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 Rap1p-D, 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-terminal domain (CTD), is both necessary and sufficient for restoration of silencing. Curiously, the LexA-Sir3p303 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 nonessential for initiation domain (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 positive regulatory domain (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 HML 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 telomere-binding protein Rap1p to high-affinity sites embedded within the telomeric poly(TG)1_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 rap1p 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-
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 (Gott schling 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<sup>4205</sup>, 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<sup>3205</sup> (Lustig et al. 1996) with transcription driven from the ADH1 promoter. LexA-SIR3 (1–356) was constructed by cleaving pBTM-SIR3 with EagI and Asp718, “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 Eagl and KpnI and filling in the ends with Klenow and T4 DNA polymerase. The plasmid lacking the Eagl/KpnI 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 AgeI and XhoI, 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 of Sir3p 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 AgeI and Asp718. The resulting vector was treated with Klenow and 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 Eagl/AgeI fragment from Sir3p into a BamHI-cleaved and blunt-ended pBMT vector. The resulting construct is in-frame with LexA and contains an N-terminal extension of 17 amino acids preceding a stop codon. LexA-SIR3 (1–835) and LexA-SIR3<sup>3205</sup> (1–835) were constructed by cleaving the respective full-length plasmids with Asp718, 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<sup>3205</sup> (1–835; 945–978) were constructed by cleaving the respective full-length plasmids with Asp718 and XhoI, 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 pBMT-SIR3 with BamHI and Eagl, filling in the 5’ overhangs with Klenow, and ligating the resulting blunt ends. LexA-SIR3 (481–978) was constructed by cleaving pBMT-SIR3 with SalI and AgeI, filling in the 5’ overhangs with Klenow, and ligating the blunt ends. LexA-SIR3 (835–978) was constructed by digesting pBMT-SIR3 with BamHI and Asp718 and filling in the 5’ overhangs with Klenow. The fragment lacking the region from BamHI to Asp718 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 diagrammatically in Figure 2.

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

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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/rap1-21Sir3. CLY3/rap1-21Sir3 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 but not Ura3− strains either producing LexA alone or lacking LexA transcription. Since all available markers in the To elucidate the minimal domains of Sir3p required for tethering and examined the ability of cells to grow on minimal 5-FOA media. With one exception, neither haploid can grow on this 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-Sir3pR17p(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 (VII). 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 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-Sir3pR17p(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.

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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 (Gott 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 Pilus 1998). To investigate the role of the extreme C terminus in tethered silencing, we constructed a fusion protein, LexA-Sir3p (1–356) 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 frequencies 238-fold relative to the wild-type fusion protein. Similarly, an in-frame deletion extending from amino acids 836–944 [LexA-Sir3p (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 |

<table>
<thead>
<tr>
<th>Protein</th>
<th>β-Gal units/ mg¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>LexA</td>
<td>4.5 (3)</td>
</tr>
<tr>
<td>LexA-Sir3p</td>
<td>77 ± 24 (6)</td>
</tr>
<tr>
<td>LexA-Sir3p (1–835)</td>
<td>53 ± 4 (3)</td>
</tr>
<tr>
<td>LexA-Sir3p (1–835; 945–978)</td>
<td>24 ± 7.4 (3)</td>
</tr>
<tr>
<td>LexA-Sir3p (356–978)</td>
<td>96 ± 7.4 (3)</td>
</tr>
<tr>
<td>LexA-Sir3p (481–978)</td>
<td>207 ± 0.72 (3)</td>
</tr>
<tr>
<td>LexA-Sir3p (356–481)</td>
<td>3.7 (3)</td>
</tr>
<tr>
<td>LexA-Sir3p (1–356; 836–978)</td>
<td>5.3 (3)</td>
</tr>
<tr>
<td>LexA-Sir3p (1–481; 835–978)</td>
<td>2.5 (3)</td>
</tr>
<tr>
<td>LexA-Sir3p (835–978)</td>
<td>1.9 (3)</td>
</tr>
<tr>
<td>LexA-Sir3p¹ (356; 9205)</td>
<td>44 ± 0.7 (3)</td>
</tr>
<tr>
<td>LexA-Sir3p¹ (1–835)</td>
<td>25 ± 3.6 (3)</td>
</tr>
<tr>
<td>LexA-Sir3p¹ (1–835; 945–978)</td>
<td>16 ± 3.1 (3)</td>
</tr>
</tbody>
</table>

