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The Yeast Silent Information Regulator Sir4p Anchors and Partitions Plasmids

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Circular plasmids containing telomeric $TG_{1–3}$ arrays or the *HMR* E silencer segregate efficiently between dividing cells of the yeast *Saccharomyces cerevisiae*. Subtelomeric X repeats augment the $TG_{1–3}$ partitioning activity by a process that requires the *SIR2*, *SIR3*, and *SIR4* genes, which are also required for silencer-based partitioning. Here we show that targeting Sir4p to DNA directly via fusion to the bacterial repressor LexA confers efficient mitotic segregation to otherwise unstable plasmids. The Sir4p partitioning activity resides within a 300-amino-acid region (residues 950 to 1262) which precedes the coiled-coil dimerization motif at the extreme carboxy end of the protein. Using a topology-based assay, we demonstrate that the partitioning domain also retards the axial rotation of LexA operators in vivo. The anchoring and partitioning properties of LexA-Sir4p chimeras persist despite the loss of the endogenous *SIR* genes, indicating that these functions are intrinsic to Sir4p and not to a complex of Sir factors. In contrast, inactivation of the Sir4p-interacting protein Rap1p reduces partitioning by a LexA-Sir4p fusion. The data are consistent with a model in which the partitioning and anchoring domain of Sir4p (PAD4 domain) attaches to a nuclear component that divides symmetrically between cells at mitosis; DNA linked to Sir4p by LexA serves as a reporter of protein movement in these experiments. We infer that the segregation behavior of telomere- and silencer-based plasmids is, in part, a consequence of these Sir4p-mediated interactions. The assays presented herein illustrate two novel approaches to monitor the intracellular dynamics of nuclear proteins.

In the yeast *Saccharomyces cerevisiae*, large autonomously replicating plasmids (*ARS* plasmids) are unstable due to asymmetric distribution of the extrachromosomal elements at mitosis (56). When grown on selective media, a small fraction of cells accumulate many plasmid copies (50 to 100 copies), whereas the remainder of the population is plasmid free and destined to die. Incorporation of centromere sequences (*CEN*) or the *REP3* partitioning locus of the endogenous 2μm episome rectifies the segregation defect, as expected given that both loci segregate genetic material in their endogenous contexts (15, 33, 36). Surprisingly, the *HMR* E silencer and telomeric sequence tracts also possess a robust partitioning activity (38, 43). Although it is not clear how these sequences promote partitioning, an important role for telomeres in transporting chromosomes to, or retaining chromosomes at, assigned nuclear positions has been inferred from an expanding body of cytological evidence. During meiotic prophase in *Schizosaccharomyces pombe*, chromosome movement is led by telomeres which cluster near the spindle pole body (the yeast equivalent of the centrosome) (12). During stages of mitotic growth, by contrast, centromeres lead chromosome movement and telomeres cluster into groups at the nuclear periphery (21), an arrangement observed for chromosome ends during interphase in numerous cell types of many organisms, including budding yeast, trypanosomes, and fruit flies (14, 50, 57), but notably absent in others (29, 66).

Transcriptional repression of otherwise functional *Saccharomyces* genes occurs when they are positioned near the silent mating-type loci (46) or adjacent to a telomere, a phenomenon termed telomere position effect (67). Similar *cis*-acting and *trans*-acting factors are required for repression at both locations, indicating that the mechanisms of repression are related. The silent mating-type loci *HML* and *HMR* contain two silencers each, E and I, that consist of different combinations of binding sites for Rap1p, Abf1p, and the eukaryotic replication initiator, ORC (46). By comparison, telomeres contain numerous Rap1 binding sites within a 300- to 600-bp terminal tract of $(TG_{1–3})_n/(C_{1–3}A)_n$ where the protein binds in vivo (17). Binding sites for Abf1p and ORC (the *ARS* consensus) are located within arrays of larger subtelomeric repeats, termed X and Y′, although the repeats are not necessary for chromosome maintenance or telomeric silencing. Transcriptional repression requires the coordinated action of three *SIR* gene products, Sir2p, Sir3p, and Sir4p (2, 39, 59), which interact with one another to form a complex (52, 53, 64). The proteins are constituents of a repressive chromatin structure (28, 64) that blocks the access of various probes of DNA accessibility (26, 45, 61). Sequence-specific DNA binding by the silencing factors has not been achieved in vitro; however, Sir3p and Sir4p interact with peptides corresponding to histone tails (27). The proteins also interact with DNA-bound Rap1p, which targets the silencing complex to silencers and telomeres (28, 54). Recruitment at silencers is aided by the association of Sir4p with Orc1p via another *SIR* gene product, Sir1p (11, 65). Nevertheless, the role of Rap1p in silencing can be bypassed if Sir3p and Sir4p are tethered to DNA directly utilizing fusions to the Gal4p DNA binding domain (47, 48).

The similarity in requirements for transcriptional silencing and plasmid partitioning is striking. Rap1p is critical as evidenced by the loss of stabilization of *HMR* E plasmids lacking the Rap1 binding site and the elimination of telomere-based partitioning in selected *rap1* mutants (37, 43). The Sir proteins are also essential for partitioning *HMR* E plasmids, and they

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provide added stabilization to plasmids containing TG_{1-3} tracts, if the telomeric sequences are accompanied by an X repeat (20, 38, 44). Sir-dependent augmentation by the subtelomeric element requires the resident Abf1 site and *ARS* consensus, as well as the *ABF1* gene product. On the basis of these observations, Enomoto et al. proposed that Abf1p, in conjunction with Rap1p and ORC, recruits the Sir proteins to plasmids (20).

