The EMBO Journal Vol.16 No.11 pp.3243–3255, 1997

Localization of Sir2p: the nucleolus as a compartment for silent information regulators

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In wild-type budding yeast strains, the proteins encoded by *SIR3*, *SIR4* and *RAP1* co-localize with telomeric DNA in a limited number of foci in interphase nuclei. Immunostaining of Sir2p shows that in addition to a punctate staining that coincides with Rap1 foci, Sir2p localizes to a subdomain of the nucleolus. The presence of Sir2p at both the spacer of the rDNA repeat and at telomeres is confirmed by formaldehyde cross-linking and immunoprecipitation with anti-Sir2p antibodies. In strains lacking Sir4p, Sir3p becomes concentrated in the nucleolus, by a pathway requiring *SIR2* and *UTH4*, a gene that regulates life span in yeast. The unexpected nucleolar localization of Sir2p and Sir3p correlates with observed effects of *sir* mutations on rDNA stability and yeast longevity, defining a new site of action for silent information regulatory factors.

Keywords: nuclear organization/nucleolus/Sir2p/Sir3p/telomere

Introduction

Regulation of gene expression by alterations in chromatin structure is a universal mechanism in eukaryotic cells, responsible for maintaining patterns of gene expression throughout the development of multicellular organisms (Orlando and Paro, 1995), for position effect variegation in flies (Henikoff, 1992) and for the variable expression of foreign genes integrated into mammalian chromosomes (Martin and Whitelaw, 1996). In the budding yeast *Saccharomyces cerevisiae*, gene repression at the silent mating type loci (*HML* and *HMR*) and the variegated expression of genes inserted near the poly($TG_{1–3}$) tracts at telomeres reflect a chromatin-dependent silencing mechanism in which the accessibility of a chromosomal domain to DNA-modifying enzymes is significantly reduced (reviewed in Thompson *et al.*, 1993). This transcriptionally silent domain spreads inward from telomeres and is limited by the dosage of components required to form the 'silenced' chromatin state, similar to the spread of centromeric heterochromatin in flies (Renauld *et al.*, 1993; Hecht *et al.*, 1996; reviewed in Sandell and Zakian, 1992).

A number of proteins are required for both telomeric and mating type loci repression. These include repressor activator protein 1 (Rap1), the silent information regulators (Sir) 2–4 and the N-termini of histones H3 and H4 (Ivy *et al.*, 1986; Rine and Herskowitz, 1987; Kayne *et al.*, 1988; Aparicio *et al.*, 1991; Sussel and Shore, 1991; Kyrion *et al.*, 1993; Thompson *et al.*, 1994). Sir3p and Sir4p are both able to form homo- and heteromultimeric complexes (Marshall *et al.*, 1987; Moretti *et al.*, 1994), and both interact with the N-termini of histones H3 and H4 (Hecht *et al.*, 1995). In wild-type strains, repression is generally lost beyond 3 kb from the telomeric repeat, although in strains overexpressing *SIR3*, silencing can extend up to 20 kb from the chromosomal end (Renauld *et al.*, 1993). Immunoprecipitation and cross-linking data confirm that Sir2p, Sir3p, Sir4p, histones and the telomere repeat-binding protein, Rap1, can be co-immunoprecipitated with subtelomeric DNA in wild-type cell extracts, and that, when overexpressed, Sir3p propagates inward from the telomere (Hecht *et al.*, 1996; Strahl-Bolsinger *et al.*, 1997).

Under normal levels of expression, Sir3p, Sir4p and Rap1 form a multicomponent complex in which the stoichiometry among the proteins appears carefully balanced. Overexpression of either full-length Sir4p, or a C-terminal fragment of Sir4p, abrogates both mating type and telomeric repression (Marshall *et al.*, 1987; Cockell *et al.*, 1995). Moreover, both point mutations and the anti-*SIR* activity of the Sir4p C-terminus, as well as nonsense and missense mutations in the C-terminus of Rap1, can be suppressed by increased *SIR3* dosage (Marshall *et al.*, 1987; Liu *et al.*, 1994; M.Gotta and S.M.Gasser, unpublished results). Similarly, increased *SIR1* and *SIR4* dosage restores repression at *HMR* in a silencing-specific *rap1* mutant, called *rap1^s* (Sussel and Shore, 1991).

Combined *in situ* hybridization and immunostaining experiments in wild-type yeast cells have demonstrated that telomeres are clustered in interphase nuclei at a limited number of foci which contain Rap1, Sir3p and Sir4p immune signals (Gotta *et al.*, 1996). This focal staining pattern is lost in a range of mutants that impair telomeric silencing, suggesting that intact silencing complexes are necessary for the characteristic focal distribution of Rap1, Sir3p and Sir4p (Palladino *et al.*, 1993a; Cockell *et al.*, 1995; Hecht *et al.*, 1995; Gotta *et al.*, 1996). This, along with the observation that both *HML* and *HMR* silencers repress more efficiently when placed near telomeric repeats (Thompson *et al.*, 1994; Maillet *et al.*, 1996), suggests that the clustering of telomeres creates a

subnuclear domain that favors the establishment of repressed chromatin (discussed in Marcand *et al.*, 1996b). It is not known, however, what maintains the unequal subnuclear distribution of Sir proteins, nor is it known exactly where *HM* loci are in relation to the clustered telomeric domains.

The role of Sir2p in telomeric and mating type silencing remains poorly characterized. Sir2p was shown to interact with Sir4p *in vitro* and to co-immunoprecipitate with Sir4p from whole cell extracts (Moazed and Johnson, 1996; Strahl-Bolsinger *et al.*, 1997). Consistent with a role in the maintenance of chromatin-mediated repression (Ivy *et al.*, 1986; Rine and Herskowitz, 1987; Aparicio *et al.*, 1991), mutations in *SIR2* result in the hyperacetylation of lysines in the N-termini of histones H3 and H4 at telomeres and at *HM* loci (Braunstein *et al.*, 1993). However, mutation in *SIR2* also produces an elevated level of rDNA recombination (Gottlieb and Esposito, 1989) and loss of repression of a *URA3*-containing Ty element inserted into the rDNA of yeast (Smith and Boeke, 1997; Bryk *et al.*, 1997). These authors observe a variegated expression pattern for the transplaced *URA3* that is dependent on *SIR2*, but not on *SIR3* nor *SIR4* (Smith and Boeke, 1997). Unlike Sir3p and Sir4p, for which no homologs are known, Sir2p is part of a multi-gene family in yeast (*HST1*,*2*,*3*,*4*) that has highly conserved structural homologs in organisms ranging from bacteria to man (Brachmann *et al.*, 1995).

In *S.cerevisiae*, the rDNA locus is a stretch of at least 200 kbp containing direct repeats of a 9 kbp unit that encodes the 5S RNA and a 35S precursor rRNA, essential for ribosomal biogenesis. Both RNA polymerase I (pol I)-mediated transcription and ribosome assembly occur in the nucleolus, which is a membrane-less organelle occupying a distinctive half-moon crescent within the yeast nucleus (reviewed in Melese and Xue, 1995). Ribosome biogenesis involves the post-transcriptional processing of initial precursor transcripts, interactions with imported snRNAs and their accompanying proteins, the assembly of the rRNA with ribosomal proteins and, finally, transport of the pre-ribosomal subunits to the cytoplasm. The nucleolus itself contains morphologically distinct subdomains which reflect the different stages of this process and which organize the diverse enzymes required for these functions (reviewed in Scheer and Weisenberger, 1994).

To determine whether Sir2p, like Sir3p and Sir4p, is concentrated exclusively at telomeric foci, we have undertaken a study to localize Sir2p in both mutant and wild-type cells, using the complementary methods of immunolocalization and *in vivo* protein–DNA cross-linking, followed by immunoprecipitation and PCR analysis of the associated DNA (see Hecht *et al.*, 1996). Surprisingly, the majority of the immunodetectable Sir2p localizes to the nucleolus and Sir2p is recovered cross-linked to the rDNA locus on chromosome XII. Even less expected is the finding that Sir3p also becomes localized to the nucleolus when Sir4p is absent or mutated. This re-localization requires *SIR2* and *UTH4*, a gene that influences life span in yeast, and is consistent with the previously defined role of Sir proteins in extending yeast life span (Kennedy *et al.*, 1995). Our results provide critical evidence suggesting that the yeast nucleolus is indeed a physiologically significant site of action for Sir proteins.

Results

Immunolocalization of Sir2p

Like mutations in *SIR3* and *SIR4*, the mutation of *SIR2* eliminates transcriptional repression at both *HM* and subtelomeric loci (Rine and Herskowitz, 1987; Aparicio *et al.*, 1991). Yet recombination studies suggest that Sir2p may have a function independent of the other Sir proteins in the yeast nucleolus (Gottlieb and Esposito, 1989). This *SIR2* function could either reflect a direct role for the protein in rDNA organization or indirect effects evoked by the modulation of histone acetylation (Braunstein *et al.*, 1993) or by a drop in the mitotic stability of chromosomes, as observed for mutants in the Sir2p homologs, *HST1–4* (Brachmann *et al.*, 1995).