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

¹Mean values with standard deviations are presented with the number of transformants tested indicated in parentheses.
²Mean values between 1.9 and 5.3 indicate the absence of association and represent background values.
³Standard deviations for LexA-Sir3p were consistently higher than for other fusion proteins.
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⁺ 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 and the CTD, either alone or in conjunction with other domains, is 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 3

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

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>LexA⁺</th>
<th>Median FOA⁺</th>
<th>FOA⁺/ FOA⁺ SIR3⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBTM</td>
<td>+</td>
<td>&lt;1 × 10⁻⁶ (28)</td>
<td>&lt;0.00012</td>
</tr>
<tr>
<td>pBTM-SIR3</td>
<td>+</td>
<td>8.1 × 10⁻³ (0.9-50; 27)</td>
<td>1</td>
</tr>
<tr>
<td>pBTM-SIR3 (1-835)</td>
<td>+</td>
<td>4.9 × 10⁻³ (0-116; 21)</td>
<td>0.0042</td>
</tr>
<tr>
<td>pBTM-SIR3 (1-835; 945-978)</td>
<td>+</td>
<td>2.3 × 10⁻⁶ (0-22; 14)</td>
<td>0.00028</td>
</tr>
<tr>
<td>pBTM-SIR3 (835-978)</td>
<td>−</td>
<td>&lt;1.3 × 10⁻³ (7)</td>
<td>&lt;0.0016</td>
</tr>
<tr>
<td>pBTM-SIR3 (835-978)</td>
<td>+</td>
<td>2.0 × 10⁻³ (0.4-5.0; 14)</td>
<td>2.5</td>
</tr>
</tbody>
</table>

¹ 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.

² In this and subsequent tables, FOA⁺ 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⁺ colonies.

³ Ratios of values generated in CLY3/rap1-17 in cells containing LexA-Sir3p domain fusion relative to the value generated by LexA-Sir3p.

⁴ Lustig et al. (1996).

¹ Two samples failed to yield any FOA⁺ colonies in 53,000 and 20,000 cells plated.

⁵ Five samples failed to yield any FOA⁺ colonies in 400,000 cells plated.

### TABLE 4

**Characteristics of Sir3p domains**

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Initiation¹</th>
<th>Dependence on wild-type Sir3p²</th>
<th>Dominant negative³</th>
<th>Sir4C interaction⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBTM</td>
<td>−</td>
<td>NA</td>
<td>NT</td>
<td>−</td>
</tr>
<tr>
<td>pBTM-SIR3</td>
<td>++</td>
<td>+(4.81 × 10⁻⁶; 1)</td>
<td>−</td>
<td>++</td>
</tr>
<tr>
<td>pBTM-SIR3 2005</td>
<td>+++</td>
<td>±(2.7 × 10⁻⁵; 1)</td>
<td>−</td>
<td>++</td>
</tr>
<tr>
<td>pBTM-SIR3 (1-835)</td>
<td>−</td>
<td>+(3.8 × 10⁻⁶; 1)</td>
<td>−</td>
<td>++</td>
</tr>
<tr>
<td>pBTM-SIR3 (1-835) 2005</td>
<td>+++</td>
<td>±(1.2 × 10⁻⁶; 2)</td>
<td>−</td>
<td>++</td>
</tr>
<tr>
<td>pBTM-SIR3 (1-835; 945-978)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>pBTM-SIR3 2005 (1-835; 945-978)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>pBTM-SIR3 (356-978)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>pBTM-SIR3 (481-978)</td>
<td>−</td>
<td>+(4.81 × 10⁻⁶; 2)</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>pBTM-SIR3 (356-481)</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>pBTM-SIR3 (356-836-978)</td>
<td>+</td>
<td>+(1.5 × 10⁻⁶; 2)</td>
<td>NT</td>
<td>−</td>
</tr>
<tr>
<td>pBTM-SIR3 (481-835-978)</td>
<td>+</td>
<td>+(1.5 × 10⁻⁶; 2)</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>pBTM-SIR3 (835-978)</td>
<td>+</td>
<td>+(1.5 × 10⁻⁶; 2)</td>
<td>NT</td>
<td>−</td>
</tr>
</tbody>
</table>

NA, not applicable; NT, not tested.

¹ Initiation values were determined in CLY3/rap1-17. ++++, values exceeding 0.01; ++, FOA⁺ 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 2005 was previously reported in Lustig et al. (1996).