In the absence of a segregation mechanism, *ARS* plasmids are retained preferentially in mother cells by a process that has been linked to DNA replication (30, 55). Two models can be advanced to account for Sir-mediated stabilization of silencer- and telomere-based plasmids. One model postulates that the Sir proteins disengage plasmids from the mother to permit passive diffusion between the mother and bud nuclei. Another model postulates that plasmid transport is facilitated by the attachment of chromatin-bound Sir proteins to a nuclear component that segregates symmetrically between dividing cells. Support for this model comes from several observations regarding Sir4p. First, the protein contains a coiled-coil dimerization domain (10) that is homologous to regions within lamins A and C of the human nuclear envelope (18). Although a nuclear lamina has not been detected in yeast (9), the similarity has spurred much speculation that the protein links telomeres to a lamin-like shell at the yeast nuclear periphery. Second, overexpression of a carboxy-terminal Sir4p fragment releases Sir3p and full-length Sir4p from isolated nuclei, suggesting that the protein normally associates with an insoluble structure and is therefore not freely diffusible (16). Third, Sir4p colocalizes with Sir3p and telomeric DNA in a punctate pattern in a peripheral shell of the nuclear volume, indicating that its nuclear position is specified (24).

To gain insight into the role of Sir4p in partitioning, two assays were developed to evaluate the dynamic behavior of proteins within intact cells. Both approaches required that the protein of interest be tethered to DNA via fusion to a heterologous DNA binding polypeptide. The premise underlying both strategies is that the ability of a protein to move freely within the nucleus and to be segregated symmetrically at mitosis can be determined by monitoring the fate of a reporter DNA to which the protein is attached. Mitotic segregation was examined by measuring the ability of a DNA-linked protein to partition unstable plasmids. Restrictions on the rotational motion of the protein were assessed by measuring the ability of the DNA-bound derivative to alter DNA topology in appropriate topoisomerase mutants. We show that targeting Sir4p to DNA stabilizes plasmids, thereby circumventing the requirement for silencers and telomeric sequences in plasmid partitioning. The tethered polypeptide also restricts the rotational freedom of DNA sequences to which it is attached. We propose that Sir4p-mediated DNA anchoring and partitioning results, in part, from association of the protein with an immobile nuclear component which divides between progeny at mitosis.

MATERIALS AND METHODS

DNA constructions. Plasmids were generously provided by R. Sternglanz (State University of New York—Stony Brook), P. Linder (Biozentrum), and D. Shore (Columbia University). pKWD200 was derived from a parent excision vector, pKWD50N, by insertion of three tandem copies of a LexA binding-site oligonucleotide (5′-GGCCGCTACTGTACATATAACCACTGGTTTTATATACAGCAC-3′/5′-GGCCGTGCTGTATATAAAACCAGTGGTTATATGTAC-3′) at a unique *Not*I site (51). Each oligonucleotide contains the two LexA operators found at the colicin E1 promoter (19). The pAA plasmid series was derived from pRS426 by replacing a 2.8-kb *Sna*BI-*Not*I fragment with a 2.2-kb *Aat*II-*Not*I fragment from pRS404 (13). This exchange eliminates the *REP3* sequence of pRS426, creating the unstable *ARS* vector pAA0. A 489-bp *Not*I-*Nhe*I fragment bearing the LexA operators from pKWD200 was inserted between the *Spe*I and *Not*I sites of the pAA0 polylinker to yield pAA3. pAA6 contains six LexA operator oligonucleotides but is otherwise identical to pAA3. Plasmids expressing LexA-Sir4p fusions (LS4 plasmids) were constructed by cloning PCR-amplified *SIR4* fragments into the *Eco*RI-*Sal*I site of the full-length LexA expression vector pBTM116 (54) (see Fig. 1). pΔade2 was derived from pASZ10 (63) by deleting a 426-bp *Eco*RV-*Hpa*I fragment from within the *ADE2* gene. psir2::HisG-URA3-HisG was constructed by cloning a 3.8-kb *HisG-URA3-HisG* cassette (1) into the *Sna*BI-*Bgl*II sites of pSIR2-Yip5 (60).

Strain constructions. Strain YDS410 (*MAT*α *ade2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ura3-1 rap1-5*) has been described previously (40). MRG1 [*MAT***a** *ura3-52 leu2-Δ1 trp1-Δ63(GAL3) his3-Δ200 Δade2*] was derived from FY251 [*MAT***a** *ura3-52 leu2-Δ1 trp1-D63(GAL3) his3-Δ200*] by single-step gene replacement with pΔade2. MRG211 (*sir2::TRP*), MRG311 (*sir3::HIS3*), and MRG411 (*sir4::HIS3*) were derived from MRG1 by single-step disruptions with plasmids C369 (60), psir3::HIS3, and pMM10.7 (49), respectively (51). MRG221 (*sir2::HisG*) was derived from MRG211 (*sir2::TRP*) by transforming the strain with linearized psir2::HisG-URA3-HisG followed by selection on 5-fluoroorotic acid (5-FOA) plates for loss of *URA3*. THC1 was derived from W303-1A by replacement of the **a***1* and **a***2* genes at *HMR***a** with *URA3* (9a).

Measurements of plasmid stability. Single colonies containing a pAA test plasmid (*URA3* marker) and a LexA expression vector (*TRP1* marker) were transferred to 2 ml of SC-ura,trp and grown for 20 to 24 h at 30°C. Cultures were diluted appropriately, and equal volumes were spread on SC-ura,trp and SC-trp plates. Mitotic stability (F_0) was expressed as the number of colonies forming on SC-ura,trp plates divided by the number of colonies forming on SC-trp plates. To determine the plasmid loss rate, diluted cultures were grown for an additional 24 h in SC-trp. Again, the cells were spread on SC-ura,trp and SC-trp plates and the fraction of cells bearing both plasmids after nonselective growth for the pAA vector (F_1) was determined as above. Plasmid loss rates were determined by using F_0, F_1, and the elapsed number of doubling times on nonselective growth by the method of Longtine et al. (43). Each value represents the mean of at least four independent trials with individual transformants, unless specified otherwise. Plasmid stability measurements in the *rap1-5* strain were performed with cultures on solid media according to published procedures (43).