To address this issue, immunolocalization of Sir2p was performed on fixed yeast cells using affinity-purified antibodies against Sir2p (see Materials and methods). A strong Sir2p signal is detected within a restricted nuclear subdomain, along with a weaker punctate pattern near the nuclear periphery (Figure 1a). The shape of the large nuclear subdomain defined by anti-Sir2p staining resembles that of the nucleolus (see, for example, Uzawa and Yanagida, 1992), prompting us to test whether Sir2p co-localizes with a nucleolar marker like Nop1, a protein involved in both rRNA transcription and processing (Tollervey *et al.*, 1993). Counterstaining with the mouse monoclonal antibody (mAbA66) specific for Nop1 reveals a typical nucleolar crescent, that occupies roughly a third of the nucleus (Figure 1b). Specific secondary antibodies allow us to distinguish the Sir2p and Nop1 signals, and the merging of signals on a single focal section indicates an unambiguous co-localization (Figure 1c). Interestingly, the staining patterns are not entirely co-extensive within the nucleolus, suggesting that Sir2p and Nop1 may map to different nucleolar subcompartments (see below). The lack of anti-Sir2p signal above background on a congenic strain carrying the *sir2*::*HIS3* disruption indicates that the Sir2p signal is specific (Figure 5), while the Nop1 staining in this background remains unaltered (data not shown). These results allow us to conclude that Sir2p localizes by immunofluorescence to the nucleolar domain.

Sir2p co-localizes with foci of Rap1

In addition to the nucleolar signal, anti-Sir2p also reveals foci at or near the nuclear periphery (see arrows, Figure 1a and c), resembling those previously characterized for Sir3p, Sir4p and Rap1 (Palladino *et al.*, 1993a; Gotta *et al.*, 1996). The punctate Sir2p staining, but not its nucleolar signal, is lost in strains deficient for *SIR4* (Figure 1d). This is consistent with the loss of Sir3p foci in a *sir4* mutant, and of Sir4p foci in *sir3* mutants (reviewed in Gotta and Gasser, 1996). The punctate Sir2p staining is surprisingly weak, however, when compared with signals from Rap1, Sir3p or Sir4p antibodies. Since this could reflect the relative inaccessibility of Sir2p at telomeres, rather than its absence, we performed immunofluorescence in a buffer containing detergent micelles (see Materials and methods; Klein *et al.*, 1992; Palladino *et al.*, 1993b). This improves Sir2p detection and reveals on average 3–4 spots per nucleus and per focal section (Figure 2b).

To demonstrate that these Sir2p foci represent clusters of telomeres, we stained for Sir2p in a strain carrying an

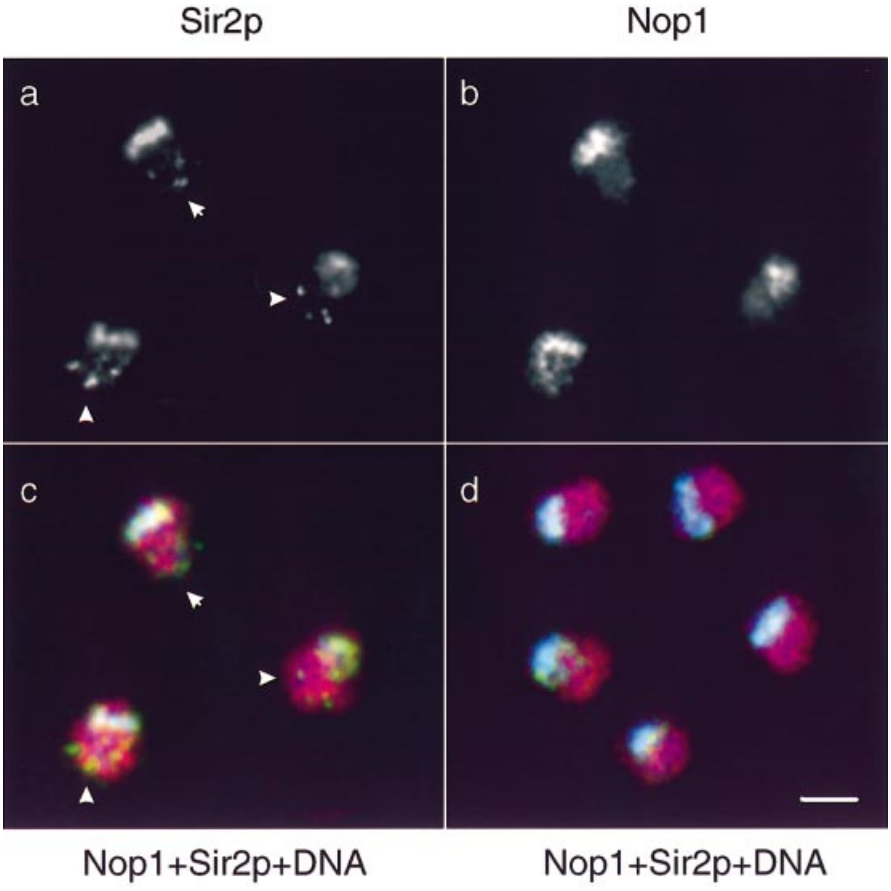


Fig. 1. Sir2p co-localizes with the nucleolar marker Nop1 in wild-type cells. A wild-type haploid strain (UCC3107, a, b and c) and an isogenic *sir4*::*HIS3* mutant strain (UCC3207, d) were double stained with anti-Sir2p, detected by a DTAF-conjugated secondary antibody, and anti-Nop1 antibodies detected by a Cy5-conjugated secondary antibody. (**a**) Anti-Sir2p staining in wild-type cells, (**b**) anti-Nop1 staining on the same cells, (**c**) the merge between Sir2p and Nop1 and the DNA staining and (**d**) the merge of Nop1, Sir2p and the DNA staining in a *sir4*::*HIS3* strain. Overlap of the three signals is white. The bar indicates 1.5 μm.

integrated fusion between Rap1 and the bacterial Green Fluorescence Protein (GFP), expressed from the the Rap1 promoter (Rap1–GFP, gift of Y.Hiraoka, Kobe, Japan). In this strain, Rap1 is detected by the combined fluorescence of the Rap1–GFP fusion and a dichlorotriazinyl aminofluorescein (DTAF)-conjugated secondary antibody recognizing a monoclonal antibody specific for GFP (see Materials and methods). Rap1 (Figure 2a) and Sir2p (Figure 2b) signals coincide at many of the perinuclear foci (see arrows on merged image, Figure 2c), confirming the presence of Sir2p at telomeres.

We next exploited conditions for immunostaining in which nucleolar RNA, but not the rDNA, is released, revealing an ethidium bromide-stained loop of genomic DNA (in red, Figure 2e). Previous *in situ* hybridization studies have shown that this loop of DNA most likely represents the tandem rDNA repeats of chromosome XII, extending from the mass of nuclear chromatin (Guacci *et al*., 1994). The Sir2p staining of the nucleolus is retained on this loop of DNA (green signal, Figure 2d). To confirm further that Sir2p associates with rDNA, and not rRNA, we analyzed by Western blot isolated nuclei extensively treated with RNase A. More than 60% of the Nop1, which binds RNA, is released efficiently after RNase treatment, while >90% of the Sir2p remains associated with the nuclear fraction (see Materials and methods, Figure 3d, lane 1, and data not shown). These results suggest that Sir2p binds both telomeric and rDNA sequences within the nucleus.

Association of Sir2p with yeast rDNA in wild-type yeast cells

An independent method for identifying sites at which chromatin factors are bound is based on *in situ* cross-linking with formaldehyde, followed by immunoprecipitation, reversal of the cross-link and PCR amplification of the associated DNA (Solomon *et al.*, 1988; Orlando and Paro, 1993; Hecht *et al.*, 1996). This technique was performed using affinity-purified anti-Sir2p antibodies to precipitate chromatin from the yeast strain AYH2.45, after sonication to an average fragment size of 0.5–1 kb (described in Hecht *et al.*, 1996). The precipitates were washed extensively and the associated DNA was analyzed by PCR, using specific primer pairs directed against regions of the rDNA loci on chromosome XII (schematically shown in Figure 3a) and the *CUP1* loci on chromosome VIII. The *CUP1* locus can be amplified from two to 14 times in the yeast genome (Fogel and Welch, 1982), and provides a control for non-specific background.

The anti-Sir2p antibodies immunoprecipitate DNA sequences from the rDNA loci preferentially when compared with the *CUP1* loci. Quantitation of the *CUP1* signal precipitated with the anti-Sir2p antibody shows that the recovered DNA produces 20 times less *CUP1* signal

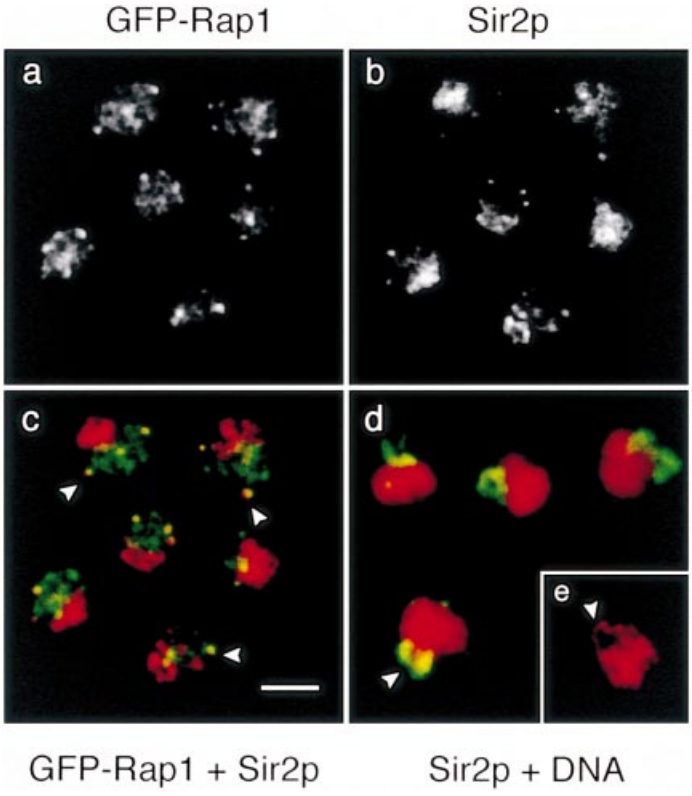


Fig. 2. Sir2p co-localizes with Rap1 at telomeric foci and with the rDNA chromatin. The diploid strain AHY111 which carries an integrated GFP–Rap1 fusion expressed from the Rap1 promoter was stained with anti-Sir2p antibody after formaldehyde fixation and incubation in 0.5% Triton X-100, 0.01% SDS (see Materials and methods). Sir2p is detected by a Texas Red-conjugated secondary antibody (red) while Rap1 is detected by both the endogenous GFP fluorescence and detection of GFP with a mouse monoclonal antibody detected by a DTAF-conjugated secondary antibody (green fluorescence). (**a**) The GFP–Rap1 signals, (**b**) the anti-Sir2p staining and (**c**) the merge of the two signals (Rap1 in green, Sir2p in red). Coincidence of the two signals is yellow. As expected, the Rap1–GFP gives both punctate, and diffuse signals. The diffuse nuclear signal is higher relative to the foci than that detected by Rap1 antibody alone [compare (a) with Figure 5]. We attribute this to a higher sensitivity of detection for the Rap1–GFP fusion, and to the fact that bivalent antibody interactions augment signals when antigen is tightly clustered. (**d**) A *SIR*$^+$ diploid strain fixed for 10 min prior incubation in Triton–SDS (see Materials and methods), reacted with anti-Sir2p and detected by a DTAF-conjugated secondary antibody (green). DNA is counterstained with ethidium bromide (red). (**e**) A SIR$^+$ diploid strain prepared for immunofluorescence at 37°C, which causes loss of the RNA from the nucleolus, revealing the rDNA loop of chromosome XII (in red, see arrow). Bar in (c) = 2 μm

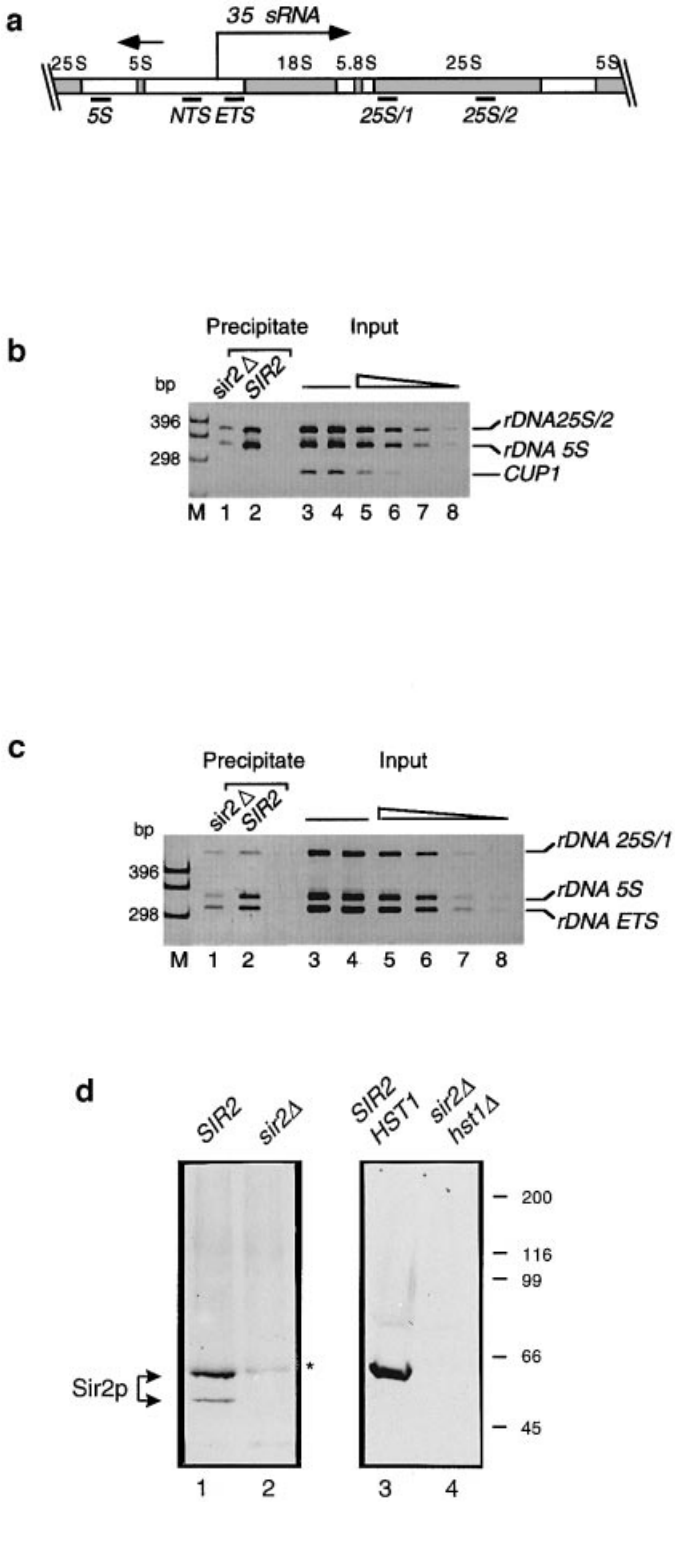
than the input material, representing the background for regions that do not bind Sir2p (Figure 3b, compare lane 2 with lanes 4–8; see also Table Ia). Using different sets of primers to identify regions of the rDNA loci that may be associated preferentially with Sir2p, we show that a region 3′ of the 5S RNA gene (5S) and the promoter of the large 35S transcript (ETS, external transcribed spacer region) are ~2- to 3-fold enriched in comparison with the 25S region (Figure 3c, compare lane 2 with lanes 4–8; see also Table Ib). Although the level of rDNA recovered by immunoprecipitation with the anti-Sir2p antibodies drops significantly in a isogenic *sir2*::*TRP1* strain (2- to 4-fold, Figure 3 and Table Ia), these antibodies still precipitate a significant amount of rDNA, as compared with *CUP1* or other non-repetitive or non-telomeric probes in the absence of Sir2p (Figure 3b and c, lane 1 and data not shown).

This background signal is apparently due to a weak immunological cross-reaction with the Hst1p, which shares 63% amino acid identity overall with Sir2p, and nearly 80% amino acid identity in the conserved C-terminal two-thirds (Brachmann *et al.*, 1995; Derbyshire *et al.*, 1995). A Western blot using affinity-purified anti-Sir2p shows strong reactions with a polypeptide at ~60 kDa, and occasionally with a breakdown product at 55 kDa (Figure 3d, lanes 1 and 3), which are both absent in the *SIR2* disruption strain (Figure 3d, lane 2). We also detect a weak reaction with a band at 61 kDa, which is the predicted size for Hst1p and which remains after *SIR2* deletion (see asterisk in Figure 3d, lane 2). In a strain deleted for both *SIR2* and *HST1*, this cross-reacting band is no longer detected (Figure 3d, compare lanes 3 and 4), suggesting that it is likely to be the *HST1* gene product. While the cross-reaction is detectable on blots, no significant signal is detected in immunofluorescence assays in the *sir2*::*HIS3* strain (Figure 5, labeled Sir2p). Since none of the smaller, less conserved Sir2p homologs (Hst2p–4p) are recognized by the Sir2p antiserum (Figure 3d, and data not shown), the cross-linking and immunofluorescence results argue strongly that Sir2p is associated with the rDNA *in vivo*.

Association of Sir2p with yeast rDNA in sir-deficient strains

In addition to the immunolocalization data presented above, we localized Sir2p to telomeric DNA by formaldehyde cross-linking studies (Figure 4; and Strahl-Bolsinger *et al.*, 1997). To compare the influence of mutations in the *SIR* genes on the association of Sir2p with telomeres and rDNA, Sir2p was immunoprecipitated after DNA

cross-linking in the wild-type strain AYH2.45, the *sir2*::*TRP1* strain STY30, the *sir3*::*LEU2* strain AYH2.8 and in the *sir4*::*TRP1* strain STY36, as described above. The precipitate was analyzed by PCR using the primer pairs for the rDNA shown in Figure 3a. The presence of Sir2p at rDNA is not affected by the deletion of either *SIR3* or *SIR4* (Figure 4, lanes 3 and 4). In a *sir2*::*TRP1* strain, the amount of rDNA recovered is reduced, but not completely lost (Figure 4, lane 1), which may again reflect cross-reaction of the anti-Sir2p serum with Hst1p. A primer set from the non-transcribed spacer (NTS) region of the rDNA confirms that this region, which includes an origin of replication and the RNA pol I promoter, is precipitated preferentially with anti-Sir2p antibodies, and that its recovery is slightly enhanced in strains lacking Sir3p or Sir4p (Figure 4, compare NTS in lane 2 with lanes 3 and 4). In contrast, the association of Sir2p with telomeres is lost whenever Sir3p, Sir4p or Sir2p are mutated (Figure 4, compare Y′ in lane 2 with lanes 3 and 4). Therefore, while Sir3p and Sir4p are required for Sir2p binding to telomeric DNA, Sir2p can bind rDNA in the absence of other Sir proteins.



Association of Sir3p and Sir4p with yeast rDNA

To examine whether other Sir proteins interact with rDNA, we tested whether the rDNA loci are enriched in anti-Sir3p and anti-Sir4p precipitates. Although telomeric regions are efficiently recovered cross-linked to these two proteins (Hecht *et al.*, 1996; Strahl-Bolsinger *et al.*, 1997), the rDNA sequences are recovered only weakly and, in comparison with anti-Sir2p precipitates, the 5S and ETS regions are not enriched (Table Ic). Immunofluorescent studies with anti-Sir3p and anti-Sir4p reveal a low level background staining of the nucleolus in wild-type cells, at a level similar to the signal in non-telomeric chromatin. In conclusion, these weak cross-linking and immunofluorescence signals may reflect either a non-specific background or a low-level binding of Sir3p and Sir4p to these regions in wild-type cells.

Rap1, Sir3p and Sir4p are delocalized from telomeric foci by SIR2 disruption

Is Sir2p an integral component of the repression complex at telomeres? In *SIR3*- or *SIR4*-deleted strains, we observe a delocalized staining pattern for Rap1 and Sir4p or Sir3p respectively, indicative of the loss of the repression

Fig. 3. Sir2p is associated preferentially with the rDNA loci on chromosome XII. (**a**) A schematic representation of the rDNA locus on chromosome XII and the location of the regions amplified by PCR in the precipitated material. (**b** and **c**) Whole-cell extracts (WCEs) were prepared from the formaldehyde cross-linked wild-type strain AYH2.45 (lanes 2 and 4) and mutant strain STY30 (*sir2*::*TRP1*; lanes 1 and 3). Immunoprecipitation was performed using affinity-purified anti-Sir2p polyclonal anibodies (for details see Materials and methods). The precipitated DNA and an aliquot of the WCE (input) were analyzed by PCR using primer pairs directed against the repetitive *CUP1* gene on chromosome VII (b) and the rDNA locus on chromosome XII (b and c). PCR products resolved on 6% polyacrylamide gels are shown. Lanes 1 and 2, PCR products from the immunoprecipitate; lanes 3 and 4, PCR products from the respective input WCEs; and lanes 5–8, 2.5-fold serial dilutions thereof. M: DNA size standard. (**d**) Analysis of affinity-purified anti-Sir2p antibodies by Western blot. Crude nuclear proteins were isolated from two pairs of related strains: UCC3107 and UCC3207, and from LPY1936 and YPH499. Lanes 1 and 2 were loaded with 100 μg of a nuclear enriched fraction of UCC3107 (*SIR*$^+$) and UCC3207 (*sir2*::*HIS3*), respectively, after extensive digestion with RNase A as described in Materials and methods. Lanes 3 and 4 were loaded with 100 μg from YPH499 (*SIR*$^+$) and LPY1936 (*sir2*::*URA3*, *hst1*::*LEU2*), respectively. After SDS gel electophoresis, proteins were transferred to nitrocellulose and probed with affinity-purified anti-Sir2p. The secondary antibody was visualized by enhanced chemiluminescence. Markers (in kilodaltons) are indicated on the right. The bold arrows indicate full-sized Sir2p and its breakdown product (lanes 1 and 3), which is absent in the *sir2*::*HIS3* strain. The asterisk indicates a weakly cross-reactive band at 61 kDa which is lost when both *HST1* and *SIR2* are deleted.

Table I. Localization of Sir2p, Sir3p and Sir4p to rDNA by formaldehyde cross-linking

a	Precipitate	Input		
rDNA 25S/2	1.0	1.0		
rDNA 5S	1.56	0.99		
CUP1	0.011	0.22		
b	Precipitate		Input	
	SIR2	*sir2*Δ		
rDNA 25S/2	1.0	1.0	1.0	
rDNA 5S	4.33	1.18	1.31	
ETS	4.96	2.41	2.16	
c	Precipitate		Input	
	Anti-Sir2p	Anti-Sir3p	Anti-Sir4p	
rDNA 25S/1	1.0	1.0	1.0	1.0
rDNA 5S	4.33	0.98	0.86	1.31
ETS	4.96	1.27	1.36	2.16

Ethidium bromide-stained PCR products were photographed, scanned and quantified using NIH Image 1.49 software. Band intensities were normalized according to the relative intensity of the rDNA 25S/1 or rDNA 25S/2 band respectively which were assigned the relative abundance of 1.0.

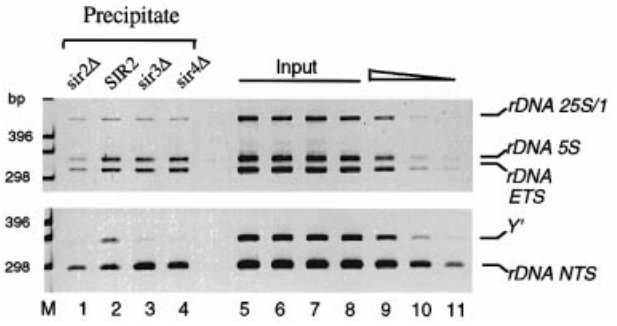


Fig. 4. Sir2p is lost at subtelomeric regions, but not at rDNA loci, upon *SIR3* or *SIR4* deletion. Sir2p was immunoprecipitated using affinity-purified anti-Sir2p polyclonal antibodies from WCEs prepared from formaldehyde cross-linked wild-type strain AYH2.45 (lanes 2 and 6) and mutant strains STY30 (*sir2*::*TRP1*; lanes 1 and 5), AYH2.8 (*sir3*::*LEU2*; lanes 3 and 7) and STY36 (*sir4*::*TRP1*; lanes 4 and 8) (for details see Materials and methods). PCR was done with primer pairs directed against repetitive subtelomeric Y′ and the rDNA locus on chromosome XII shown schematically in Figure 3a. PCR products were resolved on 6% polyacrylamide gels. Lanes 1–4 represent PCR products from the immunoprecipitate; lanes 5–8 PCR products from the input WCEs. Lanes 9–11, 2.5-fold serial dilutions thereof. M, DNA size standard.

complex (Gotta *et al.*, 1996). To see if deletion of *SIR2* also alters the focal staining patterns, we compared immunofluorescence on a SIR^+ diploid and a congenic *sir2*::*HIS3* diploid. We observe that the foci of Rap1 staining become less discrete and that Sir3p and Sir4p appear diffuse in the $sir2^-$ cells (Figure 5). This diffuse staining pattern also occurs upon deletion of *SIR3*, mutation of the Rap1 C-terminal domain, or the N-termini of histones H3 and H4 (reviewed in Gotta and Gasser, 1996), and strongly suggests that Sir2p is an integral component of telomeric chromatin, required for either assembly or stability of the repression complex.

Sir3p requires Sir2p for efficient nucleolar localization

In contrast to the generally diffuse staining pattern of Sir4p in silencing-deficient strains, a non-telomeric, but spatially restricted, staining pattern was observed for Sir3p in *sir4*-disrupted strains (Gotta *et al.*, 1996 and Figure 6a). To determine whether Sir3p might migrate to the nucleolus in the absence of Sir4p, we double stained GA202, a diploid *sir4*::*HIS3* strain, with anti-Sir3p (Figure 6a) and anti-Nop1 (Figure 6b). This reveals an unambiguous co-localization of Sir3p and Nop1 (Figure 6c; and white in the merged image, Figure 6d), whereas in wild-type cells the nucleolar Sir3p signal is very low (visualized as absence of yellow in the double-labeling experiment shown in Figure 7a). Interestingly, as observed above for Sir2p, Sir3p immunofluorescence in the *sir4*::*HIS3* strain is not entirely coincident with that of Nop1, suggesting that the two proteins may map to different subdomains of the nucleolus. Double labeling with anti-Sir2p and anti-Sir3p is not possible as we have only rabbit sera available.

Since Sir2p, Sir3p and Sir4p can be recovered as a complex in whole cell extracts, the localization of Sir3p in the nucleolus raises two questions: is Sir2p necessary for targeting Sir3p to the nucleolus? and, is association with Sir4p necessary to retain Sir3p in the non-nucleolar domain? To examine these questions, we performed double labeling for Nop1 (red) and Sir3p (green) on an isogenic set of strains carrying only the *sir4*::*HIS3* disruption (UCC3207), only the *sir2*::*HIS3* disruption (UCC3203) or both (GA617), and on the isogenic wild-type background (UCC3107). In the absence of Sir2p (Figure 7b), the superposition of Sir3p and Nop1 labeling patterns reveals a diffuse Sir3p stain that is largely excluded from the nucleolus. Immunostaining for Sir3p in the *sir2 sir4* double mutant gives a very similar pattern (Figure 7d), allowing us to conclude that Sir2p is essential for an efficient nucleolar localization of Sir3p, and that Sir4p is not necessary to retain Sir3p in the non-nucleolar compartment. Sir4p is necessary, however, to ensure that Sir3p is properly assembled in telomeric foci in silencing-competent strains (Cockell *et al.*, 1995; Gotta *et al.*, 1996). In conclusion, we find that in the absence of Sir4p, Sir3p is redirected to the nucleolus in a manner dependent on Sir2p.

A second factor cooperates for Sir3p relocalization

Is Sir2p sufficient to direct Sir3p to the nucleolus upon perturbation of the telomeric silencing complex? To find other genes that might influence Sir3p distribution, we examined a gene implicated in aging or life span determination in yeast, a process recently shown to be *SIR* dependent (Kennedy *et al.*, 1995). A truncated form of Sir4p, encoded by the *SIR4-42* allele, is a gain-of-function mutation that results in an extended life span phenotype in a stress-sensitive yeast strain, BKy1-14c (Kennedy *et al.*, 1995). Mutation of a second gene, *UTH4*, shortens life span, and complementation of the mutation by a wild-type *UTH4* allele to restore a normal life span also requires *SIR2*, *SIR3* and *SIR4*, but is independent of telomere-proximal repression (Kennedy *et al.*, 1997). Thus it was predicted that *UTH4* might influence Sir protein localization.

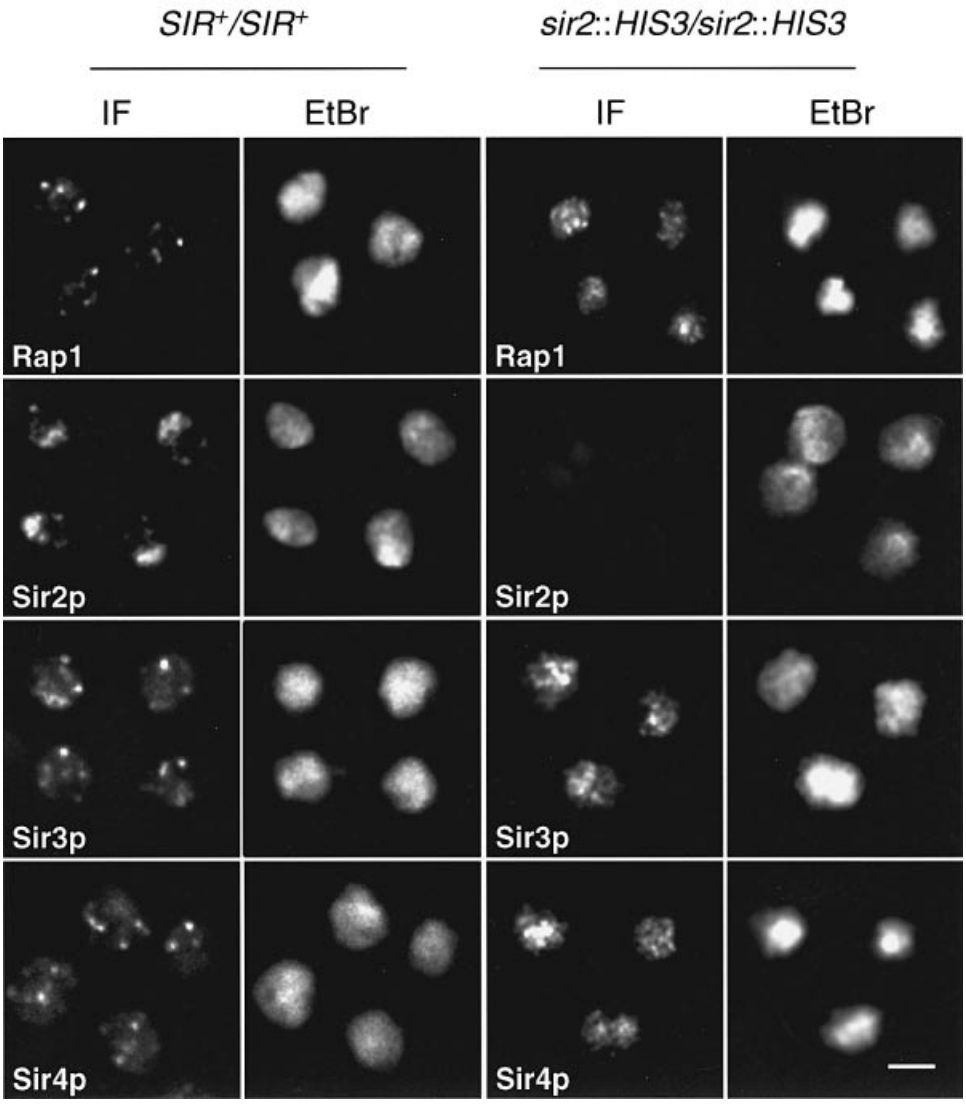


Fig. 5. Rap1, Sir3p and Sir4p foci are delocalized and diffuse in the absence of Sir2p. The congenic diploid strains GA225 (*SIR*$^+$/*SIR*$^+$) and GA194 (*sir2*::*HIS3*/*sir2*::*HIS3*) were stained with affinity-purified anti-Rap1, anti-Sir2p, and-Sir3p and anti-Sir4p antibodies detected by a DTAF-conjugated secondary antibody, as indicated. The DNA is visualized by staining with ethidium bromide. A 0.3 μm section of a field of spheroplasts is shown for each antibody reaction, with the corresponding DNA stain. IF, immunofluorescence; EtBr, ethidium bromide. Bar = 2 μm.

To test this hypothesis, we have stained for Sir3p (green) and Nop1 (red) in an isogenic set of strains (GA697, GA629, GA654) that carry deletions of either *SIR4*, *UTH4* or both. As expected, mutation of *SIR4* alone results in a Sir3p staining that largely coincides with Nop1, indicated by a high coincidence of red and green signals (yellow in Figure 7f). However, mutation in both *UTH4* and *SIR4* results in a diffuse Sir3p staining, with the majority of the protein excluded from the nucleolus (absence of yellow signal in Figure 7g). This could indicate either that Uth4p itself influences Sir3p targeting, or else that Sir2p is mislocalized or degraded in the *uth4*$^-$ strain, producing the same effect on Sir3p localization as a *SIR2* disruption. The latter possibility was eliminated by demonstrating that anti-Sir2p stains the nucleolus efficiently in the *uth4 sir4* double mutant strain (Figure 7h). In conclusion, the *UTH4* product itself is implicated in the redistribution of Sir3p.

Mutation of the *UTH4* gene in a *SIR*$^+$ background improves telomeric silencing (B.K.Kennedy, data not shown), and, in all cases examined to date, telomeric silencing correlates with a localization of Sir proteins to telomeric foci (Gotta and Gasser, 1996). Consistently, Sir2p, Sir3p and Sir4p were found coincident with telomeric foci in the *uth4*$^-$ strains (results shown for Sir3p, Figure 7e). In summary, the integrity of Sir2p and Sir4p, but not Uth4p, is required for the assembly of Sir3p at telomeric foci, while both Sir2p and Uth4p are necessary to achieve nucleolar localization of Sir3p, in the absence of Sir4p (summarized in Figure 8).

Discussion

Using complementary immunolocalization and *in vivo* formaldehyde cross-linking techniques, we demonstrate here that Sir2p is localized to two distinct subnuclear domains. First, like Rap1, Sir3p and Sir4p (Gotta *et al.*, 1996), Sir2p is found in discrete foci which correspond to clusters of telomeres. Second, Sir2p is found within a subdomain of the nucleolus, associated with the rDNA.

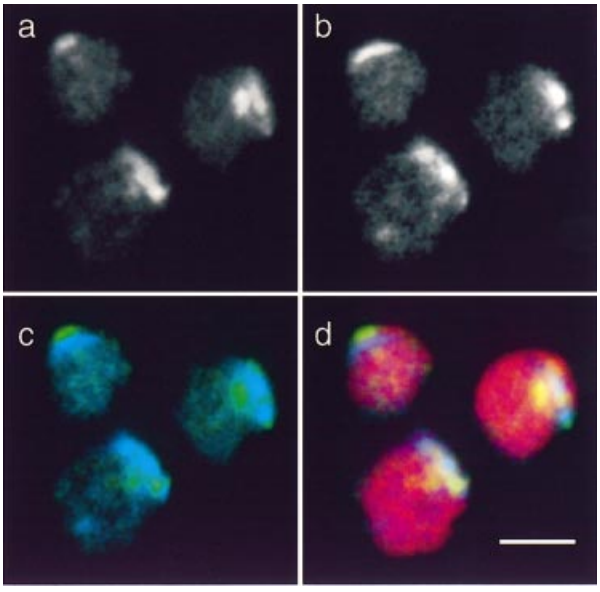


Fig. 6. Sir3p localizes to the nucleolus in a *sir4*::*HIS3* strain. In (a–d), the diploid strain GA202, which carries a double disruption for *SIR4* (*sir4*::*HIS3*) was stained with both anti-Sir3p (detected by a DTAF-conjugated secondary antibody, green) and anti-Nop1 (detected by a Cy5-conjugated secondary antibody, blue). The nuclei were counterstained with ethidium bromide for the DNA (red). (**a**) Anti-Sir3p alone; (**b**) anti-Nop1 alone; (**c**) the merge of Sir3p and Nop1 staining, in which the coincidence of the green and blue is a pale blue; (**d**) the merge of Sir3p, Nop1 and the DNA staining. Coincidence of all three signals is white. Bar = 2 μm.

In addition, we show that Sir3p can be distributed to different nuclear compartments depending on the cell's genotype: it is concentrated in telomeric foci in Sir^+ yeast cells, in the nucleolus in *sir4* mutants and it is diffuse but largely excluded from the nucleolus in strains lacking Sir2p.

Sir2p is an integral component of the telomeric repression complex

The present data extend previous observations showing that Sir3p, Sir4p, Rap1 and the Y′ subtelomeric DNA co-localize in a discrete number of foci in wild-type yeast cells (Gotta *et al.*, 1996). Mutations in *SIR3*, *SIR4* and in the Rap1 C-terminus that eliminate mating type and telomere-proximal silencing also disrupt this focal staining pattern. Consistent with an essential role for Sir2p in telomeric position effect (Aparicio *et al.*, 1991), we map Sir2p to telomeric foci and show that this localization is lost in *sir3* and *sir4* mutants. Finally, deletion of *SIR2* itself results in a diffuse, delocalized staining pattern for Sir3p and Sir4p. This, together with cross-linking and co-immunoprecipitation data (Strahl-Bolsinger *et al.*, 1997), establishes Sir2p as an integral component of the repression complex.

Roles for nucleolar Sir2p

In contrast to its localization at telomeres, the association of Sir2p with the nucleolus is observed in the absence of Sir3p and Sir4p. In 1989, Gottlieb and Esposito suggested that *SIR2* might influence chromatin organization within the nucleolus, because mutation of *SIR2* led to hyper-recombination between *LEU2* alleles integrated into the rDNA. This recombination phenotype was not dependent on *SIR3* or *SIR4* (Gottlieb and Esposito, 1989). More recently, two research groups have shown that RNA pol II genes inserted at the rDNA cluster are repressed in a variegated manner, and that this rDNA silencing, like the suppression of recombination, is again dependent on *SIR2*, but not on *SIR3* or *SIR4* (Smith and Boeke, 1997; Bryk *et al.*, 1997). In neither study was it clear whether Sir2p plays a direct or an indirect role at the rDNA locus. By localizing Sir2p to the nucleolus, we lend strength to the hypothesis that Sir2p influences rDNA organization directly.

Sir2p could help repress both recombination and pol II-mediated transcription within the rDNA repeats by inducing a compact chromatin structure. This repressed chromatin structure must differ from that formed at telomeres and *HM* loci, since it does not require Sir3p and Sir4p. It is not clear whether Sir2p can create this predicted higher-order chromatin structure on its own, or whether it requires other factors, such as the members of the *HST* family (*H*omolog of *SIR2*; Brachmann *et al.*,1995). Interestingly, it has been shown that the ability of the *HOT1* sequence to stimulate recombination at the rDNA depends on its ability to promote transcription (Stewart and Roeder, 1989). It is possible, therefore, that Sir2p represses transcription at the rDNA and thereby suppresses recombination. At a molecular level this mechanism remains ill-defined.

What directs or sequesters Sir2p to the rDNA?

Although both cross-linking and RNase digestion data suggest that Sir2p is associated with DNA, and not with the rRNA, Sir2p has no obvious DNA-binding motif, and no DNA-binding activity has been demonstrated to date. Consistent with the cross-linking data, which suggest a preferential association with the NTS of the rDNA repeat, the *URA3*-tagged Ty insertions that display the most severe *SIR2*-dependent repression map to the NTS, close to the promoter of the 35S rDNA (J.S.Smith and J.D.Boeke, submitted). This suggests that Sir2p might associate with factors that have binding sites within the transcriptional control region, such as Reb1p, Rap1, Abf1p and Origin Recognition Complex (ORC) (Buchman *et al.*, 1988; Ju *et al.*, 1990; Bell *et al.*, 1993; Kang *et al.*, 1995). All but the first of these are also implicated in silencer-mediated repression at *HM* loci (Buchman *et al.*, 1988; Sussel and Shore, 1991; Foss *et al.*, 1993).

It appears unlikely that Sir2p will be targeted to the nucleolus through association with Rap1, because immunofluorescence and formaldehyde cross-linking experiments reveal very little Rap1 in the nucleolus. If Reb1p, Abf1p or ORC were to mediate the association of Sir2p with this region, it must be explained why Sir2p does not bind such ligands at their many other recognition sites throughout the genome. All three of these sequence-specific DNA-binding factors are encoded by genes essential for viability, and the factors act at multiple sites to stimulate transcription and/or DNA replication (Buchman *et al.*, 1988; Ju *et al.*, 1990; Bell *et al.*, 1993). If Sir2p is targeted to the NTS of the rDNA through any of these factors, other unidentified elements would be required to confer specificity for the nucleolar subpopulation of the given factor.

It is also possible that Sir2p recognizes a unique feature

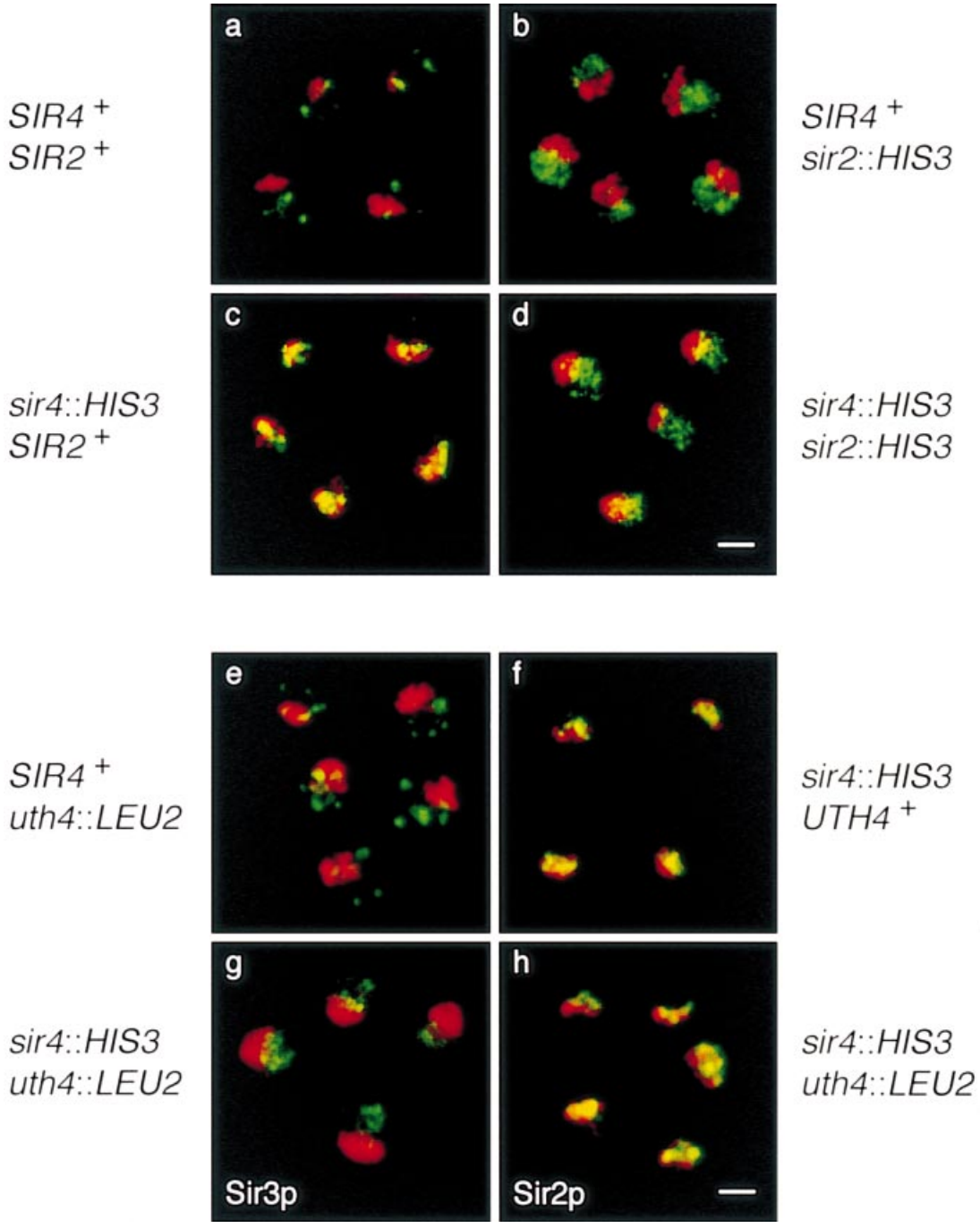


Fig. 7. *SIR2* and *UTH4* are necessary for the localization of Sir3p to the nucleolus in a *sir4*::*HIS3* strain. In (a–d), the haploid strains UCC3107 (*SIR*$^+$, **a**), UCC3203 (*sir2*::*HIS3*, **b**), UCC3207 (*sir4*::*HIS3*, **c**) and an isogenic strain carrying *sir2*::*HIS3* and *sir4*::*HIS3* alleles (GA617, **d**) were stained with both anti-Sir3p (detected by a DTAF-conjugated secondary antibody, green) and anti-Nop1 (detected by a Cy5-conjugated secondary antibody, red in the picture). Coincidence of the two signals is yellow. Bar =1.5 μm. In (**e**–**g**), the haploid strains GA629 (*SIR4*$^+$ *uth4*::*LEU2*), GA697 (*sir4*::*HIS3 UTH4*$^+$) and GA654 (*sir4*::*HIS3 uth4*::*LEU2*) were double stained with anti-Sir3p (detected by a DTAF-conjugated secondary antibody, in green) and anti-Nop1 (detected by a Cy5-conjugated secondary antibody, red in the picture). Coincidence of the two signals is yellow. (**h**) The double staining of anti-Sir2p (detected by a DTAF-conjugated secondary antibody, in green) and Nop1 (detected by a Cy5-conjugated secondary antibody, in red) on GA654 (*sir4*::*HIS3 uth4*::*LEU2*). Coincidence of the two staining patterns is yellow. Bar in (h) = 1.5 μm.

of the rDNA itself, such as its repetitive nature, rather than being targeted by protein–protein interactions. In yeast there are 20–50 repeats of the 9 kb rDNA unit, which comprises the most extensive series of direct repeats in the yeast genome (Petes *et al.*, 1977). Furthermore, the repetitive nature of the rDNA array seems to be essential for silencing, since repression of transcription does not occur if the reporter gene flanked by single copy rDNA sequences is inserted at the *LEU2* locus (Smith and Boeke, 1997). This adds to the accumulating evidence that suggests a role for repeated DNA sequences in higher-order chromosomal functions, such as gene inactivation in *Ascobolus* (reviewed in Rossignol and Faugeron, 1995), achiasmatic pairing in *Drosophila melanogaster* (McKee and Karpen, 1990; Merrill *et al.*, 1992; Karpen *et al.*, 1996; for a review, see Renauld and Gasser, 1997) and

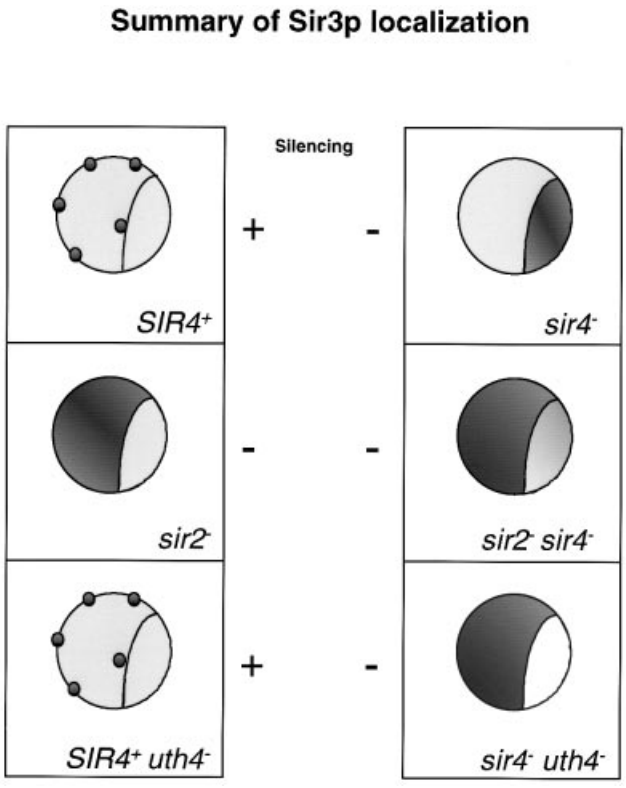


Fig. 8. Schematic representation of Sir3p distribution. The distribution of Sir3p between the nucleus (large circle) and the nucleolus (small crescent) in the various mutants analyzed here is presented schematically by the gray shaded color. In a wild-type (*SIR4*$^{+}$) nucleus, Sir3p is localized at telomeric foci (shown as gray circles). In a *sir2*$^{-}$ nucleus, Sir3p is mostly excluded from the nucleolus (shown as shaded gray). In a *sir4*$^{-}$ nucleus, Sir3p is localized mostly to the nucleolar subdomain of the nucleus. In a *sir2*$^{-}$ *sir4*$^{-}$ nucleus, Sir3p is largely but not completely excluded from the nucleolus (weak nucleolar staining shown as light gray). In a *uth4*$^{-}$ nucleus, Sir3p is localized to telomeric foci (again shown as a gray circle), while in a *uth4*$^{-}$ *sir4*$^{-}$ Sir3p is mostly excluded from the nucleolus. The presence (+) or absence (–) of the variegated, SIR-dependent repression of a gene inserted near the telomeric repeat is indicated, based on published data, except for the *uth4* strains (Kennedy, 1996). As previously observed, there is a correlation between telomere silencing and the focal staining pattern of Sir3p.

heterochromatin-mediated repression in *Drosophila* (reviewed in Hilliker *et al.*, 1980). It is intriguing that in *Drosophila*, long repetitive insertions of reporter gene constructs become spontaneously variegated or repressed, in a manner dependent on proteins implicated in centromeric hetechromatin and its associated chromatin-mediated repression (Dorer and Henikoff, 1994; Sabl and Henikoff, 1996). These results suggest that in flies an extended array of direct repeats might be sufficient to create a unique, repressed chromatin structure, although it does not exclude a requirement for a protein like Sir2p in this process.

The nucleolus as a compartment for SIR function

The two well-characterized sites of Sir protein function are *HM* loci and subtelomeric sequences. However, only silencing at the mating type loci, which ensures the mating ability of haploid strains, is clearly of physiological importance to yeast. Several lines of evidence suggest that there is competition between the subtelomeric regions and the *HM* loci for the limiting supply of Sir3p–Sir4p complex (Stone *et al.*, 1991; Stavenhagen and Zakian, 1994; Buck and Shore, 1995; Lustig *et al.*, 1996; Maillet *et al.*, 1996), and Sir1p appears to help assemble or target the Sir2p–Sir3p–Sir4p complex to the *HM* loci, thus favoring tight mating type repression (Chien *et al.*, 1993; Triolo and Sternglanz, 1996). Consistent with this idea, several groups have proposed that the clustering of telomeres creates a reservoir of silencing factors, from whence they can be redirected to other loci, such as *HML* or *HMR*, when needed (Lustig *et al.*, 1996; Maillet *et al.*, 1996; Marcand *et al.*,1996a,b).

Is the nucleolus itself a second, physiological site of Sir function, competing for the telomeric pool of Sir proteins? Intriguingly, to date, only mutations in *SIR2* have been shown unambiguously to influence nucleolar function; the deletion of *SIR3* has little effect on either the recombination phenotypes described by Gottlieb and Esposito (1989) or on the *SIR2*-dependent repression of the *URA3*-tagged Ty insertions (Smith and Boeke, 1997; Byrk *et al.*, 1997). Consistently, our immunofluorescence and cross-linking data suggest that Sir3p and Sir4p are rare in the rDNA of rapidly growing, wild-type cells. However, when Sir4p is not available to form the functional repression complex at telomeres, Sir3p is accumulated efficiently in the nucleolar compartment, in a Sir2p-dependent fashion.

What role could Sir3p play in the nucleolus? The nucleolus does not appear to be a simple default compartment for Sir proteins that cannot bind telomeres and *HM* loci, since Sir4p is not localized preferentially to the nucleolus in a *sir3* mutant. Instead, Sir4p shows a diffuse localization throughout the nucleoplasm (Gotta *et al.*, 1996). However, recent results show that in a *SIR3*$^{+}$ strain, a truncated form of Sir4p (encoded by *SIR4-42*) that has lost a C-terminal Rap1-binding domain, is found along with Sir3p, enriched in the nucleolar compartment (Kennedy *et al.*, 1997). The *SIR4-42* mutation is a recessive loss-of-function mutation for telomeric silencing, but is a dominant mutation for the extension of the yeast life span. Intriguingly, the extension of life span by the *SIR4-42* allele requires both Sir3p and Sir2p, implicating a role for chromatin organization or transcriptional repression in the phenotype (Kennedy *et al.*, 1995). This lends credence to the idea that Sir proteins have a physiological function within the nucleolus, although this function may only be manifest in aging cells, as a means to counteract the detrimental side effects of growing old.

Consistently, a second gene implicated in yeast longevity, *UTH4*, is shown here to be necessary for proper targeting of Sir3p to the nucleolus, when the association of Sir3p with the telomere is impaired (i.e. in the absence of Sir4p). Uth4p is a member of a family of proteins containing putative RNA-binding motifs (Barker *et al.*, 1992), among which are the *Drosophila* gene *pumilio*, and a second yeast gene of unknown function, called *YGL023*. Deletion of either *UTH4* or *YGL023* results in increased telomeric silencing (Kennedy, 1996), consistent with the idea that these proteins play a role in directing the Sir complex away from the telomere. In view of the homology between *UTH4* and *YGL023*, it is likely that these factors share overlapping or perhaps antagonistic functions. Further studies are underway to shed light on

Table II. Yeast strains used

Diploid strains	
GA192	(*MAT***a**/*MAT*α, *ade2-1/ADE2, trp1-1/TRP1, his3-11,15/his3, ura3-1/ura3-52, leu2-3,112/LEU2, LYS2/lys2-6, can1-100/CAN1, sir3*::*TRP1/sir3*::*LYS2*)
GA202	(*MAT/MAT*α, *ade2-1/ADE2, trp1-1/trp1, his3-11,15/his3, ura3-1/ura3-52, leu2-3,112/LEU2, lys2-6/LYS2, can1-100/CAN1, sir4*::*HIS3 /sir4*::*HIS3*)
GA225	(*MAT***a**/*MAT*α, *ade2-1/ADE2, trp1/trp1, his3-11,15/his3, ura3-1/ura3-52, can1-100/can1-100*)
GA194	(*MAT***a**/*MAT*α, *ade2-1/ADE2, trp1/trp1, his3-11/his3, ura3-1/ura3-52, can1-100/can1, leu2-3,112/LEU2, sir2*::*HIS3/sir2*::*HIS3*)
AHY111	(*MATa/MAT*α, *ho*::*LYS2/ho*::*LYS2, ura3/ura3, lys2/lys2, leu2*::*hisG/leu2*::*hisG, arg4-nsp/arg4-bgl, RAP1-GFP-LEU2*::*rap1/RAP1-GFP-LEU2*::*rap1*)
Haploid strains	
UCC3107	(*MAT***a**, *ade2*::*hisG, can1*::*hisG, his3-11, leu2, trp1, ura3-52, V_R*::*ADE2-Tel*)
UCC3203	(*MAT***a**, *ade2*::*hisG, can1*::*hisG, his3-11, leu2, trp1, ura3-52, sir2*::*HIS3, V_R*::*ADE2-Tel*)
UCC3207	(*MAT***a**, *ade2*::*hisG, can1*::*hisG, his3-11, leu2, trp1, ura3-52, sir4*::*HIS3, V_R*::*ADE2-Tel*)
GA617	(*MAT***a**, *ade2*::*hisG, can1*::*hisG, his3-11, leu2, trp1, ura3-52, sir2*::*HIS3, sir4*::*HIS3, V_R*::*ADE2-Tel*)
Bky125	(*MAT***a**, *ade2-101, leu2-3,112, lys2-801, his3-200, ura3-52, adh4*::*ADE2-Tel*)
GA629	(*MAT***a**, *ade2-101, leu2-3,112, lys2-801, his3-200, ura3-52, uth4*::*LEU2, adh4*::*ADE2-Tel*)
GA654	(*MAT***a**, *ade2-101, leu2-3,112, lys2-801, his3-200, ura3-52, uth4*::*LEU2, sir4*::*HIS3, adh4*::*ADE2-Tel*)
GA697	(*MAT***a**, *ade2-101, leu2-3,112, lys2-801, his3-200, ura3-52, sir4*::*HIS3, adh4*::*ADE2-Tel*)
AYH2.8	(*MAT***a**, *ade2-101, his3-Δ200, leu2-3, -112, lys2-801, trp1-Δ901, ura3-52, adh4*::*URA3*TelVII-L, *sir3*::*LEU2*; Hecht *et al.*, 1996);
AYH2.45	(*MAT***a**, *ade2-101, his3-Δ200, leu2-3, -112, lys2-801, trp1-Δ901, ura3-52, adh4*::*URA3*TELVII-L, *sir3*::*SIR3HA/HIS3;* Hecht *et al.*, 1996)
STY30	(*MAT***a**, *ade2-101, his3-Δ200, leu2-3, -112, lys2-801, trp1-Δ901,ura3-52, adh4*::*URA3*TELVII-L, *sir3*::*SIR3HA/HIS3, sir2*::*TRP1*)
STY36	(*MAT***a**, *ade2-101, his3-Δ200, leu2-3, -112, lys2-801, trp1-Δ901, ura3-52, adh4*::*URA3*TELVIII-L, *sir3*::*SIR3HA/HIS3, sir4*::*TRP1*).
YPH499	(*MAT***a**, *ade2-101, his3Δ200, leu2Δ1, lys2-801, trp1Δ63, ura3-52*)
LPY1936	(*MAT***a**, *ade2-101, his3Δ200, leu2Δ1, lys2-801, trp1Δ63, ura3-52, hst1Δ1*::*LEU2, hst2Δ1*::*TRP1, sir2Δ1*::*URA3*)

