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Cloning and Characterization of Four *SIR* Genes of *Saccharomyces cerevisiae*

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Mating type in the yeast *Saccharomyces cerevisiae* is determined by the *MAT* (a or α) locus. *HML* and *HMR*, which usually contain copies of α and a mating type information, respectively, serve as donors in mating type interconversion and are under negative transcriptional control. Four *trans*-acting *SIR* (silent information regulator) loci are required for repression of transcription. A defect in any *SIR* gene results in expression of both *HML* and *HMR*. The four *SIR* genes were isolated from a genomic library by complementation of *sir* mutations in vivo. DNA blot analysis suggests that the four *SIR* genes share no sequence homology. RNA blots indicate that *SIR2*, *SIR3*, and *SIR4* each encode one transcript and that *SIR1* encodes two transcripts. Null mutations, made by replacement of the normal genomic allele with deletion-insertion mutations created in the cloned *SIR* genes, have a Sir$^-$ phenotype and are viable. Using the cloned genes, we showed that *SIR3* at a high copy number is able to suppress mutations of *SIR4*. RNA blot analysis suggests that this suppression is not due to transcriptional regulation of *SIR3* by *SIR4*; nor does any *SIR4* gene transcriptionally regulate another *SIR* gene. Interestingly, a truncated *SIR4* gene disrupts regulation of the silent mating type loci. We propose that interaction of at least the *SIR3* and *SIR4* gene products is involved in regulation of the silent mating type genes.

The third chromosome of the budding yeast *Saccharomyces cerevisiae* (Fig. 1) contains three loci, all capable of determining cell type by regulating the expression of other cell type-specific genes. Normally, however, only *MAT* (either *MAT***a** or *MAT*α) is expressed, whereas *HML* and *HMR* remain unexpressed. The role of *MAT* in determining cell type in *S. cerevisiae* has been reviewed extensively (11, 15, 30, 37, 48). Briefly, haploids and diploids homozygous at *MAT* mate efficiently with cells of opposite mating type, but cells of the same mating type rarely mate. *MAT***a**/*MAT*α diploids do not mate but can sporulate when starved for nitrogen. Heterothallic (*ho*) strains possess a stable mating ability. Homothallic (*HO*) strains interconvert the *MAT* alleles at high frequency during mitotic growth and rapidly give rise to a population containing cells of both mating types, which can mate to form *MAT***a**/*MAT*α diploids (16, 49). Mating type interconversion is the result of the transposition of a copy of sequences from *HML* or *HMR* (normally containing α and **a** sequences, respectively) to *MAT* (17, 24, 25, 27, 32, 39, 51). Thus, the two silent loci serve as donors and are said to contain cassettes of information.

The two silent *HM* loci contain complete copies of mating type information (17, 39, 51) but are kept transcriptionally inactive by the products of four genes identified by mutational analysis and known as *MAR* (mating type regulator; 26), *SIR* (silent information regulator; J. Rine, Ph.D. thesis, University of Oregon, Eugene, 1979; 42), or *CMT* (change of mating type; 12). The four *SIR* genes are unlinked to the *HM* loci and encode gene products which act in *trans* to regulate the silent cassettes. Mutation in any one of the *SIR* genes results in expression of *HML* and *HMR*. Usually this results in the expression of both **a** and α information and the nonmating phenotype normally associated with *MAT***a**/*MAT*α diploids. In addition to regulating expression of the *HM* loci, Sir regulation also maintains the donor versus recipient distinction between *HML* or *HMR* and *MAT* (29). This is achieved, apparently, by blocking formation of a double-stranded DNA cut at *HML* and *HMR*. Such a cut at the *MAT* locus is thought to initiate mating type interconversion (31, 50).

Sir regulation of *HML* and *HMR* is thought to be mediated through *cis*-acting elements which flank each *HM* locus (2, 10). Sequences essential for regulation (termed the E site) lie to the left of each cassette, and sequences important for regulation (termed the I site) lie to the right of each cassette. Mutation of either allows expression of the adjacent cassette. The difference between the E and I sites is operationally defined and correlates with different levels of silent cassette expression. Comparison of the DNA sequences of the E and I sites shows limited sequence homology. All four sites share two different 11-base-pair (bp) sequences, and the two E sites share one additional 11-bp sequence (10). One striking feature of this system is that the E and I sites are at least 1,000 bp removed from the promoters at both *HML* and *HMR*. In fact, because the promoters for the two divergent transcripts of both **a** and α are within the **a**- and α-specific Y regions (Fig. 1), the silent cassettes and *MAT* share at least 800 bp of homology 5′ to every mating type transcript. Sir regulation, therefore, must act over a distance.

Three investigations of the nature of the regulation of the silent mating type loci have been reported. Nasmyth (38) has demonstrated a difference in the sensitivity to DNase I and micrococcal nuclease of *HM* chromatin isolated from Sir$^+$ and Sir$^-$ strains. Abraham et al. (1) have shown that the negative superhelical density of cloned *HMR***a** and *HML*α loci is greater in Sir$^+$ strains than in Sir$^-$ strains, whereas the negative superhelical density of a cloned *MAT*α locus does not change. Finally, it appears that cells must pass through DNA synthesis to establish Sir regulation of the silent cassettes (35). Yet much of the molecular mechanism of the negative regulation of *HML* and *HMR* remains unknown. To begin an analysis of *SIR* gene action, we cloned the four *SIR* genes. A similar analysis (46) of *SIR2* and *SIR3* has recently been published. In this paper we present evidence bearing on