Plasmid copy number determinations. Cells carrying pAA6 and LS4(839–1358) were grown overnight in 5 ml of SC-ura,trp. Total DNA was isolated by glass bead lysis, digested with *Hin*dIII, fractionated on 0.8% agarose gels, and transferred to Zeta Probe GT membrane (Bio-Rad). Blots were probed with a 505-bp *URA3 Acc*I fragment that hybridizes to the plasmid marker and the chromosomal *ura3-52* allele equally well. The plasmid copy number was estimated from the ratio of the hybridization signals from plasmid and chromosomal fragments, as determined by phosphorimager analysis.

Assay for DNA immobilization. DNA-anchoring experiments were performed with strain MRG6 [*MAT*a Δ*top1::GAL1-R top2-4 ura3-52 leu2-Δ1 trp1-Δ63(GAL3) his3-Δ200 Δade2*] and procedures described by Mirabella and Gartenberg (51), with the exception that cells were also transformed with LexA-Sir4 fusion plasmids. *sir* derivatives of MRG6 were also used: MRG231 (*sir2::TRP1*), MRG331 (Δ*sir3::HIS3*), and MRG431 (Δ*sir4::HIS3*) (51).

Derepression assay. Tests for anti-sir4 activity were performed with strain THC1, which bears a repressed *URA3* gene at *HMR***a**. Cells expressing different LexA-Sir4p fusions were grown to an absorbance at 600 nm of 0.3 in SC-trp. Equal volumes of serially diluted culture were spotted on SC and 5-FOA plates. Plates and autoradiograms were scanned with a UMAX Vista S-12 scanner, and the images were processed with Adobe Photoshop 4.0 for publication.

RESULTS

Targeting Sir4p improves plasmid partitioning. If Sir4p segregates equally between dividing cells, anything bound to the protein will also be partitioned, including plasmid DNA. By examining the persistence of unstable plasmids in culture, it should be possible to ascertain whether associated proteins are partitioned symmetrically. To link Sir4p to DNA, fragments of the protein were fused to the bacterial repressor LexA. The persistence of LexA operator plasmids in cultured cells was gauged by measuring two parameters, mitotic stability and plasmid loss rate. The mitotic stability is equivalent to the percentage of plasmid-bearing cells in a population growing under conditions that select for the plasmid. The parameter depends on the segregation efficiency of the plasmid, although it is influenced by other factors such as the replication efficiency and life span of the cell following the loss of plasmid-borne genes (55). The plasmid loss rate is defined as the rate at which each dividing cell produces a plasmid-free progeny under nonselective conditions. This parameter is influenced only by the replication and segregation efficiencies of the plasmid. In our system, conditions that result in increased mitotic

TABLE 1. A LexA-Sir4p fusion increases the stability of LexA operator plasmids

Plasmid	No. of LexA sites	LexA fusion	Mitotic stability (%)[a]	Plasmid loss rate[a]
pAA6	6	LS4(839–1358)	44.1 ± 2.8	0.14 ± 0.03
pAA3	3	LS4(839–1358)	35.1 ± 1.9	0.18 ± 0.01
pAA0	0	LS4(839–1358)	5.5 ± 2.6	0.24 ± 0.04
pAA6	6	LexA only	12.5 ± 6.8	0.36 ± 0.07
pAA3	3	LexA only	12.6 ± 2.0	0.42 ± 0.01
pAA0	0	LexA only	6.3 ± 1.3	0.44 ± 0.06
pAA6	6	No LexA	10.8 ± 4.9	0.37 ± 0.17

[a] Mean ± standard deviation.

stabilities always lead to diminished plasmid loss rates. For both measurements, the fraction of plasmid-bearing cells was determined by scoring for the presence of a plasmid-borne marker.

Plasmid stability determinations were made with the pAA family of reporter vectors that contain the *URA3* gene, the 2μm *ARS*, and tandemly arrayed LexA operators. Like other *ARS* vectors, these plasmids are extremely unstable in yeast due to the absence of a partitioning mechanism: in strain MRG1, a plasmid with six adjacent LexA sites (pAA6) was present in only 10.8% of the viable cells under uracil selection and had a loss rate of 0.37 (Table 1).

Moreover, expression of LexA protein from the yeast *ADH1* promoter in the 2μm-based plasmid pBTM116 did not reverse the instability. In contrast, a LexA-Sir4 fusion protein that links the carboxy-terminal end of Sir4p [amino acids 839 to 1358, designated LS4(839–1358)] to full-length LexA improved the persistence of pAA6 measurably; the mitotic stability of pAA6 increased almost fourfold, and there was a corresponding drop in the plasmid loss rate (Table 1).

To examine the specificity of Sir4p-mediated stabilization, we measured the persistence of reporter plasmids that differed by the number of resident LexA operators. If stabilization involved targeting Sir4p to plasmid DNA, constructs with no LexA sites would be lost more frequently. A pAA6 derivative without LexA operators (pAA0) was not stabilized by the expression of LS4(839–1358) in MRG1 (Table 1). The low mitotic stability and high plasmid loss rate of this plasmid were comparable to values obtained for pAA6 in cells lacking the Sir4 fusion protein. A construct with a single LexA operator gave similar results (data not shown). In contrast, reducing the number of operators in pAA6 from six to three (pAA3) had a minor effect. Thus, stabilization of plasmids containing LexA operators by the LexA-Sir4p chimera requires targeting of the fusion protein to plasmid DNA.

