The Carboxy Termini of Sir4 and Rap1 Affect Sir3 Localization: Evidence for a Multicomponent Complex Required for Yeast Telomeric Silencing

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Abstract. The Silent Information Regulatory proteins, Sir3 and Sir4, and the telomeric repeat-binding protein RAP1 are required for the chromatin-mediated gene repression observed at yeast telomeric regions. All three proteins are localized by immunofluorescence staining to foci near the nuclear periphery suggesting a relationship between subnuclear localization and silencing. We present several lines of immunological and biochemical evidence that Sir3, Sir4, and RAP1 interact in intact yeast cells. First, immunolocalization of Sir3 to foci at the yeast nuclear periphery is lost in *rap1* mutants carrying deletions for either the terminal 28 or 165 amino acids of RAP1. Second, the perinuclear localization of both Sir3 and RAP1 is disrupted by overproduction of the COOH terminus of Sir4. Third, overproduction of the Sir4 COOH terminus alters the solubility properties of both Sir3 and full-length Sir4. Finally, we demonstrate that RAP1 and Sir4 coprecipitate in immune complexes using either anti-RAP1 or anti-Sir4 antibodies. We propose that the integrity of a tertiary complex between Sir4, Sir3, and RAP1 is involved in both the maintenance of telomeric repression and the clustering of telomeres in foci near the nuclear periphery.

THE regulation of gene expression by alterations in chromatin structure is a universal phenomenon in eukaryotic cells, and is responsible for the proper activation and inactivation of genes in the developmental program of multicellular organisms (Paro, 1993; Tartof and Bremer, 1990), for position effect variegation in flies (Eissenberg, 1989; Henikoff, 1990), and the variable expression of foreign genes integrated into chromosomes (e.g., Butner and Lo, 1986). In the yeast *Saccharomyces cerevisiae*, gene repression at the silent mating type loci (*HML* and *HMR*, collectively termed the *HM* loci) appears to involve a reduction in the accessibility of the entire domain to the transcription machinery, the yeast endonuclease HO, and to other modifying enzymes (for review see Laurenson and Rine, 1992). Similarly, the transcription of Pol II genes positioned adjacent to the poly($TG_{1\text{-}3}$) tracts at yeast telomeres was found to be metastable, switching between repressed and derepressed states in a process called telomeric position effect or silencing (Gottschling et al., 1990). Interestingly, like position effect variegation in *Drosophila*, where the condensed higher order structure of heterochromatin "spreads" into adjacent euchromatin, the transcriptionally inactive telomeric domain spreads inward from the telomere and is limited by the dosage of components involved in forming a "closed" chromatin state (Renauld et al., 1993; for review see Sandell and Zakian, 1992).

The yeast system has provided the genetic means to identify *trans*-acting factors and *cis*-acting sequences required for both the metastable repression of gene expression in subtelomeric regions and the repression of mating type genes at *HML* and *HMR*. Both repression events are sensitive to mutations in many of the same genes, including *SIR2*, *SIR3*, *SIR4*, *NAT1*, and *ARD1* genes, and deletions in the NH_2 termini of histones H3 and H4 (Ivy et al., 1986; Rine and Herskowitz, 1987; Mullen et al., 1989; Whiteway et al., 1987; Park and Szostak, 1992; Aparicio et al., 1991; Thompson et al., 1994). Interestingly, mutations in a fourth *SIR* gene, *SIR1*, result in metastable transcriptional states at *HML* similar to those observed near the telomeric repeat, but has no effect on telomeric silencing (Aparicio et al., 1991). The involvement of histone H3 and histone H4 NH_2 termini in both processes and the inaccessibility of both *HM* and telomeric domains to ectopically expressed bacterial *dam* methylase (Singh and Klar, 1992; Gottschling, 1992) support the notion that telomeres and the *HM* loci nucleate a closed chromatin state that confers transcriptional repression. Interestingly, yeast telomeres replicate late in S-phase, reminiscent of the late-replicating, transcriptionally silent Giemsa bands in mammalian chromosomes (Goldman et al., 1985; McCarroll and Fangman, 1988; Ferguson and Fangman, 1992), and cytological studies in many species have identified telo-

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meres as heterochromatic (see Lima-de-Faria, 1986, for a review).

At both *HML* and *HMR*, a single relatively short *cis*-acting silencer element is sufficient to confer silencing on appropriate promoters, although in the chromosome each locus is flanked by two silencer elements (Brand et al., 1987; Mahoney and Broach, 1989). Like enhancers, these silencers function in either orientation and at variable distances from the targeted promoter. Besides the genes products mentioned above, *trans*-acting factors that bind sequences in the silencer elements have been implicated in the mechanism of repression. These include Repressor Activator Protein 1 ([RAP1][1]; Shore and Nasmyth, 1987), ABF1 (ARS Binding Factor 1; Buchman et al., 1988), and the origin recognition complex (ORC, Bell et al., 1993; Foss et al., 1993; Micklem et al., 1993; Li and Herskowitz, 1993), which recognizes the ARS consensus sequence at silencers in vitro.

The abundant transcriptional regulator RAP1 has high affinity-binding sites both in the silencer elements and within the telomeric $(TG_{1\text{-}3})_n$ repeat (Longtine et al., 1989; Buchman et al., 1988). Within telomeric sequences, RAP1 binds in vitro as frequently as once per 18 bp, predicting 25–30 RAP1 molecules at each chromosomal end (Gilson et al., 1993*b*). The association of RAP1 with yeast telomeres in vivo was first suggested by the copurification of RAP1 with the yeast telosome, a nonnucleosomal chromatin structure present at telomeric $(TG_{1\text{-}3})_n$ tracts (Wright et al., 1992), and by the fact that mutations in *RAP1* confer substantial effects on telomere tract size (Lustig et al., 1990; Conrad et al., 1990; Kyrion et al., 1992). Confirmation of its role at telomeres was obtained from immunofluorescence studies of yeast meiotic cells, in which a large fraction of RAP1 was found at the ends of pachytene chromosomes (Klein et al., 1992). Immunofluorescence studies have also suggested that RAP1 is associated with telomeres in vegetatively growing cells. Approximately eight intense clusters of RAP1 are present close to the nuclear periphery of haploid interphase cells, and these foci are lost concomitantly with the loss of $(TG_{1\text{-}3})_n$ repeats in *est1* mutants (ever-shorter telomeres 1; Palladino et al., 1993). Moreover, the clusters of RAP1 staining colocalize with subtelomeric repeat probes in double immunofluorescence and in situ hybridization experiments (Scherthan, H., T. Laroche, and S. M. Gasser, manuscript in preparation). In strains deleted for *SIR3* or *SIR4*, this clustered, peripheral localization of RAP1 is lost, suggesting that yeast telomeres interact with each other and position themselves near the nuclear envelope in a Sir3- and Sir4-dependent manner (Palladino et al., 1993). These data have led to the speculation that transcriptional silencing may depend on the association of telomeres with the nuclear envelope.

The effects of mutations in *RAP1* and *SIR* genes implicate these gene products directly in the repression mechanism, possibly through physical interactions among these proteins (for review see Laurenson and Rine, 1992; Palladino and Gasser, 1994). For instance, deletions of 28 aa or more from the COOH terminus of RAP1 abrogate telomeric silencing (Kyrion et al., 1992; Liu et al., 1994). Point mutations within the COOH-terminal 20 aa of RAP1 have a similar effect, and certain of these missense mutations are suppressed by Sir3 overproduction, while the truncation alleles are not (Liu et al., 1994). This raises the possibility of an interaction between Sir3 and RAP1 mediated through the extreme COOH-terminal residues of RAP1 (Liu et al., 1994; see Fig. 1). In contrast, another class of *rap1* mutations (termed the *rap1^s* alleles) defines a 20–amino acid region ~80 aa from the COOH terminus, and provokes derepression of genes at *HMR* when flanked by a weakened silencer, but does not decrease the efficiency of telomeric silencing. The mutant rap1^s protein is unable to interact with RIF1 (RAP1-Interacting Factor), a nonessential nuclear protein identified in a two-hybrid screen (Hardy et al., 1992*b*). Consistent with the importance of this interaction in vivo, *rif1* null alleles, like *rap1^s* alleles, were found to weaken *HM*, but not telomeric, silencing. This *rap1^s* phenotype can be suppressed by elevating levels of either Sir1 or Sir4 (Sussel and Shore, 1991).

Further genetic evidence for interaction among silencing factors include the observation that overexpression of *SIR3*, in addition to suppressing certain *rap1* alleles, suppresses mutations in *sir4* (Marshall et al., 1987), and that overexpression of a carboxy-terminal fragment of Sir4, containing a lamin-like coiled-coil domain (Diffley and Stillman, 1989), abrogates transcriptional repression at *HM* loci, a phenotype similar to that observed in a *sir4$^-$* strain (Marshall et al., 1987). Such a dosage-dependent phenotype is consistent with a model in which the Sir4 protein associates with other components in a precisely balanced fashion.

Recent evidence suggests that the production of the *SIR2* and *SIR3* genes affect chromatin structure. Braunstein et al. (1993) have shown that the chromatin of both silent mating type loci and subtelomeric regions is hypoacetylated when compared with active chromatin, in a manner dependent on both *SIR2* and *SIR3*. In addition, overexpression of *SIR2* resulted in general hypoacetylation, suggesting that Sir2 may either be a histone deacetylase or a regulator of a histone acetylase. A structural role for Sir3 in chromatin is also suggested from the work of Renauld et al. (1993), who observed that strains overexpressing *SIR3* display silencing up to 25 kb from the terminus of the marked chromosome, while in wild-type strains silencing decreases rapidly beyond distances of 3.5 kb.

Recently, the two-hybrid system has been used to demonstrate the ability of Sir3 and Sir4 fusion proteins to associate both with the RAP1 COOH terminus and with each other (Moretti et al., 1994). These authors also reported an interaction between Sir3 and RAP1 in vitro. However, it was not demonstrated whether or not the proposed multicomponent complex between RAP1, Sir3, and SIR4 exists as part of the repression machinery in vivo. In this study, we examine the immunolocalization of RAP1 and Sir3 in various mutants of *S. cerevisiae*, in which telomeric silencing is altered due to impairment of RAP1 or Sir4 function. Our data establish that specific COOH-terminal domains of RAP1 are required for the peripheral localization of Sir3, Sir4, and RAP1/telomeric complexes. Overexpression of the COOH-terminal domain of Sir4, which disrupts silencing, delocalizes Sir3 and RAP1 from the nuclear periphery, and alters the solubility properties of Sir3 and Sir4. This is consistent with the disruption of a multicomponent complex. We demonstrate that RAP1 and Sir4 coimmunoprecipitate from nuclear ex-

1. *Abbreviations used in this paper*: 5-FOA, 5-fluoro-orotic acid; RAP1, repressor activator protein 1; Δ indicates a gene disruption.

