *ADE2* expression. The restoration of silencing in *rap1-17* cells and the stabilization of the repressed state by the LexA-Sir3p fusion proteins suggested that recruitment of telomeric Sir3p may normally be limiting for telomeric silencing in wild-type cells. To test this, we utilized a system in which LexA-Sir3p function could be assayed in the absence of a significant dominant negative effect. To this end, we constructed strains containing different derivatives of a *URA3*/*ADE2*-marked VIIL telomere (Fig. 2, middle). The order of the elements in these strains is centromere. . .*URA3*. . . *ADE2*. . . telomere. Repression of *ADE2* was monitored by the production of red colonies or sectors, while derepression was monitored by the production of white colonies or sectors. The use of this telomere has two advantages. First, wild-type silencing of *ADE2* is relatively weak, allowing enhanced silencing (which we will term hyperrepression) to be more easily detected. Second, repression of each gene can be monitored, allowing an estimation of both the degree and directionality of silencing from the site of initiation.

URA3/*ADE2*-marked VIIL telomeres were introduced into strains wild type for *RAP1* and *SIR3*. Marked telomeres that either did not contain a LexA binding site, contained a single LexA binding site (LexAS1), or contained three adjacent LexA binding sites (LexAS3) were constructed. The LexA sites were cloned directly adjacent to the telomeric tract. The results of these experiments were analyzed by assaying the color of patches and the sectoring patterns of individual colonies (Fig. 5).

In the presence of one or three LexA binding sites, introduction of LexA led to a typical wild-type phenotype, with only 1 to 2% of cells forming red colonies (26). In striking contrast, when LexA-Sir3p was introduced into cells containing one or three telomeric LexA binding sites, all cells formed red or pink colonies, reflecting transcriptional repression, with only rare white sectors (Fig. 5). Therefore, tethering of two Sir3p molecules to a telomere (after LexA dimerization) results in a hyperrepressed state in wild-type cells. Under these conditions, the *ADE2* gene appears to be maintained in a repressed state, a situation reminiscent of *HM* silencing.

Introduction of the LexA-Sir3p^{N205} fusion protein had a qualitatively similar, but more pronounced, effect (Fig. 5; data not shown). Colonies were fully red, indicating complete and stable repression of the *ADE2* gene. In the case of LexA-Sir3p^{N205}, the expression of the telomere-distal *URA3* gene, whose promoter is 4.6 kb from the telomere, was also analyzed. We found that tethering of LexA-Sir3p^{N205} led to enhanced repression of the *URA3* gene (Table 4). Production of LexA in the presence ($+_T$, Table 4) or absence ($-$, Table 4) of telomeric LexA binding sites had values within the broad range observed in wild-type strains (0.006 and 0.12%, respectively). While production of LexA-Sir3p^{N205} in the absence of a binding site stimulated FOA^r values to 2%, addition of one ($+_T$) or three ($+++_T$) telomeric LexA sites led to significantly higher FOA^r values of 12 and 40%, respectively. In all cases, FOA^r cells were Ade$^-$, as indicated by the formation of red colonies on FOA–low-adenine medium. These data are consistent with a unidirectional spreading of silencing through the *ADE2* and *URA3* genes initiated at the telomere. Thus, tethering of either LexA-Sir3p or LexA-Sir3p^{N205} to the telomere leads to hyperrepressed states, suggesting that recruitment of Sir3p to the telomere by Rap1p is limiting in wild-type cells.

Tethering of Sir3p^{N205} to a subtelomeric site enhances telomeric silencing. High-efficiency telomeric silencing was conferred by tethering either LexA-Sir3p or LexA-Sir3p^{N205} adjacent to, but physically separated from, the telomeric tract. To test the effect of larger distances between the TG_{1-3} tract and the site of Sir3p tethering on silencing, we generated an isogenic set of strains with a single LexA binding site either adjacent to the telomeric tract or between the *URA3* and *ADE2* genes, ≈0.8 and 1.0 kb from their respective promoters and 3.6 kb from the junction between telomeric and unique sequences (Fig. 2, middle). In these experiments, we transformed cells with plasmids encoding LexA-Sir3p^{N205}, since this

FIG. 5. Hyperrepression of the *ADE2* gene by telomeric tethering of LexA-SIR3p or LexA-SIR3p^{N205}. *RAP1* strains containing either the *URA3*/*ADE2*/LexAS-1-1 (right)- or *URA3*/*ADE2*/LexAS3 (left)-marked VIIL telomere were transformed with either pBTM116, pBTM116/*SIR3*, or pBTM116/*SIR3*^{N205}. The resulting strains were streaked onto tryptophan omission medium and grown for 3 days at 30°C before being shifted to room temperature for color development.

gain-of-function protein, while behaving qualitatively similar to LexA-Sir3p, provides a more sensitive assay.