The Sir4p-dependent improvement in pAA6 stability could be due to either its enhanced replication efficiency or its segregation efficiency or both. An increase in replication efficiency would be accompanied by an increase in plasmid copy number. To explore this possibility, we quantitated the DNA content of the six-LexA-operator plasmid in cells expressing LS4(839–1358) by Southern blot hybridization. The copy number of pAA6 relative to a chromosomal fragment was twofold greater in cells expressing the LexA-Sir4p fusion than in cells expressing LexA alone (data not shown). This figure was misleading because more than 50% of the cells in the population did not contain plasmid (Table 1). When the data were corrected for the presence of plasmid-free cells, the relative copy number of pAA6 was actually 30% lower in cells expressing the LexA-Sir4p fusion than in cells expressing LexA alone (data not shown). Thus, plasmid stabilization by LS4(839–1358) results from efficient segregation of plasmids between progeny cells, not from the enhanced copy number of a poorly segregating extrachromosomal element.

A Sir4p domain responsible for plasmid partitioning. Initial results with LS4(839–1358) indicated that the entire Sir4 polypeptide was not necessary for Sir4p-mediated plasmid stabilization. To identify a smaller subdomain of Sir4p responsible for enhanced partitioning, we fused successively smaller fragments of the protein to LexA. The constructs are represented graphically in Fig. 1. Western blot analysis with anti-LexA antibody showed that all of the *LexA-SIR4* gene fusions yielded polypeptides of the appropriate size at comparable expression levels (data not shown). In addition, DNA binding by the LexA chimeras in vivo was confirmed by a blocking assay (6), which exploits the ability of bound repressors to block transcriptional activation by an upstream UAS_{GAL} in a *lacZ* reporter (reference 23 and data not shown). Surprisingly, a Sir4p fragment with a truncated amino terminus, LS4(950–1358), resulted in greater stability of pAA6. In the presence of this chimera, the pAA6 loss rate was comparable to that for a telomere-based vector (43). Larger amino-terminal truncations of the Sir4p fragment, however, produced fusion proteins that did not stabilize the reporter plasmid. Segregation mediated by LS4(1050–1358), LS4(1150–1358), and LS4(1262–1358) was no greater than that for LexA alone. Deletions at the carboxy-terminal end of LS4(950–1358) were also made. Truncation of the Sir4p fragment to amino acid 1262, LS4(950–1262), had little effect on the stabilization function. This deletion removes the heptad repeat of leucines that constitutes a dimerization domain of Sir4p (10). LS4(950–1150) stabilized pAA6 less efficiently, and an even smaller Sir4p fragment, LS4 (950–1050), had no effect. We conclude that the full Sir4p partitioning domain resides between amino acids 950 and 1262 at the carboxy-terminal end of the protein.

Sir4p is anchored in vivo. If Sir4p is attached to a nuclear substructure, anything bound to the protein, including extrachromosomal DNA rings, will also be immobilized. By exam-

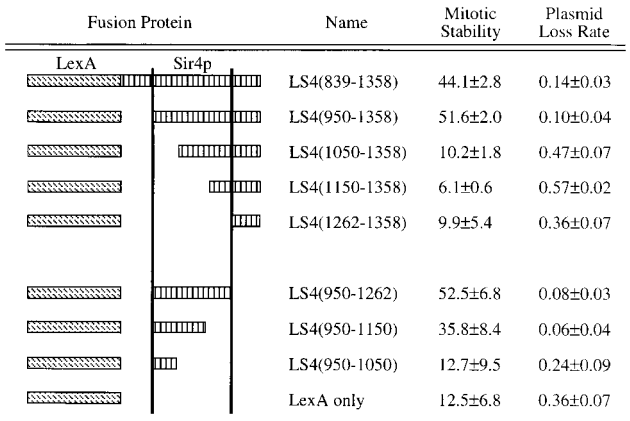

FIG. 1. A domain of Sir4p confers plasmid stability when tethered to DNA directly by LexA. A schematic diagram of LexA-Sir4p chimeras is shown. The Sir4p fragment in each construct (amino acid end points are indicated in parentheses) is linked to the carboxy terminus of full-length LexA (202 amino acids). Neither the growth rate nor the cell viability was altered by expression of any of the LexA-Sir4 fusions (unpublished results). Attempts to work with LexA fused to full-length Sir4p were thwarted by poor expression levels of the chimera. The solid vertical bars delimit the internal Sir4p region necessary for full partitioning activity. pAA6, containing six LexA operators, was used as the reporter plasmid. Numerical values for mitotic stability and plasmid loss rate represent the average of at least four trials with independent transformants of strain MRG1.

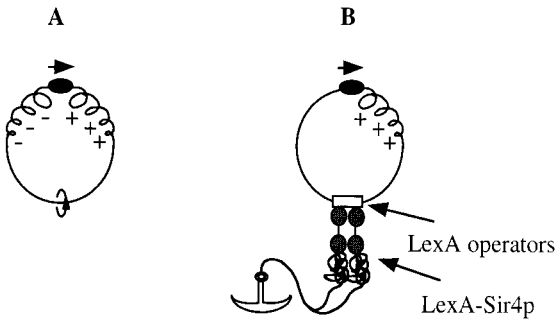

FIG. 2. Use of paired supercoiling domains to detect protein-mediated DNA anchoring. (A) Tracking of the DNA helix by proteins such as RNA polymerase (solid ellipse) generates domains of positive and negative supercoils. The arrow indicates the direction of protein movement. In a circular DNA, the twin domains of supercoiling will cancel if the DNA segments separating them are free to rotate. (B) Binding of an immobilized protein blocks DNA rotation. LexA operators (rectangle) are bound by a dimer of LexA fusion proteins (a pair of dumbbells linked to tangled chains). Expression of bacterial topoisomerase I in the absence of major yeast topoisomerase activities promotes specific relaxation of negative supercoils, resulting in accumulation of positive supercoils.

ining the intracellular dynamics of rings containing LexA operators, it should be possible to ascertain whether the fusion proteins are anchored in vivo. To this end, we used a DNA topology-based assay that identifies DNA sequences that prevent free rotation of DNA about its helical axis. The assay has been described previously and will be discussed only briefly here (22, 51). Inducible site-specific recombination by the R recombinase is used to generate simple nonreplicating DNA rings. DNA-tracking proteins, such as RNA polymerase, generate domains of positive and negative supercoils in the ring, according to the twin-domain model of transcriptional supercoiling (42). In cells lacking DNA topoisomerases I and II (enzymes which relax both positive and negative supercoils), cancellation of the domains occurs through axial rotation of the DNA segments that join the domains (Fig. 2A). If the DNA segments are not free to rotate, selective relaxation of the negatively supercoiled domain by the negative-supercoil-specific *Escherichia coli* topoisomerase I yields positively supercoiled rings. Assuming that a tracking protein provides one barrier to DNA rotation, rings with LexA operators become positively supercoiled if the LexA-Sir4 fusion protein is anchored (Fig. 2B).