Table I. Strains Used in This Study

Diploid strains
AJL306 (*MATa/MATα RAP1/RAP1 trp1/trp1 ade2-1/ade2-1 HIS3/his3 leu2-3,112/leu2-3,112 ura3-1/ura3-1 AC: URA3/SUP11*)
AJL307 (*MATa/MATα RAP1/rap1-17 trp1/trp1 ade2-1/ade2-1 HIS3/his3 leu2-3,112/leu2-3,112 ura3-1/ura3-1 AC: URA3/SUP11*)
AJL308 (*MATa/MATα rap1-17/rap1-17 trp1/trp1 ade-2-1/ade2-1 HIS3/his3 leu2-3,112/leu2-3,112 ura3-1/ura3-1 AC: URA3/SUP11*)
GA192 (*MATa/MATα ade2-1/ADE2 trp1-1/TRP1 his3-11/his3 ura3-1/ura3-52 leu2-3,112/LEU2 LYS2/lys2-6 CAN1/can1-100 sir3::TRP1/sir3::LYS2*)
GA304 (*MATa/MATα his/his3 ura3-52/ura3-52 hta1-1/hta1-1 hta2-1/hta2-1 [URA3/CEN3/ARS1/HTA1]* and *[HIS3/CEN6/ARS4/HTA2]*)
YLS554 (*MATa/MATα rap1-12::LEU2/rap1-12::URA3 ade2-1/ade2-1/ade2-1 leu2-3,112/leu2-3,112 ura3-1/ura3-1 hmr::ADE2/hmr::ADE2*)
Haploid strains
AJL275-2aVIIL (*MATα RAP1 ade2-1 his3 leu2-3,112 ura3-1* VIII::*URA3*)
AJL369-5d (*MATa rap1-17 ade2-1 HIS3 leu2-3,112 ura3-1* VIIL::*URA3*)
AJL399-4b (*MATα rap1-18 ade2-1 his3 leu2-3,112 ura3-1* VIIL::*URA3*)
AJL275-2a VR (*MATα RAP1 ade2-1 his3 leu2-3,112 ura3-1* VR::*URA3*)
CLY/rap1-21 (*MATa rap1::LEU2 trp1 ade2-1 leu2-3,112 ura3-1 his3* VIIL::*URA3 [rap1-21/CEN/HIS3]*)
GA59 (*MATa trp1 ura3-52 leu2-3,112 prb1-1122 pep4-3 prc1-407 gal2*)
GK28-3b (*MATa rap1::LEU2 trp1 ade2-1 leu2-3,112 ura3-1 his3 VIIL::URA3 [RAP1/CEN/TRP1]*)
GK23-1b (*MATα rap1::LEU2 trp1 ade2-1 leu2-3,112 ura3-1 his3* VR::*URA3 [RAP1/CEN/TRP1]*)
P17s4 (*MATα rap1::LEU2 trp1 ade2-1 leu2-3,112 ura3-1 his* VR::*URA3 [rap1-17/CEN/HIS3]*)
P17s4D-P (*MATα rap1::LEU2 trp1 ade2-1 leu2-3,112 ura3-1 his3* VR::*URA3 [RAP1/CEN/HIS3]*)
YLS529 (*MATα rif1::URA3 ade2-1 trp1-1 his3-11,15 ura3-1 leu2-3,112 can1-100 hmr::TRP1*)
YAB191 (*MATα ade2-1 trp1-1 his3-11,15 ura3-1 leu2-3,112 can1-100 sir1::LEU2*)

tracts suggesting a direct biochemical interaction between these proteins. Together our data suggest interactions among Sir3, Sir4, and RAP1 in vivo, which seem to be required for both subnuclear localization and silencing function at telomeres.

Materials and Methods

Plasmids and Yeast Strains

Overexpressing plasmids pADH-SIR4 and pADH-SIR4C were constructed from plasmid pAAH5. This 2 μm-based plasmid carries 1.5 kb of the *ADH1* promoter region upstream of a HindIII site, and 500 bp of the *ADH1* 3′ region, including polyadenylation and transcriptional termination signals, downstream of this site. A 4.4-kb ScaI–ClaI fragment containing the entire *SIR4* gene and a 1.9-kb SmaI–ClaI fragment encoding the COOH-terminal 558 aa of Sir4 were cloned into the filled-in HindIII site of pAAH5 to create pADH-SIR4 and pADH-SIR4C, respectively. For antibody purification and quantiation of Sir4 in nuclei, a GST-fusion protein was expressed in *E. coli* containing the same 558 aa COOH-terminal Sir4 domain (kind gift of M. Gotta). The single copy plasmids pRS6.3 (*SIR3* in pSEYC58) and pJR368 (*SIR4* in YCp50) were kind gifts of J. Rine; plasmid pAR16, containing the *SIR3* gene under control of the *GAL1-10* promoter, was the kind gift of J. Broach (Braunstein et al., 1993).

All strains used are listed in Table I. The diploid strains AJL306, AJL307, and AJL308 carrying a 150-kb artificial chromosome (AC), have been previously described (Kyrion et al., 1992). Haploid strains AJL275-2a VIIL and AJL399-4b carry a copy of the *URA3* gene adjacent to the left arm of chromosome VIIL as described (Kyrion et al., 1993). AJL275-2a VR is isogenic to AJL275-2a VIIL except for the presence of a *URA3* gene adjacent to the right telomere of chromosome V (Kyrion et al., 1993). For analysis of the effects of high copy numbers of Sir4 and Sir4C on telomeric silencing and positioning, plasmids pAAH5, pADH-SIR4, and pADH-SIR4C were transformed into AJL275-2a VR.

Strain P17s4 was derived from GK23-1b by a plasmid shuffle that substitutes the wild-type allele of *RAP1* with the *rap1-17* allele as previously described (Kyrion et al., 1993). P17s4D-P was derived from P17s4 by a plasmid shuffle replacing the *rap1-17* allele, after elongation of telomeric tracts, with a wild-type copy of *RAP1* as described (Kyrion et al., 1993). This strain differs from GK23-1b only in the length of their telomeres. P17s4D-P was subcultured for multiple rounds of growth on nonselective media, each round representing 25 generations of growth. CLY/rap1-21, carrying a plasmid-borne copy of *rap1-21*, was derived from GK28-3b by a plasmid shuffle as described (Liu et al., 1994).

The protease deficient strain GA59 (also called BJ2168) was used for the preparation of yeast nuclei and for immunoprecipitation experiments. The strains carrying deletions of *SIR1* and *RIF1*, and the *rap1*^{s} mutant (*rap1-12*) were kind gifts of D. Shore. Growth media and genetic manipulations with yeast are as described by Rose et al. (1990).

Antibody Production and Affinity Purification

The preparation of the anti-RAP1 antibody was described in Klein et al. (1992). The rabbit antisera against Sir3 and Sir4 were raised against full-length *lacZ-SIR3* and *lacZ-SIR4* fusion proteins (expression plasmids were gifts from L. Pillus), or were as described in Palladino et al. (1993). Standard methods for subcutaneous injection and antiserum collection were used (Harlow and Lane, 1988). All antibodies were affinity purified before use as described in Gasser et al. (1986). To obtain RAP1 for affinity purification and competition studies, amino acids 19–827 of *RAP1* were fused to phage T7 gene 10 protein and overexpressed in *E. coli* (Gilson et al., 1993*b*). The two *lacZ-SIR* gene fusions described above were used to affinity purify the specific anti-Sir antibodies.

Immunofluorescence on Yeast Spheroplasts

Immunofluorescence was performed as described in Palladino et al. (1993). After three washes in PBS, slides were mounted with 50% glycerol in PBS and 2 μg/ml DAPI, and viewed and photographed on a Zeiss Axiophot microscope using a 100× Pan Neofluar objective. For confocal microscopy either ethidium bromide staining or anti-DNA monoclonal antibodies (Chemicon International, Temecula, CA) were used to detect the nuclear DNA. Visualization was performed on an MRC 600 confocal microscope using the control software version 4.81-Beta (Biorad Microscience; Biolabs Labs, Hercules, CA) and an inverted Zeiss Axiovert microscope. An argon laser was used at wavelengths of 488–518 nm to detect both Texas red and FITC fluorochromes. On the Biorad system, images were standardly filtered using the convolve function C7B.

Yeast Nuclear Isolation and Coimmunoprecipitation Assays

Nuclei from protease deficient strains were prepared essentially according to Verdier et al. (1990) with the following modifications. After spheroplast lysis by Dounce homogenization in cell breakage buffer (18% Ficoll, 5 mM Pipes-KOH, pH 7.4, 20 mM KCl, 2 mM EDTA-KOH, pH 7.4, 0.125 mM spermidine, 0.05 mM spermine, 0.5 mM PMSF, 1% thiodiglycol, 1%

Trasylol, 1 μg/ml pepstatin, 1 μg/ml leupeptin), 20 ml of suspension (containing not more than 3 g wet weight of cells) was layered over 18 ml of 20% glycerol, 7% Ficoll, in the same buffer as above with protease inhibitors, and was centrifuged at 20,000 *g* for 30 min. The supernatant containing cytosol and vacuolar material was removed by aspiration. The pellets were resuspended in cell breakage buffer and subjected to a further 20–30 strokes of the Dounce homogenizer, and centrifuged at 3,000 *g* for 15 min. This step was repeated with the supernatant. The supernatant from the second low speed spin was recovered and further centrifuged at 20,000 *g* for 25 min. The resulting nuclear pellet was resuspended in 60% glycerol, 5 mM Tris-Cl, pH 7.4, 20 mM KCl, 2 mM EDTA-KOH, 0.125 mM spermidine, 0.05 mM spermine and protease inhibitors as described above. Nuclei were either used directly or stored at −20°C at ~20 A_{260}/ml. 1L of cells at 2.5–5 × 10^7 cells/ml, routinely yields 30–45 A_{260} of crude nuclei.

For solubility studies of RAP1 and the Sir proteins, approximately 10 A_{260} of nuclei were pelleted by brief centrifugation and resuspended in 500 μl of lysis buffer: (1% Triton X-100, 20 mM $NaPO_4$, pH 7.8, 100 mM NaCl, 20 mM NaF) containing protease inhibitors at the following concentrations; 1 mM PMSF, 0.1% Trasylol (Bayer, aprotinin), 1 μg/ml pepstatin and 1 μg/ml leupeptin. Nuclei were incubated in lysis buffer for 30 min at 4°C and subsequently were either centrifuged directly or subjected to brief sonication before centrifugation in an Eppendorf centrifuge at 4°C for 5 min. Alternatively, these nuclei were treated lightly with micrococcal nuclease, which also released a majority of the genomic DNA. The sonication method proved more rapid and was standardly used. Equivalent starting volumes of material from the insoluble pellets and the soluble supernatants were dissociated in sample buffer (Laemmli, 1970), subjected to SDS gel electrophoresis in 10% acrylamide gels, and Western blotted with antibodies directed against RAP1, Sir3, or Sir4 proteins, using enhanced chemiluminescence (ECL, Amersham) to detect antibodies. RAP1, like Sir3 and Sir4 proteins, copurifies with the nuclear scaffold, a fractionation protocol distinct from the solubilization of chromatin used here (see Cardenas et al., 1990).

Proteins were immunoprecipitated from equal volumes of the nondenatured, soluble fraction of lysed and sonicated nuclei (see above). Soluble nuclear extracts were preincubated with protein A–Sepharose beads for 30 min at 4°C. Beads were removed by brief centrifugation at 4°C and 200 μl aliquots of the supernatants were incubated overnight at 4°C with the different antisera, in the presence of 10 μg/ml DNaseI and 5 mM $MgCl_2$. Antibody-antigen complexes were precipitated by 2 h incubation with 25 μl of protein A–Sepharose beads followed by brief centrifugation. The beads were washed four times in 500 μl of lysis buffer at 4°C, and then boiled in 200 μl of sample buffer to release bound proteins. Beads were removed by centrifugation and the samples were subjected to SDS-PAGE followed by Western blot analysis by standard procedures.

Quantitation of RAP1, Sir3, and Sir4 in Yeast Nuclei

Proteins were purified from bacteria (RAP1 and Sir4) or from yeast (Sir3) carrying plasmids containing either full-length *RAP1*, a GST-Sir4C fusion protein or full-length *SIR3* under control of inducible promoters. RAP1 was purified by affinity chromatography (Gilson et al., 1993*b*), and the Sir proteins by cell fractionation. Confirmation that the induced products stained by Coomassie blue corresponded to the expected proteins was obtained by Western blotting with affinity-purified antibodies.