Cells containing the subtelomeric LexA binding site (*URA3*/LexAS/*ADE2*-marked VIIL telomere; $+_S$, Table 4) were transformed with plasmids encoding either LexA alone or the LexA-Sir3p^{N205} fusion protein (Table 4). When LexA-Sir3p^{N205} was tethered at the subtelomeric site, ≈70% of cells developed red colonies with white sectors, significantly higher than the values found in the absence of the site or in the presence of LexA alone. A major increase in the repression of the telomere-distal *URA3* gene was also observed, with ≈25% of cells forming FOA^r colonies. Subtelomeric tethered silencing is fully dependent on Sir4p, suggesting that it shares elements with the wild-type pathway of telomeric silencing (data not shown).

Subtelomeric tethering could theoretically be initiated in one of two ways. Nucleation could proceed from the tethered site and extend uni- or bidirectionally into the adjacent *URA3*

TABLE 4. Tethering of SIR3p^{N205} to telomeric and subtelomeric sites in wild-type *RAP1* cells enhances telomeric silencing

Protein	Site[a]	FOA^r			Red colony formation		
		Repression % (fold increase)[b]	Range of medians	No. of assays	Repression % (fold increase)[b]	Range of medians	No. of assays
LexA	−	0.12 (1)	0.002–0.3	5	7 (1)	2–11	6
LexA-Sir3p^{N205}	−	2 (17)	0.3–5	7	13 (1.9)	6–26	6
LexA	$+_T$	0.006 (1)[c]		1	0.2 (1)[c]		1
LexA-Sir3p^{N205}	$+_T$	12 (2,000)[c]		1	100 (500)[c]		1
LexA-Sir3p^{N205}	$+++_T$	40 (6,700)		1	100 (500)		1
LexA	$+_S$	0.07 (1)	0.01–0.13	5	4 (1)	2–6	5
LexA-Sir3p^{N205}	$+_S$	24 (340)	10–33	8	71 (18)	51–88	8

[a] $+_S$, single subtelomeric site; $+_T$, telomeric site (S2-2); $+++_T$, three telomeric LexA binding sites; −, no LexA binding site.

[b] The average of medians obtained in independent fluctuation assays is presented, together with the fold increase over the relevant LexA control. Results are presented as the percentage of cells carrying the *URA3*/*ADE2*-marked VIIL (with the indicated LexA binding sites) capable of growth on FOA medium (as an assay for *URA3* repression) and of red colony formation (as an assay for *ADE2* repression). Each fluctuation trial used at least seven independent colonies.

[c] Similar results were obtained in multiple experiments using the LexAS1-1 site (Fig. 2).

TABLE 5. Tethering of Sir3p^{N205} directs *SIR1*-stimulated silencing from the subtelomerically tethered site in cells lacking the Rap1p C terminus

rap1 allele	*sir1* allele	Protein	FOA^{r}[a]			FOA^{r} Ade^{+}[a]		
			% (fold increase)	Range	No. of trials	% (fold increase)	Range	No. of trials
RAP1	*SIR1*	LexA-Sir3p^{N205}	24[b]			<0.0003, <0.0008, <0.00001[c]		
	sir1	LexA-Sir3p^{N205}	20	18–21	3	NT		
rap1-17	*SIR1*	LexA	≤0.003[d]		4	<0.0001		1
	sir1	LexA	<0.0002		3	NT		
	SIR1	LexA-Sir3p^{N205}	(1) 7.8 (>2,600)[e]	4–12	9	2.4 (>24,000)[e]	1–5	5
			(2) 0.04 (>13)[e]	0.003–0.12	9	0.02 (>200)[e]	0.002–0.47	3
	sir1	LexA-Sir3p^{N205}	(1) <0.0001[f]		3	NT		
			(2) 0.05 (>250)[f]	0.03–0.07	2	NT		

[a] Average values of fluctuation medians obtained from multiple trials in the absence (FOA^{r} Ade^{+}) or presence (FOA^{r}) of adenine in the FOA medium are presented as described in Table 4. The fold increase over the relevant LexA control is given. NT, not tested.
[b] Value is from Table 4.
[c] Three trials are listed separately.
[d] Value derived from four trials, yielding percentages of $<1 \times 10^{-4}$, $<1.5 \times 10^{-4}$, 0.94×10^{-4}, and 1.15×10^{-3}.
[e] Transformants fell into two classes (listed as 1 and 2) having the indicated values.
[f] Values derived from two isogenic spore colonies (listed as 1 and 2).

and *ADE2* genes. Alternatively, it could facilitate initiation of silencing at the telomere, spreading sequentially into *ADE2* and *URA3* sequences. Two observations argue for the latter possibility. First, all FOA^{r} cells formed red colonies on FOA-containing low-adenine medium (data not shown). This is an expectation of unidirectional spreading from the telomere but need not be required for silencing initiated at the subtelomeric site. Second, under selection for *ADE2* expression, FOA^{r} colonies are never observed (Table 5). The dependence of *URA3* repression on *ADE2* repression is most consistent with the promotion of unidirectional silencing from the telomere by the tethering of LexA-Sir3p^{N205} to the subtelomeric LexA site.

Subtelomerically tethered Sir3p^{N205} nucleates silencing in *rap1* strains lacking the C terminus of Rap1p. Subtelomerically tethered Sir3p^{N205} might facilitate silencing by improving interactions with (a) the Rap1p C terminus, (b) factors associating with the Rap1p C terminus (e.g., Sir3p, Sir4p), (c) other domains of Rap1p, (d) other novel components of the telomeric complex, or (e) a nuclear substructure necessary for silencing. To begin to distinguish among these possibilities, we tested whether the ability of LexA-Sir3p^{N205} to enhance silencing is dependent on the C terminus of Rap1p. We constructed a *rap1-17* strain, which lacks the C-terminal 165 amino acids of Rap1p, containing a *URA3*/*ADE2*-marked VIIL telomere with a telomeric (*URA3*/*ADE2*/LexAS1) or subtelomeric (*URA3*/LexAS1/*ADE2*) LexA binding site. Silencing was assayed by measuring *URA3* repression. As expected, tethering of LexA-Sir3p^{N205} to the telomeric site resulted in suppression of the *rap1-17* silencing defect. FOA^{r} frequencies ranged between 20 and 25%, while undetectable levels were observed in cells either transformed with LexA or lacking the site (data not shown). These data indicate that a single LexA-Sir3p^{N205} (possibly dimerized by LexA) is sufficient to nucleate silencing in *rap1-17* cells. We note that FOA^{r} frequencies were independent of the length of the *ADE2*-marked telomere (data not shown).

Silencing was also observed when LexA-Sir3p^{N205} was tethered to the subtelomeric site in *rap1-17* cells (Table 5). However, its characteristics were significantly altered from those observed in the *RAP1* wild-type strain. First, unidirectionality of silencing from the telomere was no longer a condition of silencing. A relatively high frequency of FOA^{r} Ade2^{+} cells was observed (Table 5). This population was not due to mutation within the *URA3* or *ADE2* genes, since both phenotypes were reversible. Southern analysis confirmed the absence of subtelomeric rearrangements (data not shown). These data suggest that silencing can be nucleated at the subtelomeric tethered LexA-Sir3p^{N205}.

Second, the formation of FOA^{r} colonies is stimulated by Sir1p, a protein which normally does not play a role in telomeric silencing (Table 5). This effect is not the consequence of changes in LexA-Sir3p^{N205} abundance in the *sir1* alleles, since the levels of the fusion proteins are identical in Sir1^{+} and Sir1^{-} strains (data not shown).

Third, the levels of FOA^{r} cells among different transformants varied more widely than in *RAP1* strains in a manner independent of LexA-Sir3p abundance. Transformants fell into two frequency classes (Table 5) that differed by ≈200-fold in FOA^{r} frequency. The presence of two phenotypic populations in Sir1^{+} and Sir1^{-} cells precludes a precise quantitation of the degree of Sir1p stimulation.

These results indicate that, in the absence of the Rap1p C-terminal 165 amino acids, LexA-Sir3p^{N205} can direct an alternative *SIR1*-stimulated pathway in which a large fraction of silencing can be nucleated at the tethered site. These data also provide evidence for a physical association between the subtelomerically tethered Sir3p and telomeric proteins that lead to the hyperrepressed state in wild-type *RAP1* cells.

Sir3p nucleates silencing at an internal site in the presence of an 81-bp TG$_{1-3}$ tract. The ability of LexA-Sir3p^{N205} to direct silencing from a subtelomeric site led us to investigate whether it can function in an internal chromosomal context. We integrated a *URA3* gene flanked at its 3′ end with three LexA binding sites into the *HIS3* locus, located ≈200 kb from the right telomere of chromosome XV. This construct creates a direct repeat of *HIS3* separated by the *URA3* tester gene (Fig. 2, bottom). The *URA3*/LexAS construct was introduced in both the presence and the absence of an 81-bp TG$_{1-3}$ tract 3′ to the LexA site in the same relative orientation as present at the telomere. Previous studies have demonstrated that this tract alone is insufficient to nucleate silencing (18, 44). We transformed both strains with either LexA, LexA-Sir3p^{N205}, or LexA-Sir3p and monitored the level of silencing (Table 6).

In these strains, FOA^{r} colonies can arise either by recombination between the flanking *HIS3* repeats or by silencing of the *URA3* gene. To distinguish between these possibilities, all or a

TABLE 6. Internal silencing directed by tethering of SIR3p^{N205} is stimulated by truncation of the Rap1p C terminus

rap1 allele	Fusion protein	TG_{1-3} tract	FOA^r Ura^{+a}		
			% (fold increase)	Range	No. of assays
RAP1	LexA	+	<0.0003		2
	LexA-Sir3p^{N205}	+	0.004 (>13)	0.003–0.008	5
rap1-17	LexA	+	<0.0004		2
	LexA-Sir3p^{N205}	+	0.5 (>1,250)	0.005–0.7	8

[a] Average values of medians are presented as in Table 4, with fold increases over the appropriate LexA control.

subset of colonies derived from each FOA plate was plated directly onto uracil omission medium. Only cells capable of switching to the Ura3$^+$ state under inducing conditions (FOA^r Ura$^+$ colonies; Table 6) were scored as silencing events.

In the presence of the LexA-Sir3p^{N205} fusion, cells containing the 81-bp TG_{1-3} tract demonstrated a low level of silencing, forming FOA^r Ura$^+$ colonies at a rate of $\approx 4 \times 10^{-5}$ (Table 6). Lower but detectable levels of FOA^r Ura$^+$ colonies were observed when LexA-Sir3p was introduced into cells (data not shown). Southern analysis demonstrated that FOA^r Ura$^+$ colonies retained the original structure at the *URA3*-marked *HIS3* locus, indicating the absence of rearrangements. The LexA protein alone had no effect on *URA3* expression. We conclude that the presence of a TG_{1-3} tract in conjunction with tethered LexA-Sir3p (data not shown) or LexA-Sir3p^{N205} is sufficient to recruit factors necessary for internal silencing.

A higher basal recombination rate, observed only in cells lacking the TG_{1-3} tract, made an exact estimate of silencing difficult. However, none of the FOA^r colonies analyzed were capable of growth on uracil omission medium, leading us to estimate a frequency of $< 5 \times 10^{-5}$ (data not shown), even in *rap1-17* strains that increase silencing in the TG_{1-3}-containing silencer (see below). These data raise the possibility that Rap1p binding may be required for internal tract silencing.

The Rap1p C terminus antagonizes internal silencing. The ability of *URA3*/LexAS/TG_{1-3} constructs to confer low levels of silencing raised the question of the role of Rap1p in internal silencing. Five Rap1p binding sites are present within the 81-bp TG_{1-3} sequence, and as many as three can concurrently associate with Rap1p (16). To test the role of the Rap1p C terminus, we constructed an isogenic set of strains containing the *rap1-17* mutant gene in place of *RAP1*. Surprisingly, we found that FOA^r Ura$^+$ cells in strains carrying the TG_{1-3} tract were increased $\approx$100-fold in a *rap1-17* background (Table 6). LexA-Sir3p^{N205}-tethered internal silencing was independent of Sir1p in both *RAP1* and *rap1-17* alleles. This increase in silencing did not correlate with a change in abundance of the fusion protein (data not shown). These data indicate that the C-terminal 165 amino acids of Rap1p are dispensable for internal silencing nucleated by tethered Sir3p. Indeed, the data suggest that the presence of the C terminus of Rap1p, sequestered normally at the telomere, may titrate factors necessary for internal silencing.

DISCUSSION

In this study, we provide several lines of evidence that recruitment of Sir3p is a critical step in the formation of silent domains. First, tethering two molecules of LexA-Sir3p to two adjacent LexA sites at a telomere overcomes the telomere silencing defects exhibited by a Rap1 protein lacking the C-terminal 165 amino acids. Hence, tethering of Sir3p is sufficient to nucleate telomeric silencing. Second, tethering of LexA-Sir3p and LexA-Sir3p^{N205} in wild-type *SIR3* strains leads to a hyperrepressed state in which many of the metastable properties of telomeric silencing are lost. Third, recruitment of subtelomeric Sir3p^{N205} facilitates telomeric silencing even at large distances from the telomeric tract in wild-type *RAP1* cells. Fourth, in the absence of the C terminus of Rap1p, we have uncovered a novel Sir1p-stimulated process in which part or all of silencing is nucleated and spread from the site of Sir3p^{N205} tethering. Fifth, Sir3p and Sir3p^{N205} are also capable of nucleating silencing at internal positions in the presence of a short TG_{1-3} tract, an effect that is stimulated by a mutation that eliminates the Rap1p C-terminal 165 amino acids. Below, we discuss the implication of each of these findings.

The role of Rap1p in Sir3p recruitment. Our data indicate that two to three tethered Sir3p or Sir3p^{N205} molecules at the *URA3*-marked telomere (Table 2) or a single tethered molecule of Sir3p^{N205} at the *ADE2*-marked telomere (data not shown) is sufficient to nucleate telomeric silencing in *rap1-17* strains, which lack the region necessary for Sir3p association in a two-hybrid assay (33). If dimerization occurs through LexA, twice these numbers of molecules would be expected to be present at the tethered site. This result suggests that a primary function of the Rap1p C terminus in telomeric silencing is to recruit Sir3p and its associated factors to the telomere (Fig. 6A, bottom). One of these Sir3p-interacting factors is likely to be Sir4p, previously demonstrated to associate with Sir3p in two-hybrid assays (33). These data also suggest that recruitment of Sir3p to the telomere may be limiting in telomeric silencing. Only a few stably bound molecules of Sir3p are necessary to nucleate telomeric silencing in *rap1-17* strains. The dependence of this restoration of silencing on Sir2p and Sir4p suggests that tethered silencing shares mechanistic similarities with telomeric silencing, as previously characterized (1a). Importantly, tethering of Sir3p does not affect the telomere size defects in the *rap1-21* allele and therefore separates the functions of the Rap1p C terminus in telomeric silencing from size control (data not shown).

In addition to the ability of Sir3p tethering to partially or completely restore silencing in *rap1-17* strains, two additional lines of evidence argue that recruitment of Sir3p to the telomere is limiting in telomeric silencing. First, tethering of one or more LexA-Sir3p or LexA-Sir3p^{N205} molecules adjacent to a telomere in wild-type *RAP1* cells represses the telomere-adjacent gene to levels beyond that normally observed in wild-type cells (Fig. 5). Indeed, under these conditions, silencing cannot be fully overcome even under conditions of activation and exhibits stability resembling silencing at the *HM* loci.

Second, we have previously observed that elongation of telomeric tracts increases the level of repression in wild-type strains (26). These data suggest that a factor(s) limiting for telomere silencing is recruited with higher efficiency by longer TG_{1-3} tracts. Interestingly, silencing nucleated by LexA-Sir3p^{N205} is independent of telomere length in *rap1-17* cells.

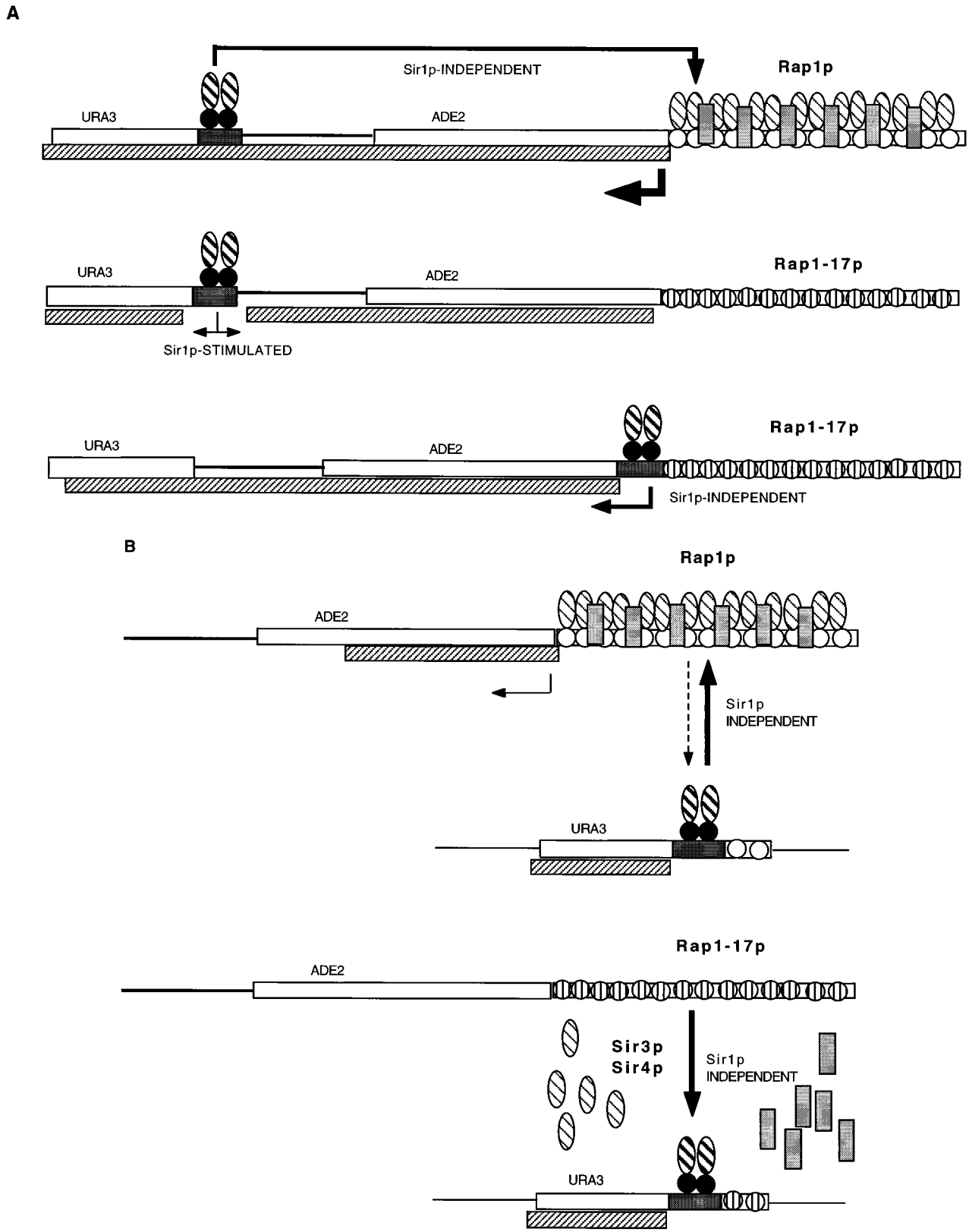

FIG. 6. Sir3p- and Sir3p^{N205}-tethered silencing at telomeric, subtelomeric, and internal sites. (A) (Top) When LexA-Sir3p^{N205} is tethered to a subtelomeric site, silencing initiated at the telomere and spreading into subtelomeric regions is hyperrepressed, presumably through interactions between the tethered LexA-Sir3p^{N205} and wild-type Rap1p, Sir3p, or Sir4p at the telomere (indicated by the arrow) in a Sir1p-independent process. (Middle) In a *rap1-17* strain, silencing can be initiated by LexA-Sir3p^{N205} in a Sir1p-stimulated fashion from the tethered site, probably because of loss of interaction with telomeric silencing proteins recruited by the Rap1p C terminus. The Sir1p stimulation of this process may occur by facilitating tethered silencing through either direct interactions with LexA-Sir3p^{N205} or a LexA-Sir3p^{N205}-recruited factor or facilitation of Sir3p and Sir4p recruitment. (Bottom) In contrast, in a *rap1-17* strain, silencing initiated by telomerically tethered

This finding suggests that the C terminus of Rap1p is necessary for the recruitment of factors, including Sir3p and Sir4p, responsible for the increased efficiency of telomeric silencing with increasing telomere tract length in wild-type cells.

That Sir3p recruitment to the telomere may be limiting for telomeric silencing was unexpected. Telomeres contain Rap1p binding sites spaced at a frequency of one in every 18 bp. Telomeres of wild-type size (≈300 bp) can associate with 17 Rap1p molecules (16), each of which is likely to be able to associate with one or more molecules of Sir3p. In addition, Sir3p is present at concentrations comparable to Rap1p in vivo (11). Hence, if Rap1p bound Sir3p efficiently, our data would suggest that silencing would be highly efficient under these conditions. Yet this is clearly not the case, even for strains containing telomeric sequences of greater length (26). Telomeric silencing is neither efficient nor stable and varies among strains, genes, and chromosomal termini examined (18).

How can the large number of Rap1p binding sites at the telomere be reconciled with the seemingly inefficient recruitment of Sir3p? One explanation is that Rap1p-Sir3p association is intrinsically weak, with Sir3p existing in an equilibrium between the associated and unassociated states (28). Given that binding of Rap1p to multiple sites is not strongly cooperative (16), a combination of two effects may facilitate Rap1p recruitment of Sir3p.

First, Rap1p is likely to act stochastically to recruit Sir3p. Multiple Rap1p binding sites may simply increase the probability of a stable association of Rap1p with Sir3p, leading to a silencing nucleation event. Second, additional protein-protein interactions may stabilize arrays of Rap1p-Sir3p association with the telomere. One possibility is that the complex is stabilized by multiple associations between Rap1p-bound Sir3p and Sir4p molecules.

Supporting this viewpoint, we have shown, in a separate study (28), that the Sir3p^{N205} suppressor acts synergistically with wild-type Sir3p to improve suppression of telomeric silencing defects conferred by some *rap1* alleles. Synergism is not observed, however, at *HML-E*, which contains only a single Rap1p binding site. A plausible interpretation of these data is that the tandem arrays of Rap1p present at the telomere nucleate the stable multimerization of Rap1p-Sir3p complexes through Sir3p-Sir3p and Sir3p-Sir4p interactions in *cis* or between clustered telomeres in *trans*. This model predicts that Sir3p has the ability to associate concurrently with Rap1p and either Sir3p or Sir4p. Although this has not been demonstrated biochemically, Sir3p and Sir4p homodimers and Sir3p-Sir4p heterodimers are able to form in two-hybrid systems (9, 33), consistent with this view. Genetic studies have also suggested the presence of interactions between Sir3p and Sir4p (32).

A large number of Sir3p molecules may be present at the telomere, therefore, not as a requirement for multiple Sir3p molecules for function per se, but as a means for stabilizing recruited Sir3p. This model would explain why only a few molecules of stably bound LexA-Sir3p or LexA-Sir3p^{N205} are sufficient to nucleate silencing in *rap1-17* cells and to hyperrepress telomeric silencing in wild-type cells.

Subtelomeric-telomeric dynamics are revealed by tethering of Sir3p^{N205} to subtelomeric positions. LexA-Sir3p^{N205} was used to investigate interactions between subtelomeric and telomeric regions. This gain-of-function protein is more efficient than Sir3p in promoting silencing from a tethered site, probably because of an enhanced ability to recruit downstream factors (28). The results detected by LexA-Sir3p^{N205} nonetheless represent a qualitative indicator of the types of processes that Sir3p can promote.

Tethering Sir3p^{N205} to a subtelomeric site 3.6 kb from the telomeric tract and approximately equidistant from the *URA3* and *ADE2* promoters leads to a hyperrepressed state in strains wild type for Rap1p. This hyperrepression has the expected characteristics of telomeric silencing, in that it is independent of Sir1p and spreads unidirectionally from the telomere sequentially into the *ADE2* and *URA3* genes.

One model which can explain these data is that tethering of Sir3p^{N205} to the subtelomeric site allows the physical association of Sir3p^{N205} with telomeric Rap1p, Sir3p, and/or Sir4p to form a stable complex at the telomere (Fig. 6A, top). This complex may recruit other factors and promote normal telomeric silencing in a unidirectional fashion. This model would predict the formation of a transient loop, allowing the association of subtelomeric and telomeric components prior to the nucleation of silencing. A similar looping mechanism may also help to explain the previous observation that the 6.7-kb Y′ elements, which are flanked by TG_{1-3} repeats, do not reduce the level of silencing of distal genes despite the movement of these genes to distances further from the telomere (37).

Supporting such a model, we observe that the nature of silencing is significantly altered when LexA-Sir3p^{N205} is tethered to a subtelomeric site in a *rap1-17* strain. Silencing can be both directed from the tethered site and stimulated by Sir1p in the absence of the Rap1p C terminus. The involvement of Sir1p in a subtelomeric context is unexpected. Possibly, the residual closed chromatin state in subtelomeric regions observed in *rap1-17* cells (26) hampers either the accessibility of LexA-Sir3^{N205} to the LexA site or the formation of a nucleation complex. Sir1p may help to overcome such an effect through facilitating the recruitment of other components of the nucleation complex or through direct alterations in chromatin structure allowing greater accessibility in this region. Alternatively, the presence of a nearby ORC may recruit Sir1p through interactions with the Orc1p subunit (44a) and facilitate silencing (10). However, the only known ORC binding site in this region is present ≈1 kb from the tethered site, immediately upstream of the *ADE2* promoter (44c). The participation of Sir1p-Orc1p interaction with the tethered LexA-Sir3^{N205} over this distance seems unlikely.

Could a mechanism of association between subtelomeric and telomeric sequences play a physiological role? Among the many aspects of Sir3p is its ability to interact with the N termini of histones H3 and H4 (20). Indeed, overproduction of Sir3p increases the distances over which silencing can be spread, probably through facilitated associations with the histones (37). Our data raise the possibility that subtelomeric Sir3p associated with histones may be able to interact with factors at the telomere. Such an interaction may provide a means to stabilize telomeric silencing. Interactions between subtelomeric and telomeric factors could form a highly folded struc-

LexA-Sir3p^{N205} does not require Sir1p and proceeds unidirectionally from the telomere. (B) Depiction of the proposed equilibrium between telomeric and internal silencers. In wild-type strains (top), Sir3p and Sir4p are sequestered at the telomere, limiting their availability for internal silencers. In contrast, in the *rap1-17* mutant (bottom), in which Sir3p and Sir4p are unable to associate with the telomere, the larger free pool of these proteins stimulates internal silencing nucleated by tethered Sir3p^{N205}. Regions are not drawn to scale. Symbols: white circles, Rap1p; striped circles, Rap1-17p; light striped ovals, Sir3p; dark striped ovals, Sir3p^{N205}; stippled boxes, Sir4p; black circles, LexA; striped box, region of subtelomeric silent chromatin. The direction and intensity of the arrows refer to the directionality and efficiency of silencing, respectively.

ture that may also explain the inaccessibility of these regions to in vivo and in vitro probes (17, 26, 34, 42).

A competition between telomeric and internal silencers? We have demonstrated that Sir3p^{N205}, and to a lesser extent Sir3p, when tethered downstream of the *URA3* gene, is capable of nucleating low levels of *SIR1*-independent silencing at internal chromosomal sites in the presence of an adjacent short TG_{1-3} tract (Fig. 6B). On the basis of these data, we propose two minimal requirements for internal silencing: (a) the ability to recruit Sir3p and (b) the ability to bind Rap1p, the latter possibly acting through alterations in DNA structure.

The efficiency of internal tethered silencing is increased in *rap1-17* cells. One formal possibility is that Rap1-17p, which binds to its cognate site with wild-type efficiency, is better able to alter DNA structure in a fashion that facilitates telomeric silencing. However, there is no evidence that Rap1-17p affects DNA structure differently than Rap1p. Therefore, we favor a simpler model in which limiting factors, including Sir3p and Sir4p, normally sequestered to the telomere, are released in *rap1-17* cells and are hence more accessible to the internal silencers (Fig. 6B).

This study helps to explain the findings of Stavenhagen and Zakian (44) that long ≈900-bp telomeric tracts (termed $C_{1-3}A$ silencers) confer internal silencing at internal sites independent of the telomere, if sufficient internal Sir3p recruitment requires such an elongated tract of TG_{1-3}. These data also may explain the enhanced level of silencing observed in that study when $C_{1-3}A$ silencers were brought into closer proximity to the telomere (44). This situation may be comparable to the hyperrepression that we observe after subtelomeric tethering of Sir3p^{N205} in wild-type cells. If true, these data would predict that $C_{1-3}A$ silencing near a telomere may be initiated at the telomere through associations between factors recruited by subtelomeric and telomeric TG_{1-3} tracts.

Telomeric silencing has been observed in a number of eukaryotic organisms including *S. pombe*, trypanosomes, and *D. melanogaster* (1, 20a, 27a, 39, 48). The studies presented in this paper provide a paradigm for (a) the function of telomere-binding proteins as recruitment factors for proteins acting in more specific functions, such as silencing; (b) the nature of subtelomeric-telomeric interactions in the silencing process; and (c) the competition between telomeric and internal silencers for limiting factors. It may also serve as a model for the mechanism by which heterochromatin can be established through the recruitment of specific proteins that nucleate and spread a closed chromatin state into adjacent regions. The tethering system presented here gives us the opportunity to dissect the domain structure of one of these proteins, Sir3p, and identify and characterize other factors that act in the silencing pathway.

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URA3/ADE2/LexAS3
marked VIIL

URA3/ADE2/LexAS1-1
marked VIIL

lexA

lexA-SIR3

lexA-SIR3N205