Galactose induction of the site-specific R recombinase in strain MRG6 (Δ*top1::GAL1-R top2-4*) produced a 2.5-kb ring, designated rKWD200, which contained six adjacent LexA operators. Subsequently, cells were shifted from 26 to 35°C to inactivate the conditional *top2-4* allele, leaving a plasmid-borne *GPD:topA* gene fusion as the predominant source of topoisomerase activity. Topoisomer distributions were examined by two-dimensional agarose gel electrophoresis in buffer containing chloroquine (see the legend to Fig. 3A for a description). Lane 1 of Fig. 3B shows that rKWD200 becomes highly positively supercoiled in cells expressing LS4(839–1358). The effect is not due simply to the binding of LexA to the ring, since rKWD200 remains negatively supercoiled in cells expressing the unmodified bacterial repressor (lane 2). Furthermore, LS4(839–1358) has no effect on the topology of rKWD50N, a ring lacking LexA operators (lane 3). These results indicate that tethering of Sir4p to LexA operators impedes the axial rotation of DNA containing the sites. To define the Sir4p domain required for DNA immobilization, we examined the supercoiling potential of each of the LexA-Sir4p deletion derivatives described above. Like LS4(839–1358), LS4(950–1358) yielded high levels of positive supercoiling (Fig. 4A). However, rKWD200 displayed a mixture of negatively and positively supercoiled topoisomers in cells expressing LS4(1050–1358), and the ring accumulated few or no positive supercoils in cells expressing LS4(1150–1358) or LS4(1262–1358). Examination of carboxy-terminal deletion derivatives of Sir4p showed that LS4(950–1262) yielded positive supercoils efficiently whereas LS4(950–1150) yielded a mixture of negatively and positively supercoiled topoisomers and the most severe truncation, LS4(950–1050), did not lead to accumulation of any positive supercoils (Fig. 4B). These results show that a domain spanning amino acids 950 to 1262 is fully sufficient for DNA immobilization. The anchoring domain correlates well with the domain required for increased partitioning of plasmids containing LexA operators.

Conceivably, DNA rotation could be hindered without physical attachment to a nuclear substructure; DNA binding proteins with sufficient mass and cross section could retard the rotation of DNA by providing frictional drag within a viscous medium, such as the nucleoplasm (42). This does not appear to be the case for Sir4p, based on comparison to other LexA chimeras. A 312-amino-acid Sir4p domain (positions 950 to 1262) was sufficient for DNA anchoring, whereas an overlapping, 308-amino-acid, nonpartitioning piece of the protein (positions 1050 to 1358) did not impede DNA mobility. In addition, DNA immobilization was not observed when LexA was fused to a 672-amino-acid fragment of Sir3p (positions 307 to 978) (data not shown). These data strongly favor a model in

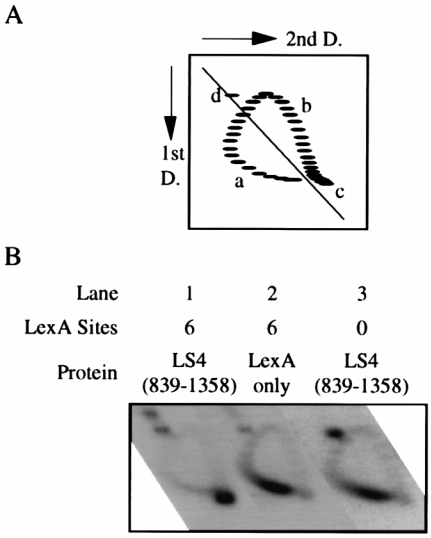

FIG. 3. LexA-Sir4p mediates immobilization of DNA rings containing LexA operators. (A) Topoisomer distributions were resolved by electrophoresis in two dimensions differing by the concentration of chloroquine in the buffer. Topoisomers with progressively higher linking numbers align in an arc (58): (a) negatively supercoiled rings; (b) relaxed rings; (c) highly positively supercoiled rings; (d) nicked rings. In each diagonal lane, DNA topology was evaluated by comparing the intensities of regions a and c qualitatively. 1st D., 2nd D., first and second dimensions, respectively. (B) Experiments were performed in strain MRG6 (Δ*top1::GAL1-R top2-4*) expressing *E. coli* topoisomerase I constitutively from a plasmid-borne *GPD* promoter, under conditions where topoisomerase II is inactivated (51). Since common yeast plasmids already contain DNA anchors, the assay is performed with well-defined DNA rings generated by induction of the *GAL1-R* recombinase expression cassette. Each lane represents a unique combination of a LexA-Sir4p chimera and a DNA target. Lanes: 1, LS4(839–1358) and rKWD200, a 2.55-kb DNA ring containing six LexA operators; 2, LexA alone and rKWD200; 3, LS4(839–1358) and rKWD50N, a 2.45-kb DNA ring containing no LexA sites. DNA rings were visualized by hybridization with a radiolabeled 900-bp *LYS2* probe, a sequence common to all the rings used.