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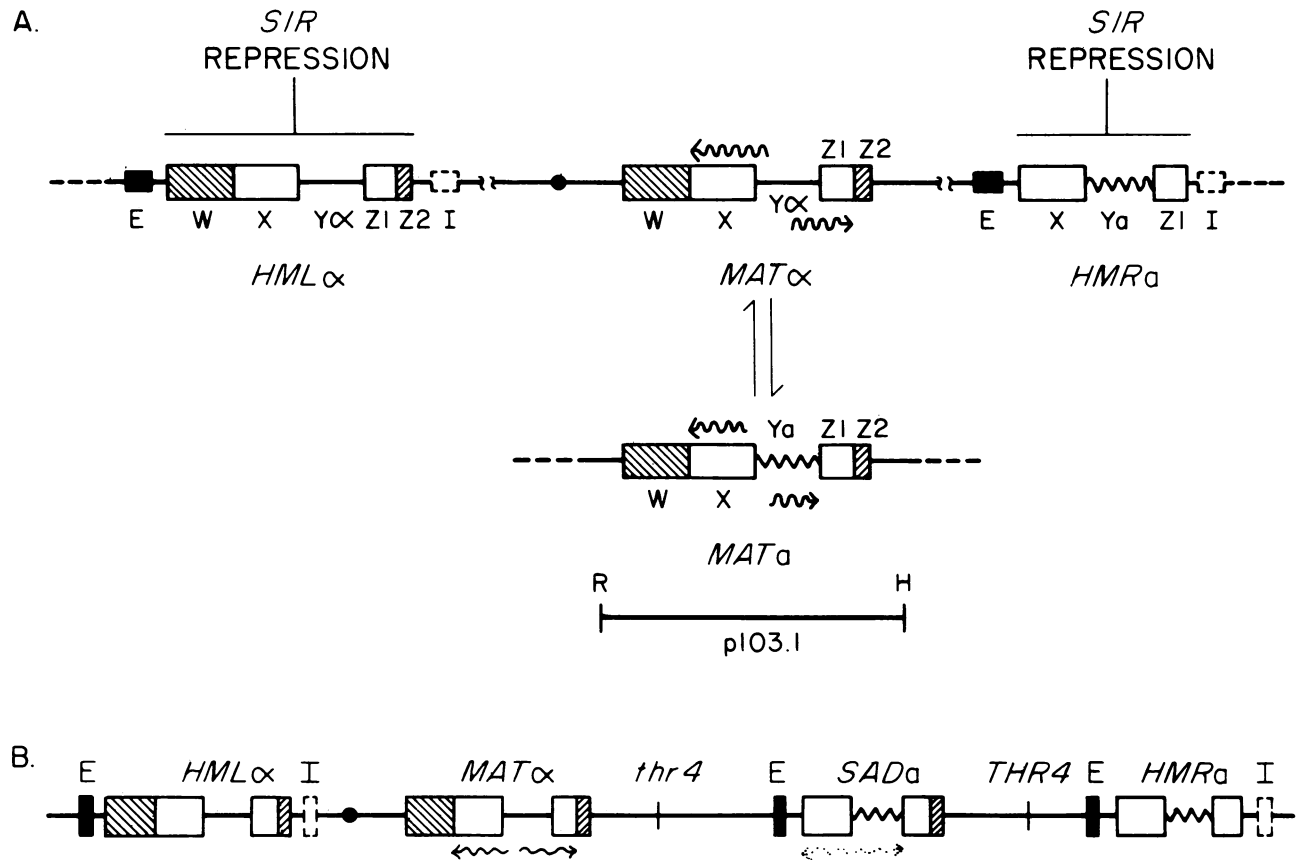


FIG. 1. (A) Chromosome III of *S. cerevisiae* (not drawn to scale) with the mating type cassettes indicated. W, X, Z1, and Z2 (723, 704, 239, and 88 bp, respectively) represent regions of homology shared by the mating type loci. Ya (642 bp) and Yα (747 bp) represent the sequences specific to a and α information, respectively. The *MAT* locus exists with either α or a information, and each encodes two divergent transcripts indicated by wavy-line arrows. Transcriptional repression of *HML* and *HMR* requires the *cis* elements E and I and the four *SIR* genes. p103.1 is a cloned *Eco*RI (R)-*Hin*dIII (H) fragment containing the *MAT*a locus that was used as a probe for mating type transcripts. (B) A rearranged third chromosome of yeast containing a duplication of the genes between *MAT* and *HMR* (e.g., the *THR4* locus is drawn as on the original isolate [18, 23]) and the fusion mating type locus *SAD*. *SAD* is flanked by only one of the two *cis* regulatory elements and produces a low level of transcript indicated by dotted, wavy-line arrows.

identities of the four clones, on their transcriptional organization, on the absence of a cascade of transcriptional regulation among the *SIR* genes, on the viability and Sir$^-$ phenotype of null mutations in the *SIR* genes, and on the ability of a truncated *SIR4* clone to disrupt regulation of the silent mating type genes. Additionally, the observation that multiple copies of *SIR3* suppress *sir4* mutations suggests an interaction between the gene products of at least these two *SIR* genes.

MATERIALS AND METHODS

Strains. *S. cerevisiae* strains used in these studies are listed in Table 1. *Escherichia coli* strains used were C600 (3) and DH1 (13). The *leuB6* mutation in C600 is complemented by the yeast *LEU2* gene, which allows for the selection of cells containing the plasmid-borne *LEU2* gene on medium lