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# Sir3p Domains Involved in the Initiation of Telomeric Silencing in *Saccharomyces cerevisiae*

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ABSTRACT

Previous studies from our laboratory have demonstrated that tethering of Sir3p at the subtelomeric/telomeric junction restores silencing in strains containing Rap1-17p, a mutant protein unable to recruit Sir3p. This tethered silencing assay serves as a model system for the early events that follow recruitment of silencing factors, a process we term initiation. A series of LexA fusion proteins in-frame with various Sir3p fragments were constructed and tested for their ability to support tethered silencing. Interestingly, a region comprising only the C-terminal 144 amino acids, termed the *C-t*erminal *d*omain (CTD), is both necessary and sufficient for restoration of silencing. Curiously, the LexA-Sir3$^{N205}$ mutant protein overcomes the requirement for the CTD, possibly by unmasking a cryptic initiation site. A second domain spanning amino acids 481–835, termed the *n*onessential for *i*nitiation *d*omain (NID), is dispensable for the Sir3p function in initiation, but is required for the recruitment of the Sir4p C terminus. In addition, in the absence of the N-terminal 481 amino acids, the NID negatively influences CTD activity. This suggests the presence of a third region, consisting of the N-terminal half (1–481) of Sir3p, termed the *p*ositive *r*egulatory *d*omain (PRD), which is required to initiate silencing in the presence of the NID. These data suggest that the CTD "active" site is under both positive and negative control mediated by multiple Sir3p domains.

ONE of the least understood facets of eukaryotic gene expression is the regional repression and derepression of transcription. Position-dependent effects on transcription have been observed in a wide variety of organisms, including *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, Drosophila, and vertebrates. Such repressive domains are associated with the remodeling of chromatin into heterochromatic-like "closed" chromatin states.

The yeast *S. cerevisiae* has served as an excellent model system to study position effects. In yeast, several discrete loci exhibit context-dependent effects on transcription (Lustig 1998). These include the silent *HM* loci (*HML* and *HMR*) that encode cryptic mating-type information and sequences adjacent to telomeres, which are the protein-DNA complexes present at the chromosomal termini.

Genes positioned in the vicinity of telomeres undergo epigenetic switching between repressed and derepressed transcriptional states (Gottschling *et al.* 1990; Aparicio *et al.* 1991). This long-range influence on transcription spreads unidirectionally from the telomere and exhibits a gradient of repression; repression is highest adjacent to the telomere and decreases rapidly with increasing distance from the telomere (Renauld *et al.* 1993). Telomeric position effect, also called telomeric silencing, serves as an ideal system to investigate both the impact of telomeric structures on neighboring sequences and the formation of heterochromatic domains.

Telomeric silencing in yeast has been investigated by genetic, molecular biological, and biochemical techniques. On the basis of these studies, telomeric silencing can be separated into at least three (not necessarily mutually exclusive) steps (Lustig 1998) analogous to those involved in higher eukaryotic heterochromatin formation: (1) the targeting or recruitment of silencing factors to the telomere, (2) the subsequent initiation of silencing, and (3) the "spreading" of a closed chromatin state unidirectionally along the chromatin fiber.

One of the central steps in targeting is the association of the telomeric-binding protein Rap1p to high-affinity sites embedded within the telomeric poly $(TG)_{1\text{-}3}$ simple sequence tract at an average frequency of once every 18 bp (Shore 1995). The Rap1p C-terminal domain, consisting of the terminal 165 amino acids of this 827-amino-acid protein, can associate with the silencing factors Sir3p and Sir4p (Moretti *et al.* 1994; Cockell *et al.* 1995; Liu and Lustig 1996). Indeed, tethering of either Sir3p or Sir4p restores silencing in *rap1* mutants encoding proteins unable to recruit these factors (Lustig *et al.* 1996; Maillet *et al.* 1996). Interestingly, the Rap1p C-terminal domain also associates with components of the telomere-size machinery, Rif1p and Rif2p, which antagonize silencing and compete with Sir3p and Sir4p for Rap1p association (Hardy *et al.* 1992; Marcand *et al.* 1997; Wotton and Shore 1997). One of the determining factors in shifting this competition toward silencing may be the presence of a microenvironment (Boscheron *et al.* 1996; Marcand *et al.* 1997) that favors formation of a telomeric structure required for the initi-

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ation of silencing, possibly through the clustering of telomeres at the nuclear periphery (Gotta *et al.* 1996) and the ability of Sir3p and Sir4p domains to homo- and heterodimerize (Chien *et al.* 1991; Moretti *et al.* 1994).

The use of *in vivo* formaldehyde cross-linking coupled with immunoprecipitation and PCR methodologies has led to a major advance in understanding the "spreading" of repressed chromatin from the telomere (Hecht *et al.* 1996; Strahl-Bolsinger *et al.* 1997). These studies have shown that Sir2p, Sir3p, and Sir4p are present in subtelomeric chromatin in a gradient that parallels the phenotypic gradient. These structural data are consistent with the finding that both Sir3p and Sir4p interact with the N-terminal tails of histones H3 and H4 *in vitro* (Hecht *et al.* 1995). An alteration in chromatin structure in subtelomeric regions is also inferred by the observation that mutations in *SIR2*, *SIR3*, and *SIR4* enhance accessibility of subtelomeric chromatin to exogenous probes *in vivo* (Gottschling 1992; C. Zhang and A. J. Lustig, unpublished data). Subtelomeric chromatin, like other silenced regions, is also associated with the presence of a specific subset of histones H3 and H4, which may be modified by specific acetyltransferases and deacetylases and/or the deposition of modified histones by chromatin assembly factors (Grunstein 1997).

The silencing factor Sir3p appears to be involved in each step of telomeric silencing (Stone and Pillus 1998). This central role may be mediated by the ability of Sir3p to associate with both Rap1p and the N termini of histones H3 and H4. Indeed, an identical dominant mutation in *SIR3* (*SIR3$^{N205}$*, *SIR3R1*), resulting in the substitution of an asparagine for an aspartic acid at position 205, was independently identified as a suppressor of defects in the Rap1p C terminus and in the N-terminal tail of histone H4 (Johnson *et al.* 1990; Liu and Lustig 1996).

As opposed to the recruitment and spreading steps, the molecular communication between the telomeric silencer and subtelomeric chromatin that initiates the silencing process is poorly understood. We have previously used a tethered silencing system to investigate early events in the silencing process (Lustig *et al.* 1996). In this system, LexA-Sir3p fusion proteins are targeted to artificially introduced LexA binding sites at the telomeric/subtelomeric junction in the presence of wild-type Sir3p to ensure subsequent spreading (Figure 1). Tethering of LexA-Sir3p to these sites overcomes the requirement for the C-terminal domain of Rap1p, suggesting that recruitment of Sir3p is a critical step in initiation.

To better understand the role of Sir3p in the initiation of silencing, we conducted a functional domain analysis of Sir3p using the tethered silencing assay as a model system for initiation. Our studies also indicate that a domain, consisting of only the C-terminal 144 amino acids, is both necessary and sufficient for the initiation of silencing; and our data suggest the presence of Sir3p domains that both positively and negatively regulate C-terminal activity in initiation.

## MATERIALS AND METHODS

**Plasmids:** All plasmids encoding LexA fusion proteins were derived from pBTM-SIR3 or pBTM-SIR3$^{N205}$ (Lustig *et al.* 1996) with transcription driven from the *ADH1* promoter. LexA-SIR3 (1–356) was constructed by cleaving pBTM-SIR3 with *Eag*I and *Asp*718, "filling in" with a Klenow fragment, and ligating the resulting blunt ends. The LexA fusion protein contains sequences in-frame with the first 356 amino acids of Sir3p, followed by an out-of-frame 8-amino-acid tail preceding a stop codon. LexA-SIR3 (1–356; 836–978) was constructed by digesting pBTM-SIR3 with *Eag*I and *Kpn*I and filling in the ends with Klenow and T4 DNA polymerase. The plasmid lacking the *Eag*I/*Kpn*I fragment was purified by gel electrophoresis, and the blunt ends were ligated. The resulting plasmid contains an in-frame deletion between amino acids 356 and 836. pBTM-SIR3 (1–481) was constructed by cleaving pBTM-SIR3 with *Age*I and *Xho*I, filling in with Klenow fragment, and ligating the resulting blunt ends. The product contains an in-frame fusion of the N-terminal 481 amino acids with an out-of-frame 9-amino-acid tail preceding a stop codon. LexA-SIR3 (1–481; 835–978) was constructed by cleaving pBTM-SIR3 with *Age*I and *Asp*718. The resulting vector was treated with Klenow and the blunt-ended products were ligated. This produces a fusion protein containing an in-frame deletion between amino acids 481 and 835. LexA-SIR3 (356–481) was constructed by ligating a blunt-ended *Eag*I/*Age*I fragment from Sir3p into a *Bam*HI-cleaved and blunt-ended pBTM vector. The resulting construct is in-frame with LexA and contains a C-terminal extension of 17 amino acids preceding a stop codon. LexA-SIR3 (1–835) and LexA-SIR3$^{N205}$ (1–835) were constructed by cleaving the respective full-length plasmids with *Asp*718, filling in the 5′ overhangs with Klenow, and ligating the blunt ends. The resulting products contain LexA in-frame with Sir3p sequences from amino acids 1 to 835 followed by an out-of-frame 7-amino-acid tail preceding a stop codon. LexA-SIR3 (1–835; 945–978) and LexA-SIR3$^{N205}$ (1–835; 945–978) were constructed by cleaving the respective full-length plasmids with *Asp*718 and *Xho*I, filling in the cohesive ends, and ligating the blunt ends. This produces an in-frame deletion of amino acids 836–944. LexA-SIR3 (356–978) was generated by cleaving pBTM-SIR3 with *Bam*HI and *Eag*I, filling in the 5′ overhangs with Klenow, and ligating the resulting blunt ends. LexA-SIR3 (481–978) was constructed by cleaving pBTM-SIR3 with *Sal*I and *Age*I, filling in the 5′ overhangs with Klenow, and ligating the blunt ends. LexA-SIR3 (835–978) was constructed by digesting pBTM-SIR3 with *Bam*HI and *Asp*718 and filling in the 5′ overhangs with Klenow. The fragment lacking the region from *Bam*HI to *Asp*718 was gel purified and the blunt ends ligated. The resulting plasmid contains an in-frame fusion between LexA and amino acids 835–978 of Sir3p. Fusion proteins are depicted diagramatically in Figure 2.

The sites of the in-frame fusion for LexA-Sir3p (835–978) and LexA-Sir3p (1–356; 836–978) were additionally confirmed by DNA sequencing. The presence of each construct in yeast was confirmed by Southern analysis. We carried out enhanced chemiluminescence (ECL) Western blot analysis (Amersham, Arlington Heights, IL) as previously described (Lustig *et al.* 1996) for each construct after transformation into CLY3/*rap1-17*.

The plasmid pCTC48, containing amino acids 839–1358 of Sir4p in-frame with the Gal4p activation domain (GAD) has been described previously (Moretti *et al.* 1994).

**Yeast strain constructions:** The strains used in this study are

**TABLE 1**

**Yeast strains**

| Strain | Genotype | Reference |
|---|---|---|
| W303a | *MAT***a** *rap1::LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1* | Kurtz and Shore (1991) |
| CLY1/*rap1-17* | *MATα rap1::LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 VIIL::URA3 pRS313/rap1-17* | Lustig *et al.* (1996) |
| CLY3/*rap1-17* | *MATα rap1::LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 VIIL::URA3 LexAS3*[a] *pRS313/rap1-17* | Lustig *et al.* (1996) |
| CLY3/*rap1-17*$^{\Delta SIR2}$ | *MATα rap1::LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 sir2::ura3 VIIL::URA3 LexAS3 pRS313/rap1-17* | Lustig *et al.* (1996) |
| CLY3/*rap1-17*$^{\Delta SIR4}$ | *MATα rap1::LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 sir4::ura3 VIIL::URA3 LexAS3 pRS313/rap1-17* | Lustig *et al.* (1996) |
| CLY/*RAP1* | *MAT***a** *rap1::LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 VIIL::URA3 pRS313/RAP1* | Lustig *et al.* (1996) |
| CLY3/*rap1-21* | *MATα rap1::LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 VIIL::URA3 LexAS3 pRS313/rap1-21* | Lustig *et al.* (1996) |
| CLY3/*rap1-21*$^{\Delta SIR3}$ | *MATα rap1::LEU2 leu2-3,112 his3 ade2-1 trp1 ura3-1 sir3::ADE2 VIIL::URA3 LexAS3 pRS313/rap1-21* | This study |
| CTY10-5d/pCTC48 | *MAT***a** *ade2 trp1-901 leu2-3,112 his3-200 gal4 gal80 URA3::lexA op-lacZ pCTC48(aa 839-1358)* | Moretti *et al.* (1994) |

[a] LexAS3 refers to the presence of three intact LexA binding sites at the telomeric/subtelomeric junction.

listed in Table 1. With the exception of CTY10-5d/pCTC48, strains are isogenic to the progenitor strain W303. All strains have been described previously as indicated in Table 1 with the exception of CLY3/*rap1-21*$^{\Delta sir3}$. CLY3/*rap1-21*$^{\Delta sir3}$ was derived by construction of a *sir3::ADE2* null allele (Liu and Lustig 1996) in CLY3/*RAP1*, followed by a plasmid shuffle to replace the *RAP1*-containing plasmid pD130 with pRS313/*rap1-21. rap1-21* encodes a Rap1p species lacking the terminal 28 amino acids that is incapable of recruiting Sir3p or supporting tethered silencing. Media and growth conditions were performed by standard techniques (Kaiser *et al.* 1994).

**Silencing assays:** 5-FOA assays for telomeric silencing were performed as described using selective media for maintaining plasmids (Lustig 1996). Except where indicated, median values were determined from at least two fluctuation tests consisting of six to seven independent colonies per fluctuation and are presented together with the range of values observed.

**Mating-type assays:** CLY3/*rap1-21*$^{\Delta SIR3}$ (*HMLα MATα HMRα*) transformants carrying the fusion proteins that were assayed for tethered silencing were also tested for α mating, a reflection of *HMR***a** silencing. Since all available markers in the transformants were utilized, we crossed each strain with W303a and examined the ability of cells to grow on minimal 5-FOA media. With one exception, neither haploid can grow on this media. However, because the *sir3* mutation is recessive, growth on 5-FOA media would be regained only if cells were capable of mating. In the case of the exception, LexA-Sir3p$^{N205}$(1–835), colonies capable of growth on 5-FOA media were tested for sporulation following mating. In control studies, the LexA fusion proteins did not interfere with mating in the CLY3/*rap1-21* strain containing the wild-type *SIR3* gene.

**Two-hybrid methodology:** To assay two-hybrid interaction of GAD-Sir4p (839–1358) with the LexA-Sir3p fusion proteins, the mean activities of β-galactosidase were determined (in Miller units) in cell extracts derived from three to six independent transformants as described (Kaiser *et al.* 1994).

## RESULTS

We have previously described a system designed to reflect the initiation of telomeric silencing (Figure 1; Lustig *et al.* 1996). This assay measures the ability of proteins tethered at the telomeric/subtelomeric junction to restore silencing in a strain carrying Rap1-17p, lacking the C-terminal 165 amino acids of Rap1p. Rap1-17p is unable to recruit either Sir3p or Sir4p, thereby abrogating telomeric silencing. In this assay, LexA binding sites were placed at the junction between telomeric tracts and a fragment containing the *URA3* gene positioned at the left end of chromosome *VII* (VIIL). Silencing is measured by the ability of cells to form colonies on 5-FOA, a uracil analog that allows growth of Ura3$^-$, but not Ura3$^+$, cells. While silencing is not regained in strains either producing LexA alone or lacking LexA binding sites, it is restored to near wild-type values in strains carrying both LexA binding sites and the LexA-Sir3p fusion protein (Lustig *et al.* 1996). These data suggest that tethering of Sir3p provides a function, defined here as initiation, that is missing in *rap1-17* cells. To elucidate the minimal domains of Sir3p required for initiation, we have taken advantage of the tethering

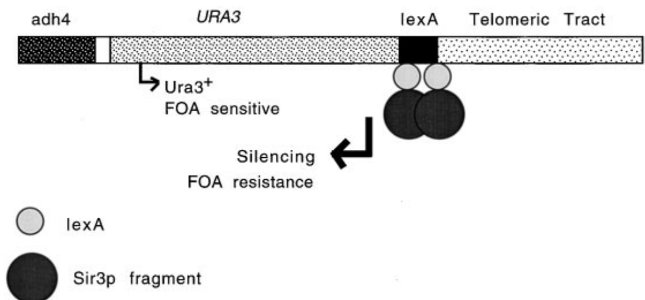


Figure 1.—Structure of the VIIL-*URA3* marked telomere. At the VIIL-*URA3* marked telomere, silencing is assayed by the frequency of 5-FOA$^r$ cells. The large arrow indicates the direction of silencing from the tethered site, while the small arrow indicates the direction of *URA3* transcription.

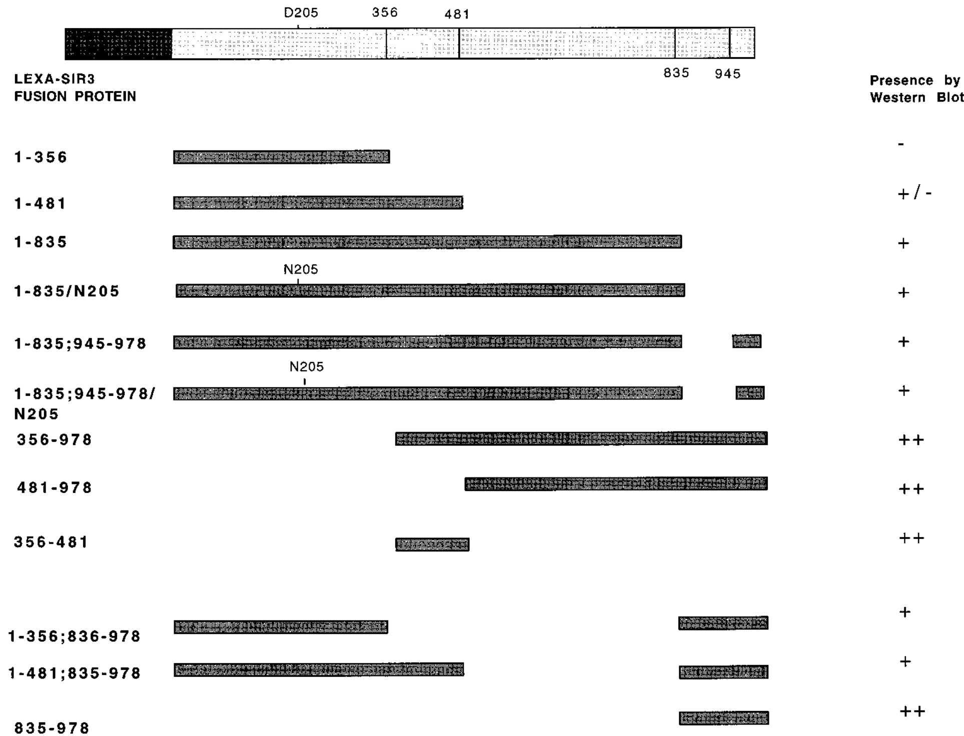


Figure 2.—LexA-fusion proteins used in this study. Bars represent the remaining region of protein present after deletion. Western blot data was derived from cell extracts containing the indicated fusion proteins in CLY3/*rap1-17*. ++ and +, the approximate relative abundance of the proteins based on ECL Western analysis; +/−, the low and variable amounts of LexA-Sir3p (1–481) on Western blots; −, the absence of LexA-Sir3p (1–356) on Western blots.

assay in the presence of wild-type Sir3p to ensure subsequent spreading. We measured two parameters: (1) restoration of silencing in *rap1-17* cells, and (2) stimulation or interference with other steps in silencing.

We tested a battery of fusion proteins (Figure 2) for their ability to confer telomeric silencing in a *rap1-17* strain. With two exceptions, fusion proteins were determined to be present based on Western blot analysis and, where relevant, by their ability to interact with the C terminus of Sir4p in a two-hybrid system (Table 2). The two exceptions were LexA-Sir3p (1–356), which was not apparent on the Western blots, and LexA-Sir3p (1–481), which was present in low and variable levels on Western blots. We note this instability of the N-terminal fragments is in agreement with the results from Susan Gasser's laboratory (Gotta *et al.* 1998) and precluded further analysis.

The cellular levels of the remaining fusion proteins do not correlate with the frequency of initiation. Hence, it is unlikely that differences in abundance among the fusion proteins can explain the observed differences in initiation.

**A "minimal" C-terminal silencer necessary and sufficient for tethered silencing:** We initially tested the importance of the Sir3p C terminus in initiation. The C terminus has been implicated in associations with histones H3 and H4, Sir3p, and Sir4p (Stone and Pillus 1998). To investigate the role of the extreme C terminus in tethered silencing, we constructed a fusion protein, LexA-Sir3p (1–835) lacking only the C-terminal 143 amino acids, a region we term the C-terminal domain (CTD; Table 3). Elimination of the CTD decreased FOA$^r$ frequencies 238-fold relative to the wild-type fusion protein. Similarly, an in-frame deletion extending from amino acids 836–944 [LexA-Sir3 (1–835; 945–978)] failed to restore silencing. Neither protein conferred a dominant negative effect in wild-type cells, indicating the absence of interference with the wild-type silencing machinery (Table 4).

**TABLE 2**

**Two-hybrid association between Sir3p domains and the C terminus of Sir4p**

| Protein | β-Gal units/mg[a] |
|---|---|
| LexA | 4.5[b] (3) |
| LexA-Sir3p | 77 ± 24[c] (6) |
| LexA-Sir3p (1–835) | 53 ± 4 (3) |
| LexA-Sir3p (1–835; 945–978) | 24 ± 7.4 (3) |
| LexA-Sir3p (356–978) | 96 ± 7.4 (3) |
| LexA-Sir3p (481–978) | 207 ± 0.72 (3) |
| LexA-Sir3p (356–481) | 3.7 (3) |
| LexA-Sir3p (1–356; 836–978) | 5.3 (3) |
| LexA-Sir3p (1–481; 835–978) | 2.5 (3) |
| LexA-Sir3p (835–978) | 1.9 (3) |
| LexA-Sir3p$^{N205}$ | 44 ± 0.7 (3) |
| LexA-Sir3p$^{N205}$ (1–835) | 25 ± 3.6 (3) |
| LexA-Sir3p$^{N205}$ (1–835; 945–978) | 16 ± 3.1 (3) |

All values were derived from β-galactosidase assays in CTY10-5d cell extracts containing the indicated protein and GAD-Sir4p (839–1358).

[a] Mean values with standard deviations are presented with the number of transformants tested indicated in parentheses.

[b] Mean values between 1.9 and 5.3 indicate the absence of association and represent background values.

[c] Standard deviations for LexA-Sir3p were consistently higher than for other fusion proteins.

**TABLE 3**

**Restoration of silencing in *rap1-17* cells directed by the Sir3p CTD**

| Plasmid | $LexA_{BS}$[a] | Median $FOA^r$[b] | $FOA^r/FOA^r$ SIR3[c] |
|---|---|---|---|
| pBTM[d] | + | $<1 \times 10^{-6}$ (28) | <0.00012 |
| pBTM-SIR3[d] | + | $8.1 \times 10^{-3}$ (0.9–50; 27) | 1 |
| pBTM-SIR3 (1–835) | + | $4.9 \times 10^{-5}$ (0–116; 21)[e] | 0.0042 |
| pBTM-SIR3 (1–835; 945–978) | + | $2.3 \times 10^{-6}$ (0–22; 14)[f] | 0.00028 |
| pBTM-SIR3 (835–978) | − | $<1.3 \times 10^{-5}$ (7) | <0.0016 |
| pBTM-SIR3 (835–978) | + | $2.0 \times 10^{-2}$ (0.4–5.0; 14) | 2.5 |

[a] The strains used in this table are CLY3/*rap1-17* (+) containing three LexA binding sites and CLY1/*rap1-17* (−) lacking any LexA binding sites. All strains carry a wild-type copy of Sir3p.

[b] In this and subsequent tables, $FOA^r$ values are presented as medians with both the range of observed values (in the exponent listed for the median) and the number of samples (in parentheses). Ranges are not listed for samples that displayed an undetectable median number of $FOA^r$ colonies.

[c] Ratios of values generated in CLY3/*rap1-17* in cells containing LexA-Sir3p domain fusion relative to the value generated by LexA-Sir3p.

[d] Lustig *et al.* (1996).

[e] Two samples failed to yield any $FOA^r$ colonies in 53,000 and 20,000 cells plated.

[f] Five samples failed to yield any $FOA^r$ colonies in 400,000 cells plated.

These results are consistent with a function for the CTD, either alone or in conjunction with other domains, in the initiation process. To distinguish between these possibilities, we constructed a fusion protein containing LexA in-frame with the C-terminal 144 amino acids [LexA-Sir3p (835–978)]. Unexpectedly, LexA-Sir3p (835–978) conferred LexA-site-dependent silencing at $FOA^r$ frequencies similar to intact LexA-Sir3p (Table 3). These data indicate that the CTD, when present in the absence of other Sir3p sequences, is sufficient for the initiation of telomeric silencing. The CTD does not act through a bypass pathway, as its ability to restore silencing is fully dependent on Sir2p (data not shown). In addition, as for LexA-Sir3p, CTD requires wild-type Sir3p for activity (Table 4). Therefore, the results obtained with this fusion indicate that the CTD is both necessary and sufficient for initiation.

**Internal deletions of Sir3p define a region nonessential for restoration of telomeric silencing:** To test whether other regions of Sir3p alter the behavior of the

**TABLE 4**

**Characteristics of Sir3p domains**

| Plasmid | Initiation[a] | Dependence on wild-type Sir3p[b] | Dominant negative[c] | Sir4C interaction[d] |
|---|---|---|---|---|
| pBTM | − | NA | NT | − |
| pBTM-SIR3 | ++ | +($<4.81 \times 10^{-6}$; 1) | − | ++ |
| pBTM-SIR3[N205] | +++ | ±($2.7 \times 10^{-2}$; 1) | − | + |
| pBTM-SIR3 (1–835) | − | +($<3.8 \times 10^{-6}$; 1) | − | ++ |
| pBTM-SIR3 (1–835)[N205] | +++ | ±($1.2 \times 10^{-2}$; 2) | − | ++ |
| pBTM-SIR3 (1–835; 945–978) | − | NT | − | + |
| pBTM-SIR3[N205] (1–835; 945–978) | − | NT | − | + |
| pBTM-SIR3 (356–978) | − | NT | ++ | ++ |
| pBTM-SIR3 (481–978) | − | +($<1.8 \times 10^{-6}$; 2) | − | +++ |
| pBTM-SIR3 (356–481) | − | NT | − | − |
| pBTM-SIR3 (1–356; 836–978) | + | +($<1.8 \times 10^{-6}$; 2) | NT | − |
| pBTM-SIR3 (1–481; 835–978) | ++ | +($<1.5 \times 10^{-6}$; 2) | − | − |
| pBTM-SIR3 (835–978) | ++ | +($<1.5 \times 10^{-6}$; 2) | NT | − |

NA, not applicable; NT, not tested.

[a] Initiation values were determined in CLY3/*rap1-17*. +++, values exceeding 0.01; ++, $FOA^r$ values falling between 0.001 and 0.01; +, values between 0.001 and 0.0001; −, any values falling below this value. The initiation activity for pBTM, pBTM-SIR3, and pBTM-SIR3[N205] was previously reported in Lustig *et al.* (1996).

[b] $FOA^r$ values determined in CLY3/rap1-21$^{\Delta sir3}$. +, dependence on Sir3p; ±, partial dependence; −, no dependence. The $FOA^r$ values and the number of fluctuations are given in parentheses.

[c] Dominant negative phenotypes were determined by $FOA^r$ values in CLY/RAP1 strains containing the indicated fusion protein.

[d] Sir4 interactions are based on data in Table 2. +++, values above 100 units; ++, values between 50 and 100 units; +, values between 15 and 45 units; −, values below 15 units.

**TABLE 5**

**The effect of Sir3p internal domains on restoration of silencing in *rap1-17* strains**

| Plasmid | Median FOA$^r$ | FOA$^r$/FOA$^r$ SIR3 |
|---|---|---|
| pBTM[a] | $<1 \times 10^{-6}$ (28) | <0.00012 |
| pBTM-SIR3[a] | $8.1 \times 10^{-3}$ (0.9–50; 27) | 1 |
| pBTM-SIR3 (1–356; 836–978)[b] | $3.0 \times 10^{-4}$ (1–14; 14) | 0.037 |
| pBTM-SIR3 (1–481; 835–978) | $1.3 \times 10^{-3}$ (0.13–24; 21) | 0.16 |
| pBTM-SIR3 (481–978) | $1 \times 10^{-5}$ (0–7.8; 14)[c] | 0.0012 |

All assays were conducted following transformation of the indicated plasmid into CLY3/*rap1-17*.

[a] Values derived from Table 3.

[b] In cells lacking the LexA binding site, microcolonies not observed in tethered derivatives occur at variable frequencies of up to $1 \times 10^{-5}$.

[c] Two samples failed to produce any FOA$^r$ colonies in $1.2 \times 10^6$ cells plated.

CTD, we sought to define the role of internal domains in silencing. We constructed two LexA fusion proteins that contained in-frame deletions between either amino acids 356 and 836 or amino acids 481 and 835 (Table 5). As expected, both fusion proteins conferred significant silencing. Fusion proteins containing the N-terminal 356 amino acids in-frame with the CTD [LexA-Sir3p (1–356; 836–978)] restored telomeric silencing to median values 25-fold lower than observed with LexA-Sir3p. More strikingly, fusion of the N-terminal 481 amino acids in-frame with the CTD [LexA-Sir3p (1–481; 835–978)] conferred FOA$^r$ values only 6-fold lower than conferred by LexA-Sir3p. No restoration of silencing was observed in the absence of LexA binding sites (data not shown).

Telomeric silencing, in this context, is dependent on both *SIR2* and *SIR4*, as expected for events occurring through the conventional silencing pathway (data not shown), and on a wild-type copy of Sir3p (Table 4). These data demonstrate that the region between amino acids 481 and 835 is dispensable for the initiation function of Sir3p in telomeric silencing. For simplicity, we refer to this region as the nonessential for initiation domain (NID).

Curiously, the fragment that contains both the NID and the CTD [LexA-Sir3p (481–978)] resulted in a protein virtually inactive in the initiation of silencing, conferring an 810-fold decrease in FOA$^r$ colonies relative to wild type. Note that this "masking" of the CTD is not due to any of the following: loss of the protein, as judged by Western analysis; inactivity, as assayed by its efficient interaction with the C terminus of Sir4p; or interference with other steps in silencing, as indicated by its lack of dominant-negative behavior in wild-type cells (Table 4).

**A Sir3p domain required for recruitment of the Sir4 C terminus overlaps the NID:** Earlier studies defined a region of Sir3p (309–978) that interacts with the Sir4p C terminus (amino acids 1204–1356; Moretti *et al.* 1994). To further define the site of Sir4 interaction within Sir3p, we conducted two-hybrid analysis using the LexA fusion proteins designed in this study (Table 2). Values obtained in this assay were similar to those previously reported for LexA-Sir3p (Moretti *et al.* 1994). Our results indicate that truncation of neither the N-terminal 481 amino acids nor the CTD reduced association with GAD-Sir4p (839–1358). In contrast, an in-frame-deletion removing the NID (amino acids 481–835) abrogated association, despite its functionality in the tethering assay. These data map a region between amino acids 481 and 835 that is responsible for interaction with the Sir4p C terminus.

The LexA-Sir3$^{N205}$ mutant protein also shows significant association with Sir4p, suggesting that the increased silencing observed in this mutant protein is unlikely to be the consequence of the failure to recruit Sir4p. Given the apparent lower association of the CTD in-frame deletion [LexA-Sir3p(1–835; 945–978)] with the Sir4 C terminus (Table 2), we cannot exclude the possibility, however, that the CTD plays a regulatory role in Sir4p association.

**The requirement for the Sir3p C terminus is dependent on N-terminal sequences:** The *SIR3$^{N205}$* (*SIR3R1*) mutation was identified as a suppressor of the silencing defects of both mutant histone H4 proteins defective in the N-terminal tail and mutant Rap1 proteins containing defects in the C-terminal 28 amino acids (Johnson *et al.* 1990; Liu and Lustig 1996). The D205N substitution in several fusion proteins provided additional insight into the interplay between the domains of Sir3p.

While LexA-Sir3p (1–835) was unable to efficiently support silencing in a *rap1-17* strain, LexA-Sir3p$^{N205}$ (1–835) displayed levels of silencing identical to LexA-Sir3p$^{N205}$ (Table 6). These data indicate that the LexA-Sir3$^{N205}$ mutant protein can overcome the requirement for the C-terminal domain. This finding suggests that sequences N-terminal to the CTD are, in some fashion, "activated" for both the initiation and spreading of silencing by the D205N amino acid substitution.

This conclusion appears to extend to fusion proteins in a *sir3* null strain. The activity of the LexA-Sir3p$^{N205}$ fusion protein was previously demonstrated to function

**TABLE 6**

**The effect of D205N on Sir3p C-terminal requirements for the restoration of silencing in *rap1-17* strains**

| Plasmid | Median FOA$^r$ | FOA$^r$/FOA$^r$ SIR3 |
|---|---|---|
| pBTM[a] | $<1 \times 10^{-6}$ (28) | <0.00012 |
| pBTM-SIR3[a] | $8.1 \times 10^{-3}$ (0.9–50; 27) | 1 |
| pBTM-SIR3 (1–835) | $4.9 \times 10^{-5}$ (0–130; 21)[b] | 0.006 |
| pBTM-SIR3 (1–835; 945–978) | $2.3 \times 10^{-6}$ (0–22; 14)[c] | 0.00028 |
| pBTM-SIR3$^{N205}$ | $36 \times 10^{-2}$ (18–52; 14) | 1 |
| pBTM-SIR3$^{N205}$ (1–835) | $24.1 \times 10^{-2}$ (6.3–48.7; 21) | 0.67 |
| pBTM-SIR3$^{N205}$ (1–835; 945–978) | $<1 \times 10^{-6}$ (14) | <0.0000028 |

All assays were conducted following transformation of the indicated plasmid into CLY3/*rap1-17*.
[a] Values from Table 3.
[b] Two samples failed to yield any FOA$^r$ colonies in 53,000 and 20,000 cells plated.
[c] Five samples failed to yield any FOA$^r$ colonies in 400,000 cells plated.

in a *sir3* mutant strain (Lustig *et al.* 1996). Interestingly, LexA-Sir3p$^{N205}$ (1–835) activity in the tethered silencing assay is only partially dependent on wild-type Sir3p, unlike the complete dependence exhibited by the other fusion proteins tested (Table 4). LexA-Sir3p$^{N205}$ (1–835) was also capable of restoring *HMR*a silencing in a MAT$\alpha$ *sir3* background as judged by the restoration of mating (data not shown).

The effect of the D205N substitution is not observed in a fusion protein containing the in-frame deletion of amino acids 836–944 [LexA-Sir3p (1–835; 945–978)]. This deletion also appears to weaken association with the upstream Sir4p C-terminal interaction site (Table 2). These data raise the possibility that, while the D205N substitution overcomes the need for the CTD, either the local C-terminal structure upstream of the CTD or portions of the CTD may influence initiation function.

**A dominant-negative region in the N terminus of Sir3p:** As noted above, decreases in tethered telomeric silencing conferred by the LexA fusion proteins could be explained by either an inability to initiate silencing or an interference with other steps in silencing. To test the latter possibility, dominant-negative activity was assayed in an isogenic strain carrying wild-type *RAP1* and *SIR3* and a *URA3*-marked VIIL telomere lacking LexA binding sites. Deviations from wild-type levels of silencing were subsequently monitored. Only one protein, LexA-Sir3p (356–978), exhibited an inhibitory effect on silencing (Table 4), reducing FOA$^r$ frequencies 1300-fold below wild type. Interestingly, LexA-Sir3p (481–978), a protein of similar abundance and Sir4p interaction ability, did not display a dominant-negative effect. LexA-Sir3p (356–481) alone does not interfere with silencing, suggesting an additional requirement of sequences C-terminal to amino acid 481 for the dominant-negative effect. None of the remaining fusion proteins tested influenced silencing in a wild-type background within more than a 4-fold range.

We have already shown that LexA-Sir3p (481–978) is virtually inactive in initiation (Table 5), while the full-length protein retains activity. Taken together, these data indicate the presence of two regions important for telomeric silencing within the N-terminal 481 amino acids: one necessary for initiation (amino acids 1–481) of silencing in the presence of the NID domain, which we term the positive regulatory domain (PRD), and a second that titrates, or interacts with, a cofactor essential for telomeric silencing.

## DISCUSSION

Numerous studies have indicated that Rap1p recruitment of Sir3p to the telomere is essential for telomeric silencing. However, very little has been elucidated concerning the role of the recruited Sir3p in initiating unidirectional silencing. We have used the tethered silencing system to determine the Sir3p domains responsible for restoration of silencing in Rap1p mutant proteins defective for Sir3p and Sir4p recruitment. This assay measures the phenotypic consequence of both initiation and subsequent spreading along the chromatin fiber. In this assay, silencing is fully dependent on the presence of the LexA binding sites. We cannot rule out, however, the possibility that differences in structure between native and fusion proteins may influence this assay quantitatively. In these experiments, wild-type Sir3p is also present so that, in the absence of interference by the fusion proteins, propagation into adjacent sequences should occur in the presence of the initiating event.

Indeed, with one exception noted below, null *sir3* mutant strains containing the fusion proteins were fully inactive in tethered silencing and were unable to overcome the *sir3* mating defect (Table 4; data not shown). This is consistent with the behavior of LexA-Sir3p, which was previously shown to be deficient in complementation of a *sir3* null allele (Lustig *et al.* 1996). In contrast, we found in these earlier studies that LexA-Sir3p$^{N205}$ could complement the *sir3* null allele. Similarly, and

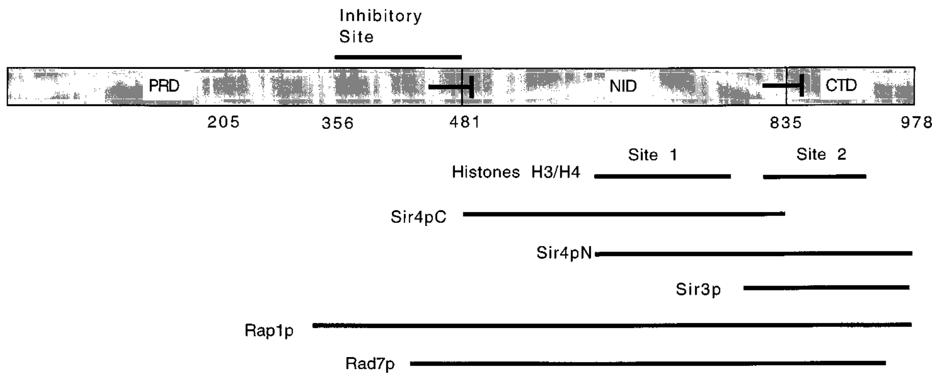


Figure 3.—A speculative model for the regulation of CTD initiation. In this model, the essentiality of the PRD (and possibly the activity of LexA-Sir3p$^{N205}$) in the presence of the NID is the consequence of the negative effect of PRD on NID inhibition (shown by the blocked arrow). The NID in turn acts to eliminate the activity of CTD in initiation. The model also raises the possibility of molecular communication between the region surrounding D205N and histone interaction site 1. The association site of Sir3p with the C terminus of Sir4p (Sir4pC; this study; Moretti *et al.* 1994) and the region necessary, but not sufficient, for the dominant-negative effect on silencing (inhibitory site) are also shown. The involvement of the CTD in the regulation of Sir4p association is additionally suggested by our data. Also shown are the Sir3p association sites for interaction with the N termini of histones H3 and H4 (Hecht *et al.* 1995), Sir3p (Moretti *et al.* 1994; P. Moretti and D. Shore, personal communication), Rap1p (Moretti *et al.* 1994; Cockell *et al.* 1995; Liu and Lustig 1996), Rad7p (Paetkau *et al.* 1994); and the N terminus of Sir4p (Sir4pN; Strahl-Bolsinger *et al.* 1997). Sir4pN requires a Sir3p region between amino acids 500 and 763 for interaction (M. Grunstein, personal communication), excluding it as a possible source of CTD initiation. CTD, C-terminal domain; NID, nonessential for initiation domain; PRD, positive regulatory domain.

in contrast to the other fusion proteins, LexA-Sir3p$^{N205}$ (1–835) was capable of conferring both tethered silencing and *HMR*a repression in *sir3* null strains.

The data presented in this study, summarized in Table 4, are consistent with the presence of three discrete regions in Sir3p acting in the initiation step of silencing (Figure 3). The primary activity responsible for the initiation function of Sir3p appears to be located in the CTD (amino acids 835–978): tethering of the CTD is both necessary and sufficient for efficient initiation of silencing.

Two additional regions appear to regulate the CTD. The first is a region nonessential for the initiation of silencing [NID (amino acids 482–834)]. Tethering of in-frame deletions lacking an internal region extending from amino acids 482 to 834 permits efficient restoration of silencing in *rap1-17* strains.

It is intriguing that the NID and Sir4p interaction domains overlap. These data suggest that association of the Sir4p C terminus is unlikely to be an early required step for initiation and, at least in some contexts, may actually repress initiation. We cannot exclude, however, the possibility that association of the Sir4p C terminus occurs after recruitment of other cofactors. The NID and Sir4p C-terminal association may well serve a more complex regulatory role in the overall function of Sir3p.

The effect of this region may actually be more extensive in the absence of the N-terminal sequences. Given the inability of LexA-Sir3p (481–978) fusion protein to initiate silencing, the NID may actually serve to mask the activity of the Sir3p CTD (Figure 3).

Indeed, the second region extending from amino acids 1–481 appears to be necessary for initiation in the presence of the NID. Because LexA-Sir3p has initiation activity, one likely role for this putative "PRD" region is abrogation of the inhibitory activity of the NID (Figure 3). This might occur by one of two general mechanisms. First, either an intramolecular folding event or an analogous set of intermolecular interactions may preclude binding of antagonistic factors with the NID. It is interesting that studies from Susan Gasser's laboratory (Gotta *et al.* 1998) have demonstrated that the N terminus, when overproduced, stimulates telomeric silencing, a possible transmanifestation of inactivation of the NID. Second, the association of specific N-terminal factors may preclude the recruitment of NID-specific factors. In this regard, the function of the PRD may also be related to its high degree of homology to the largest subunit of origin recognition complex (ORC), Orc1p (Bell *et al.* 1995).

A second finding arguing for a possible interaction between PRD and NID is the ability of the LexA-Sir3$^{N205}$ mutant protein to overcome the requirement for the CTD. This effect is not due to overall protein stability or function as judged by both Western blot and two-hybrid analysis (Figure 2; Table 4). Together with the lack of effect of the D205N substitution in the CTD in-frame deletion [LexA-Sir3p (1–835; 945–978)], these data raise the possibility that this substitution may unmask an otherwise latent site for initiation within Sir3p upstream of the CTD, the utilization of which may be dependent on C-terminal structure.

The type of modular arrangement that we observe in Sir3p has precedent in the structure of Sir4p, which appears to contain regions that positively and negatively regulate association with Sir3p (Moazed *et al.* 1997) and may be a consequence of the differing requirements for distinct Sir3p and Sir4p functions in unique steps of silencing.

During the course of these investigations, we also uncovered a second N-terminal function of Sir3p in silencing. LexA-Sir3p (356–978) expression in wild-type

cells lacking LexA binding sites confers a dominant-negative effect, resulting in the abrogation of telomeric silencing—an effect that is not observed in cells containing LexA-Sir3p (481–978). These data suggest that an N-terminal region mapping between amino acids 356 and 481 is necessary (but not sufficient) for either titration of an essential factor or interference with the structure of the silencing complex. The relationship between this region and the initiation of silencing is, at present, unclear.

We have reported that tethering of LexA-Sir3p to the telomeric/subtelomeric junction results in hyperrepression of the wild-type phenotype (Lustig *et al.* 1996). Tests of hyperrepression of the fusion proteins used in this study suggest that, while CTD is sufficient for hyperrepression (data not shown), other pathways may also to lead to hyperrepression. In support of this notion, fusion proteins that only poorly initiate silencing in *rap1-17* cells [*e.g.*, LexA-Sir3p (1–835)] partially hyperrepress wild-type *RAP1* cells. It is quite conceivable that recruitment of numerous factors including Rap1p and Sir4p may play additional roles in the hyperrepression effect in conjunction with the tethered Sir3 fusion protein in wild-type *RAP1* cells.

What protein associations may be responsible for the initiation activity of the CTD and the upstream activity uncovered in the LexA-Sir3$^{N205}$ mutant protein? One explanation is interaction of Sir3p with the LexA-Sir3p CTD. However, the mapping of the Sir3p dimerization domain between amino acids 762 and 978 (P. Moretti and D. Shore, personal communication) makes this possibility less likely, as LexA-Sir3p$^{N205}$ (1–835) is fully functional, but lacks most of this region.

In our view, the most parsimonious, albeit speculative, possibility is association of the CTD and the upstream activated site with the N-termini of histones H3 and H4. The histone interaction domains of Sir3p have been defined in two regions falling between amino acids 623 and 762 (site 1) and amino acids 808 and 910 (site 2) (Hecht *et al.* 1995), although the minimal sites have not yet been reported. The initiation conferred by the CTD may be the consequence of association of site 2 with the initial histone H3 and histone H4 N-terminal tails at the telomeric/subtelomeric junction. This association may well display a specificity to a subclass of acetylated histones H3 and H4 and initiate a cascade of Sir complex-histone interactions. A similar series of events, possibly facilitated through additional interactions, may take place in wild-type telomeric silencing.

Similar to the behavior of the CTD, the D205N substitution may increase the efficiency of site 1, thereby conferring activity to an otherwise poorly used or repressed site. Consistent with a requirement for the activation of the NID-histone interaction domain, LexA-SIR3p$^{N205}$ (1–835) can partially restore both telomeric and *HMR*a silencing in *sir3* mutants.

Such a downstream effect of the D205N substitution may explain why it was identified as a suppressor of defects in both the C-terminal tail of Rap1p, a site for Sir3p association, and the N-terminal tail of histone H4, even though the amino acid affected by the mutation lies outside of the histone interaction domain as defined *in vitro.* The mutant protein may act to amplify residual levels of silencing through an increase in the frequency of initiation. Note that the PRD may well act (albeit to a lesser extent) in an analogous fashion to the D205N substitution.

Regardless of the specific model, it is likely that initiation as assayed in tethered silencing mirrors the early events occurring in *bone fide* silencing. These studies, therefore, provide a genetic framework for the deduction of the biochemical steps involved in the regulation of Sir3p activity.

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