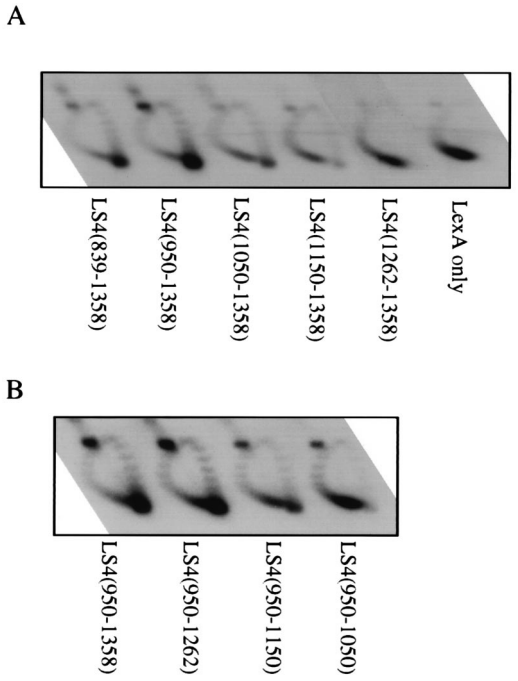

FIG. 4. DNA anchoring is mediated by a Sir4p domain spanning amino acids 950 to 1262. Experiments were performed as described in the legend to Fig. 3. Each lane represents a different LexA-Sir4p chimera in combination with rKWD200, a ring containing six LexA operators. (A) Amino-terminal truncations of the Sir4p fragment within the LexA-Sir4p fusion. (B) Carboxy-terminal truncations of the Sir4p fragment within the LexA-Sir4p fusion.

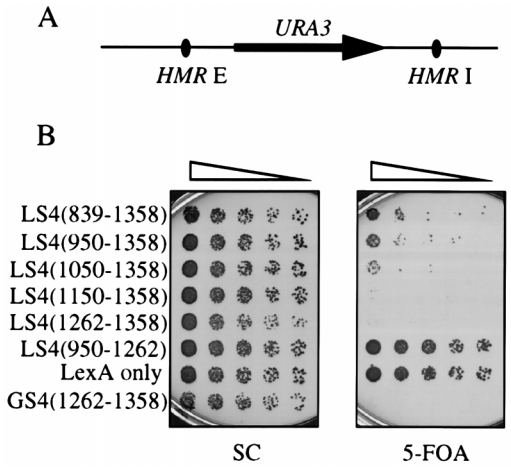

FIG. 5. Anti-sir activity of the LexA-Sir4p fusions. (A) A map of the *HMR* locus modified by replacement of **a***1* and **a***2* with *URA3* in strain THC1. (B) Each LexA-Sir4p fusion is expressed at a high level from a high-copy-number plasmid in strain THC1. Cultures were diluted serially (represented graphically by the triangle above each photograph) and spotted on both SC and 5-FOA plates. GS4 (1262–1358) denotes a Sir4p fragment from positions 1262 to 1358 linked to the Gal4p DNA binding domain instead of LexA in plasmid pCTC23 (10).

which DNA immobilization is the consequence of Sir4p binding an immobile nuclear component.

The anti-sir and partitioning/anchoring functions of Sir4p reside in separable domains. Although normal expression of *SIR4* is required to silence *HML*, *HMR*, and genes integrated near telomeres, overexpression of the full-length gene or just the carboxy-terminal end results in derepression of the loci (32, 49). This dominant negative phenomenon, termed anti-sir, has been ascribed to the sequestration of essential silencing factors by a stoichiometric excess of Sir4p. To determine whether there is overlap of the Sir4p domains required for anti-sir activity and the partitioning/anchoring functions, we tested each of the LexA fusions in a derepression assay. The LexA-Sir4p fusions were expressed in yeast strain THC1, which contains a silenced *URA3* gene at the *HMR* locus (Fig. 5A). Derepression of the *HMR*-linked nutritional marker was monitored with 5-FOA, which is converted to a toxic metabolite by the *URA3* gene product (4). If *URA3* expression occurs, the cells die. Cultures of THC1 were pregrown in media lacking tryptophan to maintain the LexA vector and plated in serial dilutions on either SC or 5-FOA plates (Fig. 5B). Overexpression of fusion proteins that retained the extreme carboxy-terminal 96 amino acids, LS4(839–1358), LS4(950–1358), LS4 (1050–1358), LS4(1150–1358), and LS4(1262–1358), derepressed the *URA3* gene and inhibited growth on 5-FOA. Similar results were obtained when the 96-amino-acid peptide (positions 1262 to 1358) was fused to the Gal4p DNA binding domain instead of LexA in GS4(1262–1358). Clearly, derepression of *HMR* can be achieved with fragments of Sir4p that lack the DNA-anchoring and plasmid-partitioning activities. More importantly, LS4(950–1262), which lacks the carboxy-terminal 96-amino-acid fragment, failed to derepress *HMR* even though it retained both the anchoring and partitioning activities. These experiments suggest that the Sir4p domain responsible for DNA anchoring and plasmid partitioning can be separated from the domain required for anti-sir activity at *HMR*.

Rap1p, not Sir2-4p, is required for partitioning by LexA-Sir4p. The corequirement for Sir2p, Sir3p, and Sir4p in silencing suggests that these proteins are coupled functionally (2, 59). In addition, Sir3p overexpression suppresses anti-sir4 activity, and Sir3p and Sir4p interact in the two-hybrid assay (49, 54). Direct interactions among the three silencing factors have been demonstrated by affinity chromatography and coimmunoprecipitation (52, 53, 64). To test whether the endogenous *SIR* genes are required for the activities of the LexA-Sir4p chimeras, plasmid partitioning and DNA anchoring were examined in the absence of *SIR2*, *SIR3*, or *SIR4*. Surprisingly, partitioning of pAA6 by LS4(950–1358) was not significantly altered in *sir2*, *sir3*, or *sir4* derivatives of strain MRG1 (Table 2). The slight destabilization of pAA6 after the loss of *SIR3* or *SIR4* occurred equally in cells expressing LexA alone and those expressing the LexA-Sir4p fusion. Moreover, DNA anchoring of rKWD200 by LS4(950–1358) in *sir* derivatives of strain MRG6 was also not affected (data not shown). Thus, enhancement of plasmid stability and immobilization of DNA by LexA-Sir4p does not require Sir2p or Sir3p or the endogenous Sir4p.