how Sir proteins are distributed among their various binding sites in wild-type cells, and what other partners regulate this balance.

Materials and methods

Yeast strains and media

All yeast strains are described in Table II. The diploid strains used for immunofluorescence studies (GA225, GA202, GA194) are congenic; and the haploid strains UCC3107, UCC3203, UCC3207 and GA617 are derived from UCC3203 and UCC3207. UCC3107 has been described elsewhere (Stone and Pillus, 1996). GA629, GA654 and GA697 were derived from Bky125. AYH2.8 and the derived *sir2*, *sir3* and *sir4* mutants have been described elsewhere (Hecht *et al.*, 1996; Strahl-Bolsinger *et al.*, 1997). LPY1936 was derived from YPH499, which is described in Brachmann *et al.* (1995). Standard genetic techniques and YPD medium supplemented with 40 mg/l adenine were used throughout.

Antibody production, purification and specificity

The preparation, characterization and affinity purification of the rabbit antisera against Rap1 (Klein *et al.*, 1992), the Sir3–βgal fusion (Cockell *et al.*, 1995) and the Sir4p–GST fusion have been described previously (Gotta *et al.* 1996). An antibody against a Sir2p–GST fusion protein (amino acids 275–562) was produced using standard methods (Harlow and Lane, 1988). Other antibodies were as follows: anti-Nop1 monoclonal antibody (gift of E.Hurt, Heidelberg; Aris and Blobel, 1988); anti-GFP monoclonal antibody (Peter *et al.*, 1996); Texas Red-conjugated secondary antibody (Jackson Immuno-research Laboratories); Cy5-coupled reagents (Milan Analytica); and fluorescein-conjugated goat anti-rabbit (Jackson Immuno-research Laboratories). Secondary antibodies were pre-adsorbed against fixed yeast spheroplasts prior to use. No cross-reactivity among these reagents has been detected, and controls using secondary antibodies alone were carried out routinely.

For Western blots, crude nuclei were isolated from the indicated strains (Verdier *et al.*, 1990), and were digested for 1 h on ice in 125 mM Tris–HCl, pH 6.8, 10 mM $MgCl_2$, 10% glycerol and 100 μg/ml RNase A. Solubilized proteins were recovered by diluting nuclei 3-fold in digestion buffer and centrifuged for 5 min at 10 000 *g*. The nuclear pellet was denatured in 2× Laemmli sample buffer (Laemmli, 1970), sonicated and heated at 95°C for 5 min. Proteins in the supernatants were precipitated by 25% trichloroacetic acid, pellets were washed in 70% acetone/10% ethanol/10 mM Tris–HCl, pH 7.4 until neutralized and similarly denatured in Laemmli sample buffer. Western blotting followed standard techniques, and signals were visualized by Enhanced Chemiluminescence (Amersham).

Immunofluorescence on yeast cells

Immunofluorescence was performed as described (Gotta *et al.*, 1996). In order to optimize Sir2p detection, fixed cells were incubated for 20 min in phosphate-buffered saline (PBS) containing 0.5% Triton X-100 and 0.01% SDS prior the antibody reaction. The weak anti-Sir2p signal at telomeres precluded use of a double fluorescence *in situ* hybridization (FISH)/immunofluorescence labeling procedure to co-localize Sir2p with telomeric DNA, since such signals do not survive the FISH procedure.

Confocal microscopy was performed on a Zeiss Axiovert 100 microscope (Zeiss Laser Scanning Microscope 410) with a 63× or 100× Plan-Apochromat objective (1.4 oil), as previously described (Gotta *et al.*, 1996). Under standard imaging conditions, no signal from one fluorochrome could be detected on the other filter set. Standardized conditions for the image capture and background subtraction (~15% of the maximum signal) were carried out uniformly on all images to allow direct comparisons.

Immunoprecipitation from fixed whole cell extracts

Fifty ml of yeast cells were grown to a concentration of 2.0×10^7 cells/ml and cross-linked with 1% formaldehyde for 15 min at room temperature. Glycine was added to a final concentration of 125 mM and the incubation continued for 5 min. After washing twice with TBS (20 mM Tris–HCl, pH 7.6, 200 mM NaCl) the cells were resuspended in 400 ml of lysis buffer [50 mM HEPES–KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM benzamidine, 0.25 mM TLCK, 50 μg/ml TPCK, 10 μg/ml aprotinin, 20 μg/ml antipain, 1 μg/ml leupeptin, 1 μg/ml pepstatin]. The cells were lysed with glass beads by vortexing on an Eppendorf shaker 5432 for 40 min at 4°C. The lysate was collected, clarified by centrifugation and sonicated according to Hecht *et al.* (1996). Immunoprecipitation and processing of the precipitates was performed as described in Strahl-Bolsinger *et al.* (1997).

PCR analyses of immunoprecipitated DNA

PCRs were carried out in a 50 μl volume with 1/50 of the immunoprecipitated material, 1/13 500 of the input material and serial 2.5-fold dilutions thereof as templates. *Taq* polymerase (Gibco-BRL) and the corresponding buffer system were used; 70 pmol of primer were added. The following gene-specific primers were designed as 24mers with 50% GC content: 25S/1: AGGACGTCATAGAGGGTGAGAATC and TTGACTTACGT-CGCAGTCCTCAGT; 25S/2: TATTTCACTGGCGCCGAAGCTCCCA

and TACGGACAAGGGGAATCTGACTGT; 5S: GAAAGGATTTG-CCCGGACAGTTTG and CTTCTTCCCAGTAGCCTGTTCCTT; ETS: AATAGCCGGTCGCAAGACGTGATT and CCACCTATTCCCTCTT-GCTAGAAG; NTS: TCGCATGAAGTACCTCCCAACTAC and TCCG-CTTCCGCTTCCGCAGTAAAA; CUP1: TCTTTTCCGCTGAACC-GTTCCAGCA and GGCATTGGCACTCATGACCTTCAT.

The PCR cycles were chosen empirically, determined by preliminary reactions with each set of oligonucleotides and the reactions stopped before reagents were exhausted. Typically, an initial denaturation for 2 min at 95°C was followed by 18 cycles with denaturation for 30 s at 95°C, annealing for 30 s at 55°C, polymerization for 60 s at 72°C and a final extension for 2 min at 72°C. PCR products were separated on 6% polyacrylamide gels and visualized with 0.1 mg/ml ethidium bromide. The gels were photographed using Polaroid film type 667 and type 55. Photoprocessing was performed using OFOTO (Light Source Computer Images) and NIH Image (version 1.49) software.

Acknowledgements

We would like to thank Dr E.Hurt (University of Heidelberg) for his generous supply of anti-Nop1 antibodies, A.Hecht for the anti-Sir2p antibodies and the Sir2p–GST fusion, Y.Hiraoka (Kobe) for the strain carrying the Rap1–GFP fusion protein, I.Davis (UCSF) for the anti-GFP antibodies, D.Gottschling (Fred Hutchinson Research Institute) and L.Pillus for strains and plasmids, and members of the Gasser laboratory for helpful discussions. Collaborative research in the Gasser and the Grunstein laboratories is funded by a Human Frontiers Grant. Research in the Gasser laboratory is supported by the Swiss National Science Foundation and the Swiss League against Cancer; in the Grunstein laboratory by the Public Health Service grant GM42421 from the NIH. M.G. thanks ISREC, H.R. thanks the European Commission, and S.S.-B. the Deutsche Forschungsgemeinschaft, for fellowships.

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Received on January 2, 1997; revised on February 12, 1997