² FOA⁺ values determined in CLY3/rap1-21 Sir3p. +, dependence on Sir3p; ±, partial dependence; −, no dependence. The FOA⁺ values and the number of fluctuations are given in parentheses.

³ Dominant negative phenotypes were determined by FOA⁺ values in CLY/RAP1 strains containing the indicated fusion protein.

⁴ Sir4C 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.
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' 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’ 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-Sir3pD205N 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 Sir4p C terminus (Table 2), we cannot exclude the possibility, however, that the Sir4p plays a regulatory role in Sir4p association.

The requirement for the Sir3p C terminus is dependent on N-terminal sequences: The Sir3pΔ205 (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-Sir3pΔ205 (1–835) displayed levels of silencing identical to LexA-Sir3pΔ305 (Table 6). These data indicate that the LexA-Sir3pΔ205 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-Sir3pΔ205 fusion protein was previously demonstrated to function

### Table 5

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Median FOA'</th>
<th>FOA'/ FOA' Sir3</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBTM*</td>
<td>&lt;1 × 10⁻⁶ (28)</td>
<td>&lt;0.00012</td>
</tr>
<tr>
<td>pBTM-SIR3*</td>
<td>8.1 × 10⁻³ (0.9–50; 27)</td>
<td>1</td>
</tr>
<tr>
<td>pBTM-SIR3 (1–356; 836–978)</td>
<td>3.0 × 10⁻³ (1–14; 14)</td>
<td>0.037</td>
</tr>
<tr>
<td>pBTM-SIR3 (1–481; 835–978)</td>
<td>1.3 × 10⁻³ (0.13–24; 21)</td>
<td>0.16</td>
</tr>
<tr>
<td>pBTM-SIR3 (481–978)</td>
<td>1 × 10⁻⁴ (0–7.8; 14)</td>
<td>0.0012</td>
</tr>
</tbody>
</table>

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

* Values derived from Table 3.

* In cells lacking the LexA binding site, microcolonies not observed in tethered derivatives occur at variable frequencies of up to 1 × 10⁻⁵.

* Two samples failed to produce any FOA’ colonies in 1.2 × 10⁶ cells plated.
TABLE 6
The effect of D205N on Sir3p C-terminal requirements for the restoration of silencing in rap1-17 strains

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Median FOA'</th>
<th>FOA'/ FOA' Sir3</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBTM</td>
<td>&lt;1 x 10^{-6} (28)</td>
<td>&lt;0.00012</td>
</tr>
<tr>
<td>pBTM-SIR3</td>
<td>8.1 x 10^{-5} (0.9-50; 27)</td>
<td>1</td>
</tr>
<tr>
<td>pBTM-SIR3 (1-835)</td>
<td>4.9 x 10^{-5} (0-130; 21)</td>
<td>0.006</td>
</tr>
<tr>
<td>pBTM-SIR3 (1-835; 945-978)</td>
<td>2.3 x 10^{-6} (0-22; 14)</td>
<td>0.00028</td>
</tr>
<tr>
<td>pBTM-SIR3D205 (1-835)</td>
<td>36 x 10^{-2} (18-52; 14)</td>
<td>1</td>
</tr>
<tr>
<td>pBTM-SIR3D205 (1-835; 945-978)</td>
<td>24.1 x 10^{-2} (6.3-48.7; 21)</td>
<td>0.67</td>
</tr>
<tr>
<td>pBTM-SIR3D205 (1-835; 945-978)</td>
<td>&lt;1 x 10^{-6} (14)</td>
<td>&lt;0.0000028</td>
</tr>
</tbody>
</table>

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' colonies in 53,000 and 20,000 cells plated.
c Five samples failed to yield any FOA' colonies in 400,000 cells plated.

d in a sir3 mutant strain (Lustig et al. 1996). Interestingly, LexA-Sir3pN205 (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-Sir3pN205 (1-835) was also capable of restoring HMR silencing in a MATα 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 VIL 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' 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) 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-Sir3pN205 could complement the sir3 null allele. Similarly, and
in contrast to the other fusion proteins, LexA-Sir3pN205 (1–835) was capable of conferring both tethered silencing and HMRa 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-Sir3pN205 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 hyper-repression 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 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-Sir3pN205 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-Sir3pN205 (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-SIR3pN205 (1–835) can partially restore both telomeric and HMRa 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 bona 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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