TABLE 2. Stabilization by LexA-Sir4p fusions does not require the *SIR* genes

LexA fusion	Strain	Genotype	Mitotic stability (%)[a]	Plasmid loss rate[a]
LS4(950–1358)	MRG1	*SIR*	51.6 ± 2.0	0.10 ± 0.04
	MRG221	*sir2::hisG*	49.6 ± 1.2	0.12 ± 0.06
	MRG311	Δ*sir3::HIS3*	34.2 ± 1.7	0.16 ± 0.01
	MRG411	Δ*sir4::HIS3*	38.3 ± 3.5	0.15 ± 0.01
LexA only	MRG1	*SIR*	12.5 ± 6.8	0.36 ± 0.07
	MRG221	*sir2::hisG*	15.9 ± 1.9	0.37 ± 0.11
	MRG311	Δ*sir3::HIS3*	9.4 ± 2.0	0.43 ± 0.05
	MRG411	Δ*sir4::HIS3*	8.0 ± 3.3	0.46 ± 0.10

[a] Mean ± standard deviation.

TABLE 3. Stabilization by LexA-Sir4p fusions requires the *RAP1* gene

LexA fusion	Strain	Temp (°C)	Genotype	Mitotic stability (%)[a]	Plasmid loss rate[a]
LS4(950–1262)	YDS410	23	*rap1-5*	41.8 ± 4.7	0.12 ± 0.04
		30	*rap1-5*	N.D.[b]	0.24 ± 0.04
LexA only	YDS410	23	*rap1-5*	14.8 ± 4.2	0.29 ± 0.09
		30	*rap1-5*	N.D.	0.26 ± 0.07

[a] Mean ± standard deviation for three independent trials.
[b] N.D., not determined.

The residual partitioning properties of telomere-based plasmids in strains lacking the *sir* genes are further reduced in selected *rap1* mutants (20, 43). In strain YDS410 containing the conditional *rap1-5* allele, partitioning of telomere-based plasmids is abolished whereas centromere- and 2μm-based plasmids are unaffected. DNA rings containing Rap1 binding sites derived from telomeric TG_{1-3} tracts are also anchored in *sir* strains (51). This suggests that Rap1p alone provides some level of plasmid transport. To test whether Rap1p plays a role in partitioning by LexA-Sir4p, we measured the loss rates of pAA6 in strain YDS410 transformed with either LS4(950–1262) or LexA alone. The stability of pAA6 was comparable to that in wild-type strains when the cells were grown at 23°C (Table 3). In contrast, the loss rate of pAA6 increased twofold when cells containing LS4(950–1262) were transferred to 30°C, the semipermissive temperature for *rap1-5*. Cells containing LexA alone were unaffected by the temperature shift. We conclude that the partitioning mechanism of LexA-Sir4p involves Rap1p.

DISCUSSION

We developed two assays for intracellular protein movement to gain insight into the role that Sir4p plays in segregating telomere- and silencer-based plasmids. One approach measures the ability of a protein to segregate between dividing cells, and the other measures the rotational freedom of the protein, an indicator of protein immobilization. In both cases, protein movement was monitored by tethering the protein to DNA via linkage to a heterologous DNA binding protein. We found that Sir4p, when fused to LexA, conferred a partitioning phenotype to unstable plasmids containing LexA sites (Table 1; Fig. 1). The chimeric protein also blocked the axial rotation of DNA rings under appropriate conditions in topoisomerase mutants (Fig. 3 and 4). These observations are consistent with a model in which Sir4p associates with an immobile nuclear component that divides equally at mitosis; attachment to the nuclear support limits the unrestricted movement of the protein, yet segregation of the support distributes the protein between dividing cells. Our data provide a rational explanation for the partitioning of circular plasmids containing silencers and telomeric sequences; symmetric segregation of these DNAs results from formation of a chromatin variant that contains Sir4p, which, in turn, facilitates DNA anchoring and plasmid transport. The data are not consistent with a role for Sir4p in promoting partitioning by passive diffusion. Otherwise, binding of the LexA-Sir4 fusion proteins would have increased the rotational freedom of unstable plasmids, not hindered it, as was observed (Fig. 3 and 4).

Attachment of eukaryotic centromeres to the mitotic spindle by kinetochore proteins provides an established example of a facilitated DNA transport mechanism. Segregation of the endogenous yeast 2μm episome appears to follow this paradigm as well (7). In this case, plasmid-encoded partitioning proteins, Rep1p and Rep2p, anchor the 2μm partitioning locus, *REP3*, to an unknown segregation apparatus (22). Given that three yeast partitioning loci, *REP3*, telomeric TG_{1-3} sequences, and centromeres, are immobilized in the DNA-anchoring assay (22, 51, 65a), we would predict that DNA rings containing silencers would also be anchored. Our attempts to detect immobilization of *HMR* E have been unsuccessful. However, a functional silencer prevents transcription and may block other DNA helix-tracking activities which are required to generate twin domains of supercoiling for the anchoring assay. In agreement with this explanation, preliminary evidence indicates that the addition of *HMR* E to rings that contain other known anchoring sequences suppresses the accumulation of positive supercoils (1a).

An accumulated body of evidence indicates that the Sir proteins function together at the chromatin level to effect silencing. It was therefore striking that partitioning and anchoring by LexA-Sir4p fusions were not influenced by disruption of *SIR2*, *SIR3*, or *SIR4* (Table 2). The simplest explanation is that the function of Sir4p as a chromatin constituent requires Sir2p and Sir3p but attachment of the protein to an immobilizing nuclear support is Sir2p and Sir3p independent. The use of LexA fusions circumvents the role of the other Sir factors in recruitment of Sir4p to DNA. With the recent exception of transcriptional and recombinational repression by *SIR2* at the *RDN1* locus (8, 25, 62), no other functions have been assigned to a Sir component that are independent of the other *SIR* genes. Nevertheless, Sir4p is not likely to be the sole factor directing the intracellular movements of telomeric DNA, nor is the protein essential for chromosome stability (57). Plasmids that contain telomeric TG_{1-3} sequences partition moderately well and are anchored efficiently in *sir* mutant strains (44, 51). Similarly chromosome ends aggregate in clusters even in the absence of the *sir* genes (24). According to one proposal, protein-protein interactions between Rap1p bound to both telomeric plasmids and chromosomal telomeres may facilitate the segregation of the extrachromosomal elements (20). In such a model, the Sir proteins would either stabilize the association between plasmids and telomeres or redirect the plasmids toward a different component that is more conducive to partitioning.

LexA-Sir4p fusions are not likely to require Rap1p for binding LexA operators, yet inactivation of the protein abrogates plasmid stabilization by the chimeras (Table 3). One explanation is that Rap1p bound at telomeres or some other nuclear location serves as the immobilization site for LexA-Sir4p linked to plasmids. This is not unreasonable, given that the two proteins coimmunoprecipitate and interact in the two-hybrid assay (16, 54). If the model is correct, Rap1p must support partitioning even when a large percentage of the protein is released from telomeres in *sir* mutants (57) (see below). An alternate explanation for the role of Rap1p is that the multifunctional protein serves indirectly as a transcriptional regulator of an additional, as yet unknown immobilization factor. A large number of other Sir4p-interacting proteins have already been identified, including Sir1p (65), histones H3 and H4 (27), the deubiquinating enzyme Ubp3p (52), and an uncharacterized 69-kDa protein isolated by affinity chromatography (52).

Colocalization of Sir3p, Sir4p, and Rap1p with discrete perinuclear foci of chromosomal telomeres requires the integrity of all three protein components (16, 24, 57). Deletion of either Sir3p or Sir4p leads to a diffuse nuclear staining pattern of the other protein, as well as Rap1p, and C-terminal truncations of Rap1p lead to dispersion of both Sir proteins. Nevertheless,

LexA-Sir4p remains anchored and partitions well in *sir* mutants (Table 2). This suggests that Sir4p immobilization sites are restricted neither to telomeric clusters nor to the nuclear periphery. Thus, an earlier proposal that Sir4p attaches telomeres to a nuclear lamina-like structure in yeast seems unlikely (18). It is now clear that the similar coiled-coil domains of Sir4p and the lamins, upon which the model was based, represent a prevalent motif that exists in a wide range of proteins. Indeed, the coiled-coil region of Sir4p (contained within positions 1262 to 1358) is not sufficient for plasmid partitioning or for DNA anchoring activity. Rather, a fragment spanning residues 950 to 1262, the PAD4 domain (partitioning and anchoring domain of Sir4p), is sufficient for both (Fig. 2, 4, and 5). Our results cannot eliminate the possibility that the PAD4 domain, in the context of full-length Sir4p, anchors exclusively to sites near the nuclear periphery. Attempts to demonstrate self-association of the PAD4 domain by the two-hybrid assay have failed (64a), and interaction with endogenous Sir4p is not essential because deletion of the *SIR4* gene does not influence the partitioning and anchoring activities of the fusion proteins (Table 2).

Interestingly, the PAD4 domain does not possess anti-sir activity, and small protein fragments with anti-sir activity neither anchor nor partition (Fig. 5). This observation indicates that the partitioning/anchoring and anti-sir activities are separable. Apparently, the PAD4 domain lacks the ability to sequester silencing factors required for the anti-sir phenotype. Whether the PAD4 domain is essential for bona fide silencing is less clear. Earlier, Marshall et al. showed that *SIR4* could be split into two intragenic complementary pieces: an allele encoding the carboxyl half of *SIR4* (amino acids 748 to 1358) rescued an unlinked allele missing the carboxy terminus of the gene (49). In contrast, a carboxy-terminal fragment beginning at position 1018, a location which interrupts the PAD4 domain, did not complement. It is thus formally possible that the PAD4 domain is important for silencing, although the evidence is only suggestive. The PAD4 domain is clearly not sufficient for silencing because the amino-terminal portion of the protein is also required (49). In addition, repression of the silent mating-type loci is lost when residues at and beyond the carboxyl junction of the PAD4 domain are removed (34). Curiously, this truncated form of the gene, the *SIR4-42* allele, lengthens the life span of yeast, as defined by an increase in the number of cell divisions before termination of growth (35). Because the effect is Sir dependent, it was proposed that the truncation permits redistribution of Sir4p and other silencing factors to affect transcriptional repression at a putative "aging" locus. If the partitioning and anchoring domain of Sir4p plays a role in silencing, it may be important for repression at the locus controlling life span, as well as the silent mating-type loci.

Immunolocalization studies of proteins in fixed samples frequently provide invaluable information about their cellular distribution and sites of action. When a protein stains in a punctate pattern within an organelle, it is generally inferred that the factor occupies an assigned position which is determined by interactions with a target or a scaffolding structure. Numerous proteins involved in DNA replication, for instance, colocalize with newly replicated DNA in discrete foci during the S phase (5, 41), and electron micrographs show that the replication centers are ovoid and attached to a network of intranuclear filaments (31). Conversely, when a protein stains in a uniform pattern within an organelle, inferences regarding its targeting and dynamics cannot be made with certainty. The factor might be freely diffusing or immobilized to a support that is well distributed. The combination of partitioning and anchoring assays provides an alternative approach to the study of the dynamics of nuclear proteins in intact cells when immunofluorescence is indecisive or inconvenient. Moreover, due to the ubiquity of the two-hybrid assay, countless proteins are already conveniently linked to heterologous DNA binding domains.

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