Partially purified GST-Sir4C and Sir3, or affinity-purified RAP1 were subjected to electrophoresis on 10% SDS-PAGE and the relevant proteins were excised and electroeluted from preparative scale gels. The concentrations of the gel-purified proteins were estimated by comparison of the Coomassie staining intensity of serial dilutions with a series of mixed molecular weight markers of known concentration. Serial dilutions of the purified Sir3, Sir4, and RAP1 at concentrations thus estimated were then subjected to electrophoresis alongside serial dilutions of yeast nuclei of known concentration (determined by cell counting and by quantitation of the DNA recovered from a given quantity of RNaseA-treated nuclei). The separated proteins were transferred to nitrocellulose and Western blotted with affinity-purified antisera to Sir4C, Sir3, and RAP1. Quantitation of proteins reacting with the primary antibodies was achieved by incubating the blots with ^{125}I-donkey α-rabbit IgG $F(ab)_2$ fragments (Amersham Inc., Buckinghamshire, UK). The relevant bands were excised and counted directly for ^{125}I-dependent radiation.

Miscellaneous Methods

The size of the majority of telomeres containing a conserved XhoI site in the subtelomeric Y′ element was determined by subjecting XhoI-digested yeast DNA to Southern analysis using a 49-nucleotide $(C_{1\text{-}3}A)_n$ probe (AT1, Lustig et al., 1990). The heterogeneous band of 1.2 kb seen in wild-type cells represents a tract length of ~300 bp. The telomere length present at the *URA3*-marked VR telomere was determined by digestion with HindIII, followed by Southern analysis using *URA3* as a probe, as previously described (Kyrion et al., 1992).

To determine the frequency of 5-fluoro-orotic acid (5-FOA)–resistant cells produced by transcriptional silencing at the telomeric *URA3* gene, cells were grown for 2–3 d under selection for the presence of the plasmids, and were subsequently inoculated into liquid selective medium. When these cultures reached mid-log phase, 25 μl of 10-fold serial dilutions were spotted onto synthetic selective medium, to determine the total number of viable cells, and on to the same medium containing 5-FOA (Gottschling et al., 1990), to determine the number of 5-FOA–resistant cells after 3–4 d growth. All cells were grown at 30°C.

Results

Repressor Activator Protein 1 is an abundant nuclear protein that binds many sites within the $(TG_{1\text{-}3})_n$ repeat at the ends of all yeast chromosomes, as well as at internal UAS and silencer elements. Serial images using a confocal microscope on yeast spheroplasts labeled with anti-RAP1 antibodies show that the RAP1-containing foci are few in number and are localized near the periphery of the nucleus (Palladino et al., 1993). In deletion mutants of *SIR3* and *SIR4*, the RAP1 staining is dispersed throughout the nucleus, distinctly unlike the small number of brightly staining foci observed in wild-type cells (Palladino et al., 1993). To investigate both the potential interdependence of RAP1, Sir3, and Sir4 in subnuclear positioning, and the relationship of their localization with telomeric silencing, we have used immunofluorescence to localize Sir3 and RAP1/telomeric complexes in a range of yeast mutants that affect telomeric silencing.

Sir1Δ, rif1Δ, and rap1^s Mutants Do Not Delocalize RAP1 Immunofluorescence

We first tested whether RAP1 delocalization is a general feature of mutations that influence telomere length and *HM* silencing. To this end, we have compared the localization of RAP1 in a parental *SIR*$^+$ strain to that in *sir1Δ*, *rif1Δ*, and *rap1^s* mutant strains, all of which disrupt mating type, but not telomeric, silencing (Aparicio et al., 1991; Kyrion et al., 1993; Liu et al., 1994). In fact, both *rif1Δ* and *rap1^s* mutations increase telomere length and the efficiency of telomere silencing, consistent with a role of RIF1 and the *rap1^s* domain of RAP1 at telomeres (Sussel and Shore, 1991; Kyrion et al., 1993; Liu et al., 1994). Mutations in *SIR1* affect neither telomeric silencing nor size control, although Sir1 can enhance telomeric silencing when artificially targeted next to a telomeric repeat (Aparicio et al., 1991; Chien et al., 1993).

Fig. 2 displays confocal fluorescence microscopy of haploid *SIR*$^+$ and *sir1Δ* yeast spheroplasts reacted with affinity-purified anti-RAP1 antibodies (panels *a–c*). In both wild-type and *sir1Δ* strains, bright spots of RAP1-dependent fluorescence are observed. The superposition of the anti-RAP1 signal on the anti–DNA-staining patterns reveals that these foci are primarily at the nuclear periphery (Fig. 2, panel *b*). Similarly, RAP1 staining in *rap1^s* (*rap1-12*) and *rif1Δ* strains appears clustered and largely perinuclear (Fig. 2, *e* and *f*), in contrast to the delocalized patterns observed in *sir3* and *sir4* deletion strains (shown for *sir3Δ*, Fig. 2 *d*). Interestingly, the intensity of the anti-RAP1 reactive foci as measured by the Biorad confocal microscope increases approximately twofold in *rap1^s* and *rif1Δ* strains when each is

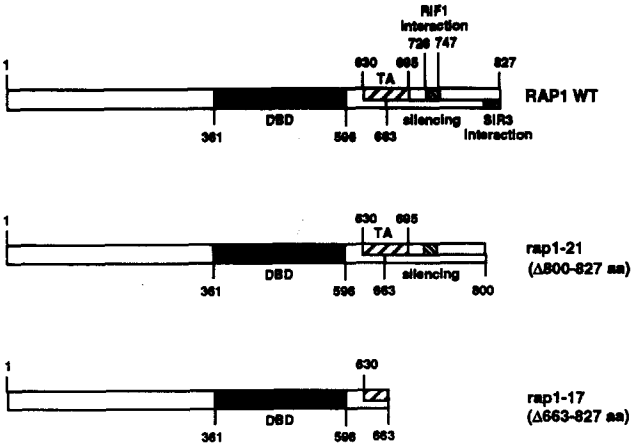

Figure 1. RAP1 and its functional domains. A schematic map of Repressor Activator Protein 1 is shown indicating the proposed functional domains based on the phenotypes of *rapl* missense and nonsense alleles (Sussel and Shore, 1991; Liu et al., 1994), and expression of individual domains (Hardy et al., 1992*a*; Henry et al., 1990). Below the wild-type RAP1 are illustrated the two COOH-terminal truncation alleles used in this study: the *rapl-21* which lacks amino acids 800–827, and the *rapl-17*, that lacks amino acids 663–827. The black box indicates the minimal DNA-binding domain (DBD, Henry et al., 1990); while the dark hatched box indicates a region defined by the *rapl*^{s} alleles as interacting with RIF1 (Hardy et al., 1992*b*). The light stippled box is the region of proposed interaction with Sir3 (see this work). The entire region from aa 663 to the end indicates a region that affects telomeric and HM silencing. TA indicates a region necessary for transactivation.

compared to its isogenic parental strain (data not shown), consistent with the observed 2–3-fold increase in the telomere tract size (Hardy et al., 1992*b*; Sussel and Shore, 1991). Thus, the correlation between telomere silencing and the clustering of RAP1 staining can be extended: when silencing is disrupted, perinuclear clustering is disrupted; when silencing is maintained, despite an extension of telomere length, perinuclear localization, and clustering of RAP1 foci are maintained.

RAP1 Staining is Aberrant in rapl^t Strains

We next examined the domain requirements for peripheral localization of RAP1/telomeric complexes. To this end, we performed immunofluorescence in strains carrying *rapl^t* alleles that truncate RAP1 by either 144 or 165 amino acids at its COOH terminus. The *rapl-17* mutation removes the COOH-terminal 165 aa of RAP1 (Fig. 1), and confers on cells high rates of chromosome loss, full derepression of telomeric silencing, and dramatic lengthening of the telomeric repeat length. It is not defective, however, for RAP1-DNA binding, gene activation, or cell viability (see Fig. 4; Kyrion et al., 1992, 1993).

The anti-RAP1 staining in the homozygous *rapl-17* mutant produces extremely bright immunofluorescence with two distinct staining patterns (Fig. 3 *a*). The anti-RAP1 staining shows either large bars, several times the length of normal RAP1 foci, still largely peripheral in the nucleus (*large arrows*, Fig. 3 *a*), or else numerous small foci distributed randomly throughout the nucleoplasm (*small arrows*, panel *a*). The same two patterns were observed in haploid strains carrying the *rapl-18* mutation, which truncates the terminal 144 amino acids of RAP1 and has phenotypes identical to *rapl-17* (data not shown; Kyrion et al., 1992, 1993). We do not know whether the two phenotypically different staining patterns in the *rapl^t* population represent two alternative telomeric states affecting all telomeres in a given cell, or possibly, distinct localization patterns at different stages of the cell cycle.

As noted above, telomeric tracts are grossly elongated in *rapl-17* alleles, with telomeres attaining sizes up to 4 kb longer than the wild-type telomeres. Two approaches have been taken to test whether the aberrant localization is the consequence of the elongated telomeres present in these cells. First, we performed a "plasmid shuffle" to exchange the *rapl-17* allele with the wild-type *RAP1* gene. In this way, we have introduced the elongated telomeres present in *rapl-17* cells (Fig. 4, *1-17s4*) into an otherwise isogenic wild-type *RAP1* background. These telomeres retain their elongated tract size, which decreases slowly over generations of growth (Fig. 4, WT_L *s0*, sl). In the shuffled strain, despite the extended length of the telomeric repeats, we observe a wild-type RAP1-staining pattern (see Fig. 3 *c*, *inset*). Thus neither chromosome stability, telomeric silencing, nor perinuclear localization is impaired in wild-type strains inheriting elongated telomeres (Kyrion et al., 1992, 1993).

Second, since *rapl-17* is semidominant for the extended telomere length, we have examined RAP1 staining in *RAP1/rapl-17* heterozygous strains. As shown in Fig. 3 (*b*), intense punctate staining is observed. These data indicate that the peripheral localization defects in these cells, like chromosome loss and silencing defects, are recessive. Both lines of evidence argue that the unusual RAP1-staining pattern in the *rapl^t* mutants is not simply due to aberrant telomere length, and suggests a role for the RAP1 COOH-terminal 144 amino acids in the peripheral localization of telomeres.

Sir3 and Sir4 Localization Is Altered in rapl^t Mutants

The loss of RAP1- and telomere-positioning observed in at least half the *rapl^t* cells resembles the delocalization observed in *sir3Δ* and *sir4Δ* strains (Palladino et al., 1993). To test the possible interdependency among RAP1, Sir3, and Sir4 in peripheral targeting, we determined the localization of the Sir proteins in the *rapl-17* strains by immunofluorescence. All antibodies were affinity purified against fusion proteins before use, and Western blots confirmed their monospecificity (for Sir4, a single polypeptide at M_r = 170, and for Sir3, one at M_r = 120, see Palladino et al., 1993). A weak, but reproducible staining of foci in the nuclei of wild-type yeast cells, similar to the staining with anti-RAP1 antibodies, was observed with each antiserum in *SIR*$^+$ cells. Previously published controls on *sir3Δ* and *sir4Δ* strains revealed no significant signals for the anti-Sir3 and anti-Sir4 antibodies, respectively, confirming the specificity of the immune reaction (Palladino et al., 1993).

The immunostaining for anti-Sir3 in the *rapl-17* diploid reveals a loss or strong diminution of the discrete punctate staining observed both in the wild-type and in the heterozygous *RAP1/rapl-17* strain (Fig. 3, *d* and *e*). Similarly, only a general nuclear staining is observed with anti-Sir4 in the *rapl-17* diploid (Fig. 3, *f* and *g*). In the isogenic parental strain, both anti-Sir3 and anti-Sir4 reveal the typical perinuclear foci seen in the heterozygotic *RAP1/rapl-17* strain

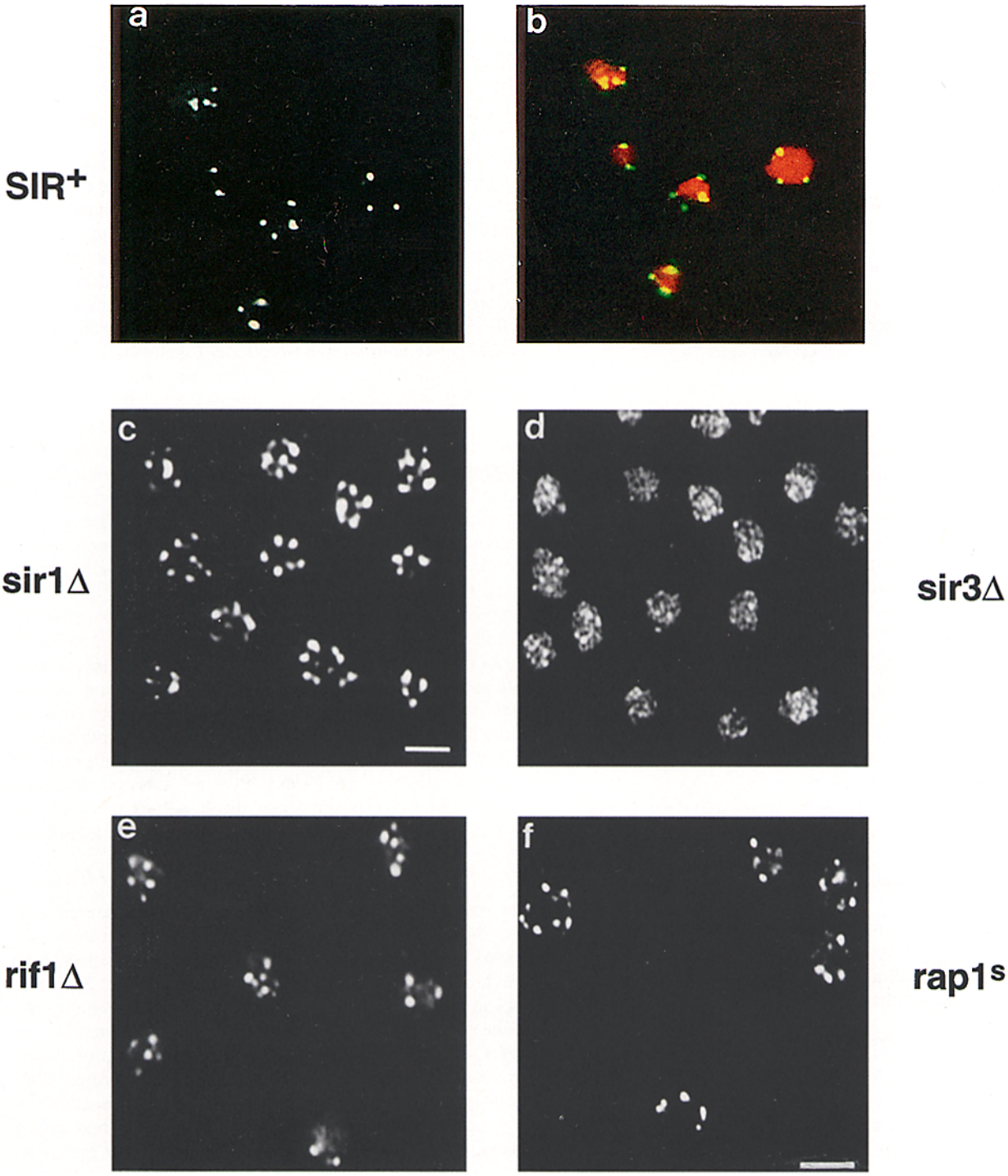

Figure 2. *SIR+*, *sir1Δ*, *rif1Δ*, and *rapl*^{s} strains stained with anti-RAP1 show discrete foci with perinuclear positioning. Immunofluorescence using affinity-purified anti-RAP1 antibodies and FITC-coupled secondary antibodies was performed on yeast spheroplasts from wild-type and mutant strains, grown overnight at 30°C in rich media. Images were visualized by confocal microscopy, and a single focal plane of 0.3 μm is shown in each case (see Materials and Methods). Panel *a* is anti-RAP1 staining alone, and panel *b* is the superposition of anti-RAP1 signal on the genomic DNA visualized by ethidium bromide, on a strain wild-type for *SIR1-4*, *RAP1*, and *RIF1* genes (GA304). The anti-RAP-1 reactive foci are largely peripheral in the nucleus. Panel *c* shows anti-RAP1 on the haploid *sir1::LEU2* deletion strain (YAB191). Superposition with anti-DNA shows that the foci are largely peripheral (data not shown). Panel *d* shows the dispersed anti-RAP1 signal in a *sir3* deletion strain (GA192). Panel *e* shows the anti-RAP1 staining of the haploid *rif1::URA3* deletion strain, YLS529, and panel *f*, the diploid *rapl*^{s} mutant (*rapl-12* in strain YLS554). Previous studies have shown that neither the anti-RAP1 preimmune serum nor the secondary antibodies alone give a signal above background (Klein et al., 1992). Panel *f* is of slightly higher magnification than the others. Bars, 2 μm.

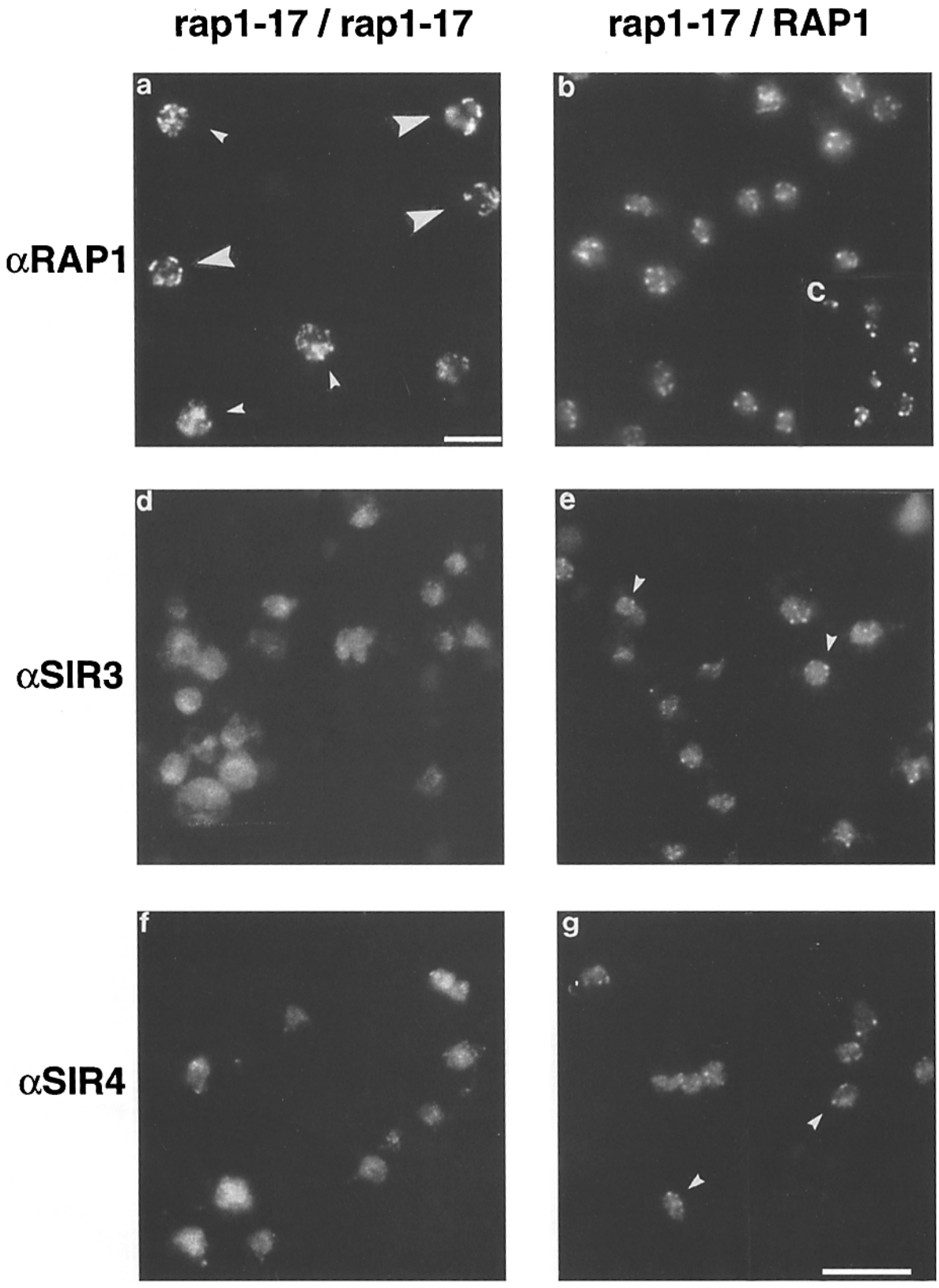

Figure 3. RAP1, Sir3, and Sir4 immunofluorescence is altered in *rapl-17/rapl-17* homozygous, but not in *rapl-17/RAP1* heterozygous strains. The isogenic diploid strains AJL308 and AJL307, which are homozygous and heterozygous for the *rapl-17* allele, respectively, were subjected to immunofluorescence with affinity-purified anti-RAP1 (panels *a* and *b*), anti-Sir3 (panels *d* and *e*) , and anti-Sir4 (panels *f* and *g*) antibodies. Shown are images taken on a Zeiss Axiophot microscope using a 100x Planapo objective. In all cases immunofluorescence signals coincide with the nucleus as detected by DAPI staining (data not shown). The inset panel *c* in panel *b* is anti-RAP1 immunofluorescence performed on the haploid P17s4D-P in which the plasmid-borne *rapl-17* allele was exchanged for a plasmid-borne *RAP1* gene (see Materials and Methods). The strain used had been subjected to one round of subculturing, and corresponds to s1 in Fig. 4; telomeres are elongated, but the RAP1 protein is wild-type (Kyrion et al., 1992). Immunofluorescence on an isogenic haploid strain wild-type for RAP1 gives an identical staining pattern. Large arrows in panel *a* indicate large, bar-like staining patterns, while small arrows show dispersed punctate staining. Small arrows in panels *e* and *g* show peripheral foci with antiSir3 and antiSir4, respectively. Bar in panel *a*, 3 μm. Bars in panels *b–f*, 4 μm.

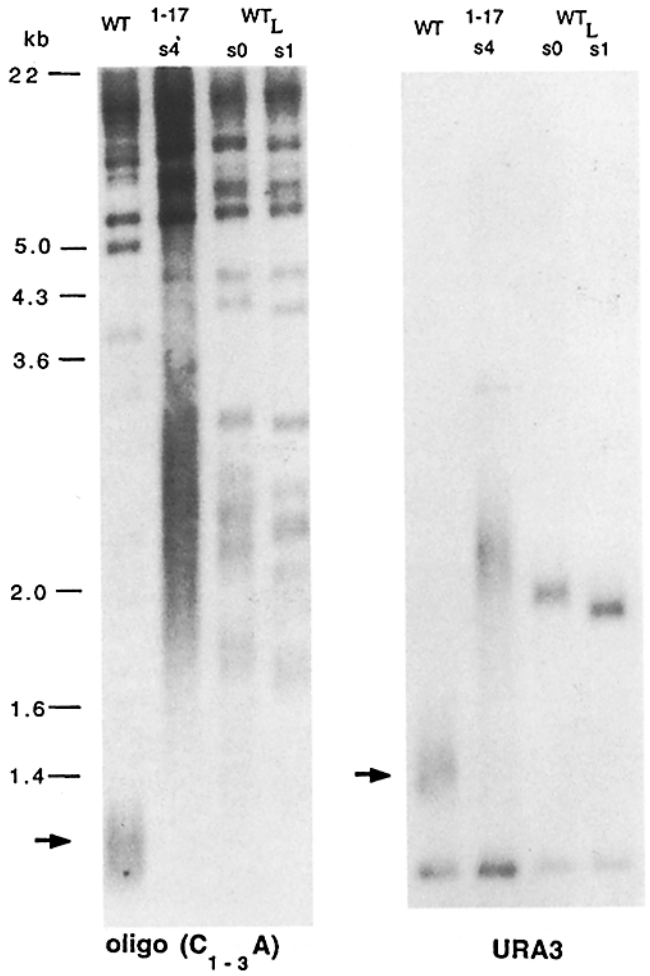

Figure 4. Elongated telomere tracts in *rapl-17* mutants are only slowly reversible when wild-type RAP1 is reintroduced. Southern blot analysis of genomic yeast DNA reveals the sizes of telomeric fragments from the Y′ class of telomeres (XhoI digest, *left*; probed with oligo $C_{1\text{-}3}A$) and of the *URA3*-marked VR telomere (HindIII digest; *right*; probed with a fragment containing *URA3*) in the shuffled strain used for the immunofluorescence assay in Fig. 3 *c*. The length of telomeres in the wild-type background is indicated with an arrow, and DNA size markers are indicated at the left of the blot. (Lanes *1*) The progenitor wild-type strain (GK28-3b); (lanes *2*) *rapl-17* strain (1-17) subcultured for four rounds (*s4*; ~100 generations); (lanes *3* and *4*) wild-type strains derived from P17 (P17D-P labeled WTL) after a plasmid shuffle before (*s0*) and after (*s1*) one round of subculturing.

(data not shown). It is noteworthy that the perinuclear foci observed for Sir4 are even more pronounced in the heterozygote than in the parental wild-type cells. Since the *rapl-17* mutation is semidominant for telomere length, the increased fluorescence signal in the heterozygote could either reflect an increased level of Sir4 associated with the longer telomeres, or else an enhanced accessibility for the antibody in the absence of the RAP1 COOH-terminal 165 aa. The apparent inaccessibility of the Sir4 epitopes in wild-type cells makes it difficult to detect this protein routinely by immunofluorescence (data not shown, Palladino et al., 1993).

The 28 aa COOH-terminal Tail Domain of RAP1 Is Required for Proper Subnuclear Localization of Sir3

It has recently been demonstrated that telomeric silencing is lost upon mutation within or truncation of the extreme COOH terminus of RAP1 (Liu et al., 1994). The *rapl-21* allele, which removes the COOH-terminal 28 aa, results in full derepression of telomeric silencing, and partial loss (40-fold) of *HML* silencing. Interestingly, overproduction of Sir3 can suppress the telomere silencing defects of some, but not all, alleles within this domain, suggesting that some alleles may be defective in binding Sir3 (Liu et al., 1994). We therefore tested whether either telomeric positioning and/or Sir3 localization were affected in the *rapl-21* mutant.

Fig. 5 shows the peripheral and punctate staining pattern of RAP1 in isogenic wild-type and *rapl-21* haploid cells. Telomeric positioning is not dramatically altered in the mutant strain, although close examination suggests a slightly higher frequency of cells with RAP1 foci that are less clustered or slightly displaced from the periphery of the nucleus (Fig. 5, *arrows* in panel *B*). Staining for Sir3, however, shows a striking delocalization of Sir3 in presence of the *rapl-21* mutation. In the wild-type strain we observe a punctate pattern of brighter spots above a low level diffuse staining throughout the nucleoplasm, while in the *rapl-21* mutant we see only the diffuse, low level staining, suggesting that Sir3 is no longer localized at telomeric domains (Fig. 5, compared panels *C* and *D*). These data indicate that the last 28 amino acids of RAP1 are required for peripheral localization of Sir3 and, combined with the allele-specific suppression of telomeric silencing by overproduction of Sir3 (Liu et al., 1994), argues that correct targeting requires direct contact between Sir3 and this domain.

Overexpression of the Sir4 COOH Terminus Derepresses Telomeric Silencing, and Delocalizes RAP1 and Sir3

Overexpression of the Sir4 COOH terminus (referred to as Sir4C) has been demonstrated to abrogate *HML* and *HMR* silencing, suggesting the titration of an essential factor or disruption of a multimeric complex (Marshall et al., 1987). To test the effect of Sir4C overproduction on telomeric silencing, we introduced high copy number plasmids expressing either full-length Sir4 or the Sir4 COOH terminus into a strain carrying a *URA3* gene adjacent to the telomeric tract on the right arm of chromosome V (see Fig. 6). Both expression constructs are under control of the *ADH1* promoter, which induces a strong constitutive level of transcription. Telomeric silencing was monitored by the efficiency of growth on 5 fluoro-orotic acid (5-FOA), which is toxic to these cells unless the telomere proximal *URA3* gene is repressed (Boeke et al., 1987). Quantitation by Western blots reveals a 40–50-fold excess of the Sir4 COOH-terminal domain over endogenous Sir4 (Fig. 9), while the full-length Sir4 under *ADH1* control was less efficiently expressed (data not shown). Overexpression of either full-length Sir4 or its COOH-terminal domain, results in derepression of the *URA3* gene, shown here as increased sensitivity to 5-FOA (Fig. 6). In contrast, the 5-FOA resistance in control cells carrying the pADH vector alone was identical to that of wild-type cells (FOA^r = 0.1 or 10%; Fig. 6).

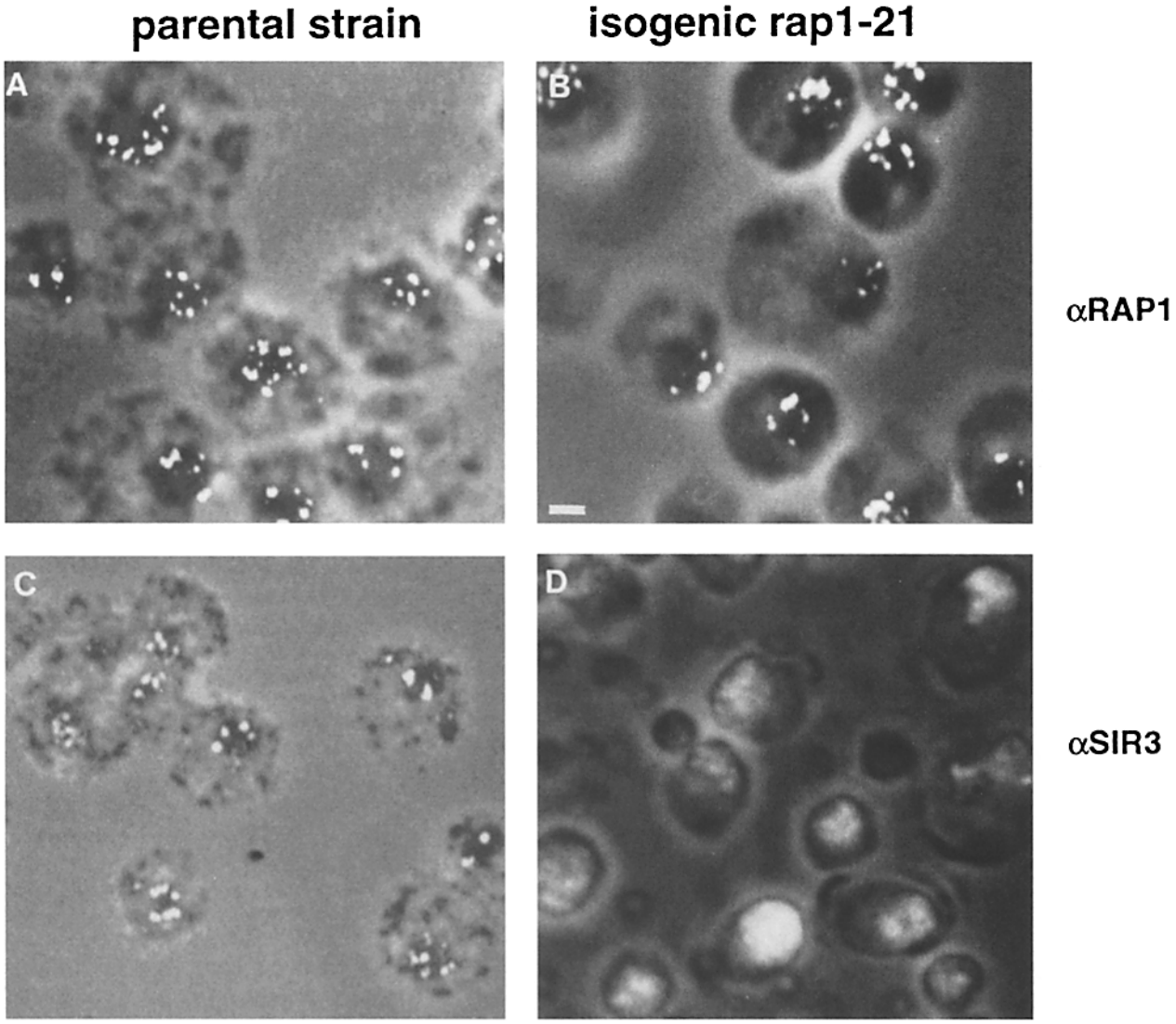

Figure 5. Deletion of the terminal 28 aa of RAP1 is sufficient to cause delocalization of Sir3. Immunofluorescence was performed as described in Materials and Methods on the haploid strains GK28-3b, containing the wild-type *RAP1* allele (panels *A* and *C*), and on CLY/rap1-21 (panels *B* and *D*), which contains the *rap1-21* mutation and is derived from GK28-3b by a plasmid shuffle (see Liu et al., 1994). The formaldehyde-fixed spheroplasts were reacted with affinity-purified anti-RAP1 or anti-Sir3, and the primary antibodies were visualized by FITC-coupled secondary antibodies. Images of the fluorescence signal are superimposed on the direct light images, both taken on the Biorad MRC600 confocal microscope, as described in Materials and Methods. Note that a threshold function removes the diffuse staining of the nucleoplasm by anti-Sir3 in panel *C* (see Fig. 3, panel *e*). In panel *D*, there are no bright foci above the general nuclear staining. The fluorescence signal in all panels is a white signal superimposed above the gray-tone phase microscope image. Bar, 1 μm.

To determine whether Sir4C overproduction also disrupts the localization of RAP1 staining, we have performed immunofluorescence on cells transformed with either the pADH plasmid or the plasmid overexpressing Sir4C. Immunolocalization of RAP1 shows a pronounced delocalization of the RAP1 foci upon Sir4 overexpression, although, in contrast to the *sir4Δ* strain, the staining is still somewhat clustered in foci (Fig. 7 *b*). Interestingly, in contrast to the normal punctate anti-Sir3 staining in wild-type cells (Fig. 7 *c*), anti-Sir3 fluorescence in strains overproducing Sir4C display a diffuse, delocalized staining, similar to the staining of nuclear DNA (see Fig. 7 *d*). This weak, diffuse staining could either represent the specific masking of the Sir3 epitopes at the peripheral foci, or, more likely, reflect a true delocalization of Sir3 from these foci. The latter interpretation would suggest that the COOH-terminal domain of Sir4 titrates a factor that is limiting for proper localization of both RAP1/telomeric complexes and Sir3. Alternatively, overproduction of Sir4C may disrupt a multicomponent complex containing both RAP1 and Sir3, possibly by aberrant associations of the Sir4 COOH-terminal coiled-coil domain with other factors. These data further support the correlation between Sir3 localization and telomeric silencing. We note that overproduction of neither Sir3 nor Sir4C significantly alters telomere tract length, indicating that telomere size control does not require the peripheral localization of RAP1/telomere complexes (data not shown).

Solubility Properties of Sir3 and Sir4 in Isolated Nuclei

Another way to demonstrate that Sir3, Sir4, and RAP1 form

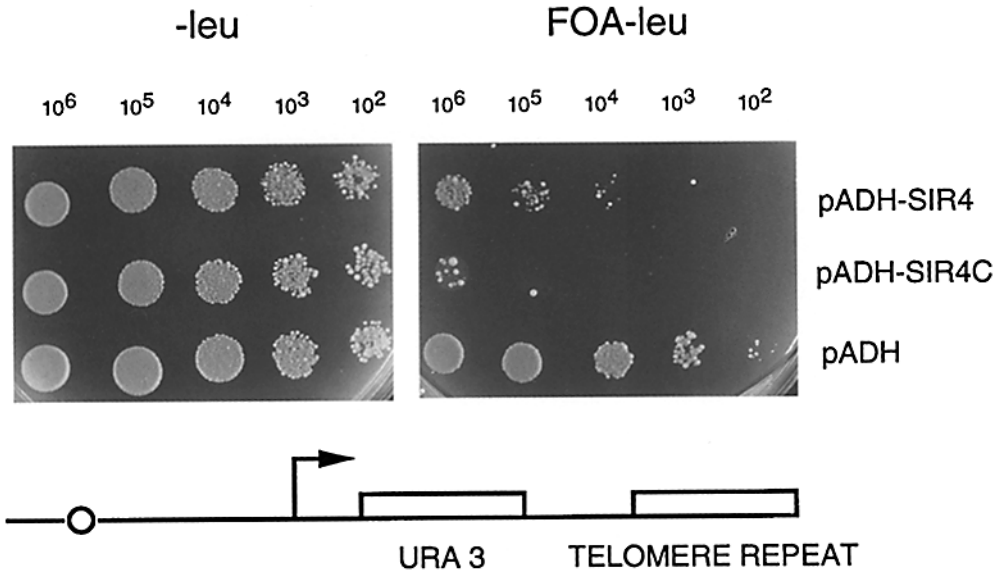

Figure 6. Overexpression of the COOH terminus of Sir4 or full-length Sir4 derepresses telomeric silencing. Cells from strain AJL275-2aVR, carrying plasmids pADH, pADH-SIR4, or pADH-SIR4C, were grown to mid-log phase in synthetic media lacking leucine, which selects for the plasmid. 10-fold serial dilutions in H_2O were plated onto selective medium with or without 5-FOA (see Materials and Methods). The approximate number of cells plated is shown above each dilution. The fraction of cells that are 5-FOA^r in the control strain carrying the pADH vector alone is ~10%. Overexpression of Sir4C results in a 1,000–10,000-fold decrease in FOA-resistant cells. Below the panel is a schematic representation of the telomere-proximal *URA3* gene used in this assay.

a complex in vivo, is to coimmunoprecipitate these proteins from a soluble nuclear extract. To monitor the solubility of these proteins, we treated isolated yeast nuclei with a light nuclease digestion followed by centrifugation (see Materials and Methods), to separate soluble chromatin from the residual nucleus. Under these conditions both Sir3 and Sir4 are relatively insoluble, sedimenting with the residual nuclei, while the majority of RAP1 is recovered in the supernatant (data not shown). The same partitioning is observed when nuclei are treated with nonionic detergent (Triton X-100) and sonication, followed by Western blot analysis, probing pellet, and supernatant for RAP1, Sir4, and Sir3 (P2 and S2 in Fig. 8). When an identical fractionation was performed on the strain overexpressing Sir4C, significant fractions of both Sir3 and Sir4 were recovered in the supernatant after treatment with nonionic detergent and sonication (pADH-SIR4C, lanes *S2* for Sir4 and Sir3, Fig. 8). Although roughly half of the full-length Sir3 and Sir4, and the overexpressed Sir4 COOH-terminal fragment, remain insoluble, these conditions nonetheless allow us to recover sufficient amounts of the proteins in a soluble form to perform coimmunoprecipitation experiments.

Sir4 and RAP1 Coimmunoprecipitate from Extracts of the Sir4C Overexpressing Strain

Having obtained both RAP1 and the Sir proteins in a soluble nuclear fraction, we tested whether any of the proteins coimmunoprecipitate under native conditions. In the experiment shown in Fig. 9, extracts were prepared from the Sir4C overexpressing strain as described above, and the various antibodies directed against either RAP1 or Sir4, or several preimmune rabbit sera, were used to precipitate proteins from the soluble extract. Treatment of the immune complex with DNaseI was included in the protocol to eliminate any coprecipitation of proteins mediated by DNA.

Western blot analysis of either anti-RAP1 or anti-Sir4 immunoprecipitates shows that anti-RAP1 antibodies can precipitate RAP1 (Fig. 9 *A*, lanes *4–6*), and anti-Sir4 can precipitate the overexpressed Sir4 COOH-terminal fragment, as expected (Fig. 9 *B*, lanes *3* and *4*). Interestingly, in the anti-Sir4 complex we also recover RAP1 (Fig. 9 *A*, lanes *2* and *3*), and, conversely, the anti-RAP1 coprecipitates the overexpressed Sir4C (Fig. 9 *B*, lanes *1* and *2*). Control antibodies failed to bring down any of the proteins (Fig. 9 *A*, lanes *7–9*, and 9 *B*, lanes *5* and *6*). Attempts to investigate association between native Sir3 and RAP1 failed. This does not rule out that the interaction exists, however, since we were also unable to detect Sir3 by Western blot after precipitation with anti-Sir3 antiserum (data not shown). These results suggest that RAP1 and Sir4 interact in nuclear extracts, and may be present as a complex in vivo. This could be a direct physical interaction or one mediated by another protein, such as Sir3.

Taken together, the results presented here suggest that Sir3, Sir4, and RAP1 form a complex in vivo, responsible in some way for establishment or maintenance of telomeric silencing. Sir3 and Sir4 also interact with the NH_2-terminal tail of histones H3 and H4 (Hecht et al., 1995). To aid in the interpretation of these results, we have determined the stiochiometry of Sir3, Sir4, and RAP1 in normal cells, by probing known amounts of the purified proteins alongside titrations of yeast nuclei on Western blots (see Materials and Methods). We estimate molecular ratios among the three proteins as roughly 1::1::1. This reflects the relative molar amounts in bulk nuclear protein. Since neither immunoprecipitation nor solubilization is 100% efficient, we do not

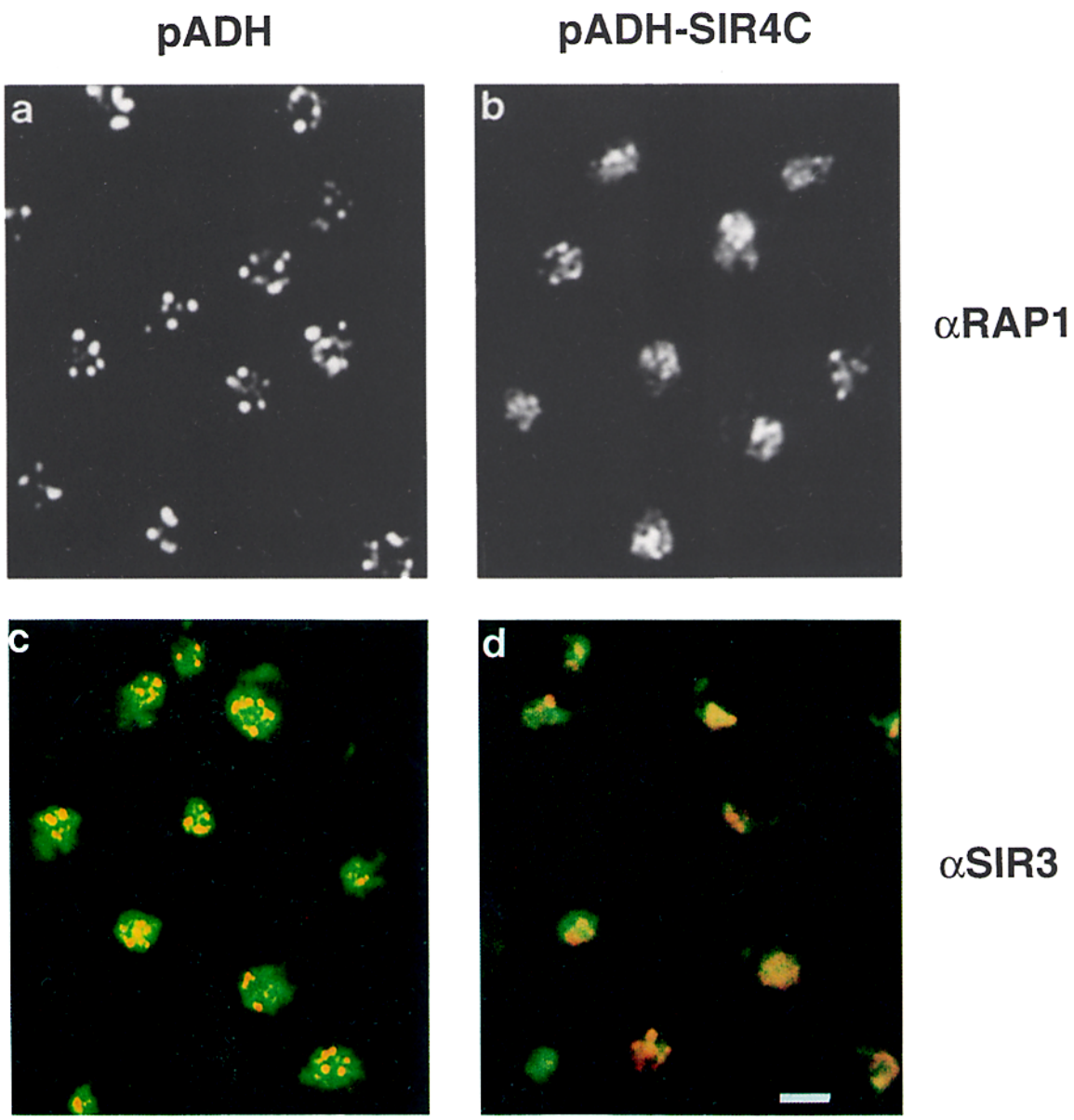

Figure 7. RAP1 and Sir3 are delocalized in strains overexpressing the COOH-terminal domain of Sir4. Cells from strain AJL275-2a VR, carrying plasmid pADH (panels *a* and *c*) or pADH-SIR4C (panels *b* and *d*), were grown to mid-log phase in synthetic media maintaining selection for the plasmid. The cells were converted to spheroplasts and subjected to the immunofluorescence protocol described in Materials and Methods, using affinity-purified antibodies. In panels *a* and *b*, the RAP1-dependent signals are shown for a number of yeast spheroplasts. In panels *c* and *d*, the anti-Sir3 signal (in *red*) was superimposed on the anti-DNA staining (shown in *green*), achieved with an anti-DNA monoclonal (Chemicorp. Inc., Temecula, CA). Yellow or orange signals show colocalization. All direct fluorescent images have been passed through the identical filtration program (C7B) on the Biorad 600 confocal imaging system. Bar, 2 μm.

know what fraction of each participates in the putative complex.

Discussion

Repression of the silent mating type loci in yeast and the related phenomenon of telomere-mediated gene silencing provide two of the best characterized examples of chromatin structure affecting gene expression. As discussed above, genetic and biochemical studies implicate the products of the *RAP1* and the *SIR2-4* genes in both instances of transcriptional repression (for review see Laurenson and Rine, 1992). Although RAP1 appears to exert its influence by binding directly to its consensus sites, there is no evidence that any of the *SIR* gene products bind DNA directly. The observation that the Sir proteins act as modifiers of chromatin structure both at the mating type loci and at telomeres (Nasmyth, 1982; Gottschling et al., 1990; Aparicio et al., 1991; Gottschling, 1992; Singh and Klar, 1992), suggests that these proteins target or nucleate the formation of a repressed, less accessible chromatin structure. Consistent with this notion, it was recently demonstrated that both Sir3 and Sir4 bind

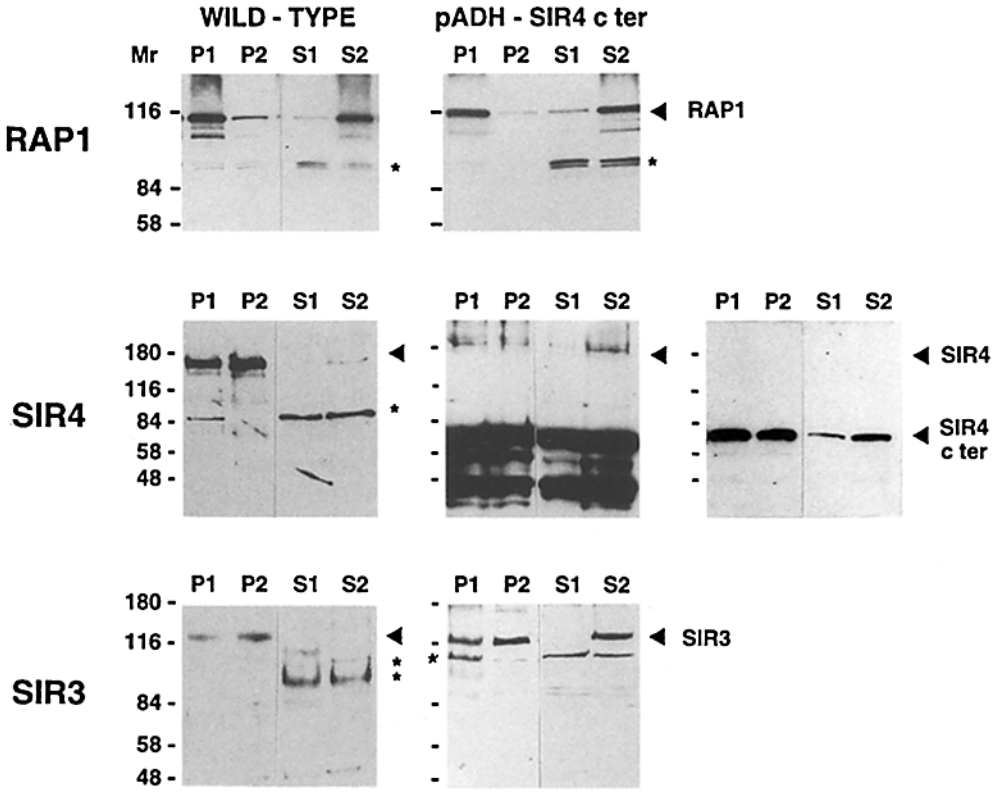

Figure 8. Overexpression of Sir4 COOH terminus changes the solubility of Sir3 and full-length Sir4 in isolated nuclei. Insoluble pellet (*P*) and supernatant (*S*) fractions from a protease deficient yeast strain GA59 and from the same strain carrying the plasmid pADH-SIR4C (labeled pADH-SIR4 c ter), were generated from isolated nuclei before (*P1* and *S1*) and after (*P2* and *S2*) brief sonication and treatment with the nonionic detergent Triton X-100, as described in Materials and Methods. Equivalent aliquots from each fraction were blotted by standard techniques using primary antibodies to RAP1, Sir3, and Sir4 as indicated to the left of the panels. The positions at which the full-length RAP1, Sir3, Sir4, and the plasmid-encoded Sir4 COOH-terminal fragment migrate are indicated by arrowheads. Bands marked with an asterisk represent cross-reacting bands. In the case of RAP1 this is a degradation product, while in the anti-Sir3 and anti-Sir4 blots these bands are not recognized by affinity-purified antibodies (data not shown), and are not specific to the *SIR* genes. A shorter exposure of the anti-Sir4 blot is shown to depict more clearly the high fraction (~50%) of the truncated Sir4C protein that remains in the pellet (*P2*). The Sir4C fragment is about 40-fold more abundant than the wild-type Sir4 protein. Western blots show no alteration in Sir3 or RAP1 levels in these cells (data not shown).

specifically to the unmodified NH_2 termini of histones H3 and H4 in vitro (Hecht et al., 1995).

In this study, we present several novel lines of evidence suggesting the presence of functional interactions among RAP1, Sir3, and Sir4 in vivo, which influence both subnuclear positioning of telomeres and telomeric silencing. First, the ability to detect Sir3 and Sir4 in perinuclear foci is dependent on a COOH-terminal domain of RAP1. Mutations in *RAP1* that truncate as little as the COOH-terminal 28 amino acids, abrogate telomeric silencing, and result in the loss of Sir3 localization to foci near the nuclear periphery. Detection of Sir4 foci is also lost in *rap1* alleles that truncate the terminal 165 amino acids of RAP1. Second, the localization of Sir3, and to a lesser extent that of RAP1, are disrupted by overproduction of the COOH terminus of Sir4, suggesting the titration of a component essential for subnuclear targeting or for the silencing complex. Third, overproduction of the Sir4 COOH terminus results in alterations in the solubility properties of Sir3 and the endogenous Sir4, suggesting that Sir4C can disrupt interactions either between these two proteins or within a multicomponent complex. Finally, intact RAP1 coimmunoprecipitates with the COOH terminus of Sir4, consistent with the presence of RAP1/Sir4 complexes in nuclei. Although we do not know the stoichiometry with which these components interact, their relative abundance in the nucleus is fully consistent with their formation in vivo.

Our immunological results confirm the importance of interactions detected in a two-hybrid system between the COOH terminus of RAP1 (amino acids 635-827), a Sir3 fusion protein (amino acids 307-978), and a COOH-terminal Sir4 fusion protein (amino acids 1204-1358) (Moretti et al., 1994). Coimmunolocalization has not been possible for RAP1 and Sir proteins, since all available antisera are from rabbits. Nonetheless, the comparison of immunofluorescence in mutant and wild-type cells argues strongly for the functional relevance of these interactions, and are consistent with recent genetic studies indicating that overproduction of Sir3 suppresses the telomeric silencing defects of COOH-terminal RAP1 mutations in an allele-specific fashion (Liu et al., 1994). In *Drosophila* the inactivation of genes through chromatin structure also appears to be achieved through a multiprotein complex that folds the chromatin of target genes into a condensed, inactive form (Paro, 1993). This model is based upon the genetic synergism of various members in a family of proteins, the *Polycomb* group, their dosage dependence, colocalization by immunofluorescence on polytene chromosomes, and the fact that mutation of one member causes the mislocalization of other *Polycomb* members (Rastelli et al., 1993).

Our studies provide the first insight into the RAP1 domain requirements for RAP1, Sir3, and Sir4 localization. Both the *rap1-17* and *rap1-21* alleles are defective in telomeric silencing, yet show distinctly different patterns of RAP1 localization. In *rap1-21* cells, the RAP1/telomeric complexes are largely peripheral in the nucleus, while in *rap1-17* and *rap1-18*

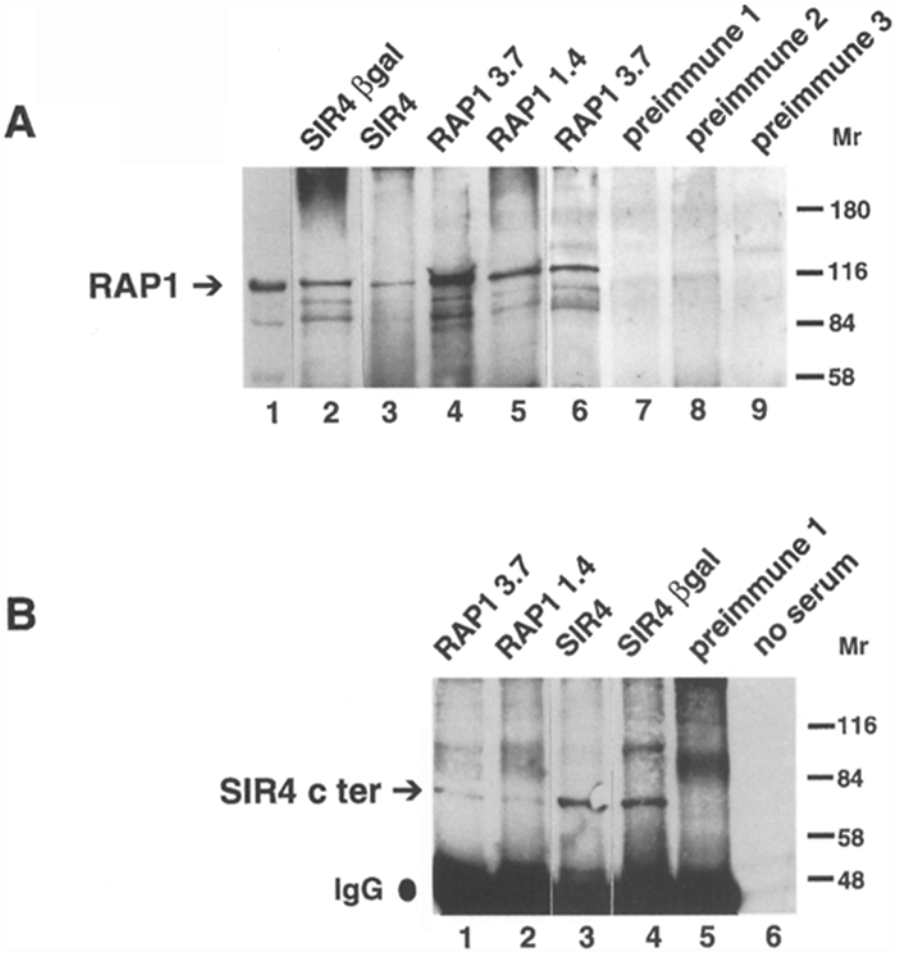

Figure 9. RAP1 and the COOH terminus of Sir4 can be coimmunoprecipitated from nuclear extracts. (*A*) A Western blot is shown using anti-RAP1 as primary antibody against total yeast nuclear proteins (lane *1*) or against proteins immunoprecipitated from equal aliquots of the soluble fraction S2 derived from nuclei isolated from the strain overexpressing the Sir4 COOH-terminal domain (GA59 carrying plasmid pDAH-SIR4C; see Fig. 8). The immunoprecipitation was achieved with antisera raised against a full-length Sir4-β galactosidase fusion protein (lane *2*, SIR4 βgal), Sir4 COOH terminus (lane *3*, SIR4), full-length RAP1 (RAP1 3.7, lanes *4* and *6*), the NH_2-terminal third of RAP1 (RAP1 1.4, lane *5*) or with three preimmune sera (lanes *7–9*). (*B*) Western blots using anti-Sir4 as the primary antibody against proteins immunoprecipitated from the same S2 fraction by antisera raised against a full-length or NH_2-terminal fragment of RAP1 (RAP1 3.7 and RAP1 1.4, lanes *1* and *2*, respectively), Sir4 COOH terminus (lane *3*), a Sir4-β-galactosidase fusion protein (SIR4 βgal, lane *4*) or antisera from a preimmune serum (lane *5*). Proteins precipitated by protein A beads in the absence of added antiserum were also blotted (lane *6*), showing little or no background due to the beads alone. The migrations of molecular weight standards are indicated at the left of each gel, and that of full-length RAP1, and the Sir4 COOH-terminal fragment are indicated with arrows. The secondary antibody also recognizes the rabbit IgG used in the immunoprecipitation (labeled IgG, part *B*; excised in part *A*).

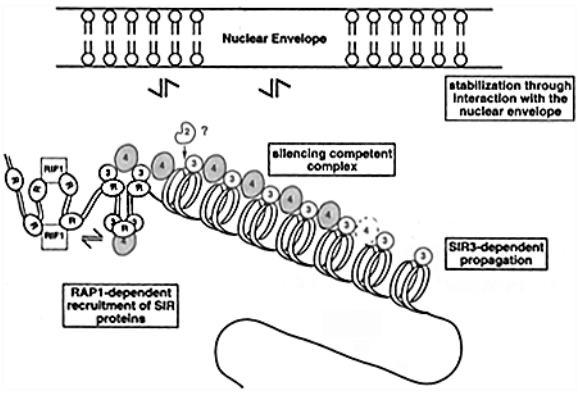

Figure 10. A model of protein–protein interactions involved in the nucleation and propagation of chromatin-mediated gene repression near telomeres in yeast. This model depicts several events involved in telomere-mediated silencing, as suggested by results in this paper and those of other laboratories. One element common to both the mating type and telomeric gene repression is the Repressor-Activator Protein RAP1, (indicated by an oval marked *R*) that binds multiple sites in the telomeric repeat. RAP1 may nucleate the binding of the Sir3-Sir4 complex (circles labeled *3* and *4*) through direct interaction between the COOH-terminal 165 amino acids of RAP1 with Sir3 and Sir4 (Moretti et al., 1994; Liu et al., 1994; Figs. 3 and 5). This first event is termed "recruitment of SIR proteins."

cells RAP1 is found either throughout the nucleus or in aberrant structures near the periphery. This does not appear to be simply the consequence of the grossly increased telomere size, since wild-type cells inheriting elongated telomeres display normal patterns of localization. This phenotype is

RIF1 may compete with Sir proteins for these sites of interaction (Kyrion et al., 1993; Moretti et al., 1994). Sir3 and Sir4 can also interact directly with NH_2-terminal tails of histones H3 and H4 (Hecht et al., 1995), forming a "silencing competent complex," perhaps with other yet unidentified partners. Since the overexpression of *SIR3* can extend telomeric silencing (Renauld et al., 1993), it is limiting in this context (see "SIR3-dependent propagation"). Its association with nucleosomes may not require, but be stabilized by Sir4. The site of Sir2 (circle labeled *2*) action is unknown, although it may facilitate the deacetylation of histone NH_2 termini, which in turn may enhance the interaction of Sir3, Sir4, and histones. In view of the localization of telomeric DNA (Scherthan, H., T. Laroche, and S. M. Gasser, unpublished), RAP1, Sir3, and Sir4 to foci near the nuclear periphery, we propose that the entire telomere-RAP1-Sir protein complex interacts with the nuclear envelope by means of unknown intermediaries (*large arrows*). Peripheral localization depends on the presence of Sir3 and Sir4, and may either help stabilize a condensed, inaccessible chromatin structure, or be a prerequisite for its formation.

distinct from that observed in *sir3* and *sir4* null mutants, which display a more random localization of RAP1/telomeric complexes (Palladino et al., 1993). These results suggest that between the *rap1-18* and *rap1-21* breakpoints (amino acids 683 and 799, respectively) are sequences critical for the proper subnuclear organization of RAP1-telomere complexes. For Sir3, we observe a diffuse staining pattern lacking foci at the nuclear periphery, in both *rap1'* and *rap1-21* mutants. Similarly, the *rap1'* truncations lead to delocalization of Sir4. Two general conclusions can be drawn from this data: (*a*) peripheral localization of RAP1-telomeric complexes is not sufficient to confer a fully repressed state and (*b*) mutations in RAP1 can alter Sir3 and Sir4 localization, without altering its own immunofluorescence pattern, which presumably reflects foci of telomeric DNA.

It is clear from the *rap1-17* results and from the positioning of telomeres in wild-type cells, that the clustering of telomeres near the nuclear periphery is not sufficient to confer repression on telomere-proximal genes. In wild-type cells the vast majority of cells shows a clear perinuclear localization of RAP1, yet the frequency of repression of *URA3* near the VR telomere is only ~10%. It is possible, however, that the juxtaposition of telomeres at the envelope is a prerequisite to establishment of a stable repressed chromatin complex. This may be influenced both by the mitotic cell cycle (Aparicio and Gottschling, 1994), as well as by the presence of Sir3 and Sir4 (Palladino et al., 1993). The detection of Sir3 in foci near the nuclear envelope correlates best with the formation of the repressed chromatin state. We assume that these foci reflect the stable association of Sir proteins both with RAP1 at the telomeric tract and with the NH_2 termini of histones H3 and H4 in subtelomeric regions, after nucleation of repressed chromatin (Johnson et al., 1990; Hecht et al., 1995). The observation that RAP1/telomeric complexes are still partially peripheral in *rap1-17* and *rap1-21* mutants, although Sir3 is delocalized, suggests that telomeres are able to cluster, and even localize to the nuclear periphery (perhaps in a transient manner) in these cells. However, because the COOH-terminal domain of RAP1 is compromised, the telomeric complex is unable to attract a sufficient concentration of Sir3 and/or Sir4 to seed the formatin of the stably repressed chromatin complex. Consistently, in cells carrying deletions of the NH_2 termini of histones H3 and H4, mutations which also abrogate telomeric silencing, we also observe the loss of Sir3 foci while telomeres remain clustered (Hecht et al., 1995).

Thus, in contrast to the complete absence of Sir3, its delocalization correlates with only a partial delocalization of RAP1 complexes, even though telomeric silencing is fully derepressed. This partial delocalization is consistent with the characteristics of subtelomeric chromatin structure in strains carrying the *rap1-17* allele: although telomeric silencing is lost in this mutant, the level of accessibility of chromatin to ectopically expressed *dam* methylase is intermediate between the complete protection observed in wild type and the lack of protection observed in *sir3* or *sir2* mutants (Gottschling, 1992; Kyrion et al., 1993). This partially closed chromatin state may reflect the ability of RAP1 to recruit factors sufficient to anchor the RAP1-telomere complex, but insufficient to establish fully repressed chromatin, which would require the stable association of Sir3 and Sir4 to adjacent subtelomeric regions.

These various stages in the establishment and/or maintenance of a telomere-proximal repressed chromatin state are depicted in Fig. 10. Based on the requirement of the COOH terminus of RAP1 for Sir3 and Sir4 localization, we propose that one of the functions of RAP1 is to recruit Sir3- and Sir3-associated factors to the telomere. This complex may nucleate the creation of a silent, less accessible chromatin which requires Sir3, Sir4, and the NH_2 termini of histones H3 and H4. The targeting of the telomeric complex to the nuclear periphery could be important for either the propagation of repressed chromatin, or for its stabilization. Part of the establishment process might be the recruitment of a lysine deacetylase to the telomere, which could modify the NH_2 termini of histones H3 and H4, rendering them appropriate substrates for interaction with Sir3 and Sir4 (Hecht et al., 1995). Evidence for this model comes both from the finding that the binding of Sir3 to an HN_2-terminal fragment of histone H4 is obliterated by mutation of histone H4 lysine 16 to a glutamine residue, mimicking the acetylated state, as well as evidence implicating Sir2 in histone deacetylation (Thompson et al., 1994; Braunstein et al., 1993; Hecht et al., 1995). Finally, the spreading of this closed chromatin state may require interactions between Sir3 and histones, since Sir3 appears to be limiting in this process (Renauld et al., 1993).

There is no strong correlation between mutations that influence telomere silencing and those that determine telomere length. For instance, point mutations mapping within the RAP1 COOH terminus cause comparable increases in telomere tract size, independent of the severity of silencing defects. Furthermore, telomeric silencing is disrupted upon truncation of either the terminal 28 or 165 amino acids of RAP1, yet the two truncations have vastly different effects on telomere length (Kyrion et al., 1992; Liu et al., 1994; Sussel and Shore, 1991). Similarly, deletion of either the *SIR3* or *SIR4* gene fully derepresses silencing, but shortens the telomeric repeat only slightly (Palladino et al., 1993), and overproduction of Sir3 or Sir4C has no effect on telomere tract size at all (data not shown), although telomeric silencing is either enhanced or eliminated, respectively. Nonetheless, in a wild-type *RAP1* background, longer telomeres correlate with enhanced telomeric repression (Kyrion et al., 1992), possibly by providing more sites for the association of RAP1 and RAP1-interacting factors.

Are telomeric complexes directly associated with the nuclear envelope? Focal plane sectioning of immunostained wild-type cells, clearly shows RAP1 foci in a perinuclear domain (Palladino et al., 1993). Both the clustering and the localization are dependent on Sir3 and Sir4. Nonetheless, our data cannot distinguish between some sort of spatial exclusion from the core of the nucleus and a direct association with elements at the nuclear periphery. Indeed, since the volume of the periphery of a spherical nucleus is larger than its core, there is a larger chance to find any given element near the periphery. The fact that mutants alter this pattern suggests that the mutated gene products are directly involved in subnuclear localization, although other interpretations are possible. The loss of telomeric clustering alone might increase the chance to find a given telomere at the nuclear core. Higher resolution techniques will be required to establish whether yeast telomeres actually contact the nuclear envelope.

In high resolution, three-dimensional reconstruction of *Drosophila* nuclei, constitutively inactive, heterochromatic regions of *Drosophila* polytene chromosomes were found near the nuclear envelope (Mathog et al., 1984; Hochstrasser et al., 1986). This positioning of heterochromatin is also consistent with electron microscopic studies of differentiated mammalian cells, in which heterochromatic regions of the genome were shown to be juxtaposed to the nuclear lamina (e.g., Bouteille et al., 1974). Similarly, localization of telomeres to the nuclear periphery also has parallels in other organisms (for review see Gilson et al., 1993*a*). Over a hundred years ago, Rabl observed telomeres abutting the nuclear envelope in *Salamander* salivary gland cells (Rabl, 1885). More recently in fission yeast, the clustering of telomeres at the nuclear periphery was observed in G2, although it was disrupted in mitosis (Funabiki et al., 1993). In view of the homology between the COOH terminus of the Sir4 protein and the coiled-coil domains of lamins A and C (Diffley and Stillman, 1989), it is tempting to speculate that Sir4 mediates the attachment of telomeres to the nuclear envelope in a lamin-like manner, perhaps also showing metaphase-specific dissociation. Indeed, the coiled-coil domain of Sir4 was demonstrated by the two-hybrid system to dimerize (Chien et al., 1991). Exploiting the genetic and molecular tools of yeast will facilitate the testing of such hypotheses, to reveal the interrelationships between telomeric silencing, condensed chromatin, and subnuclear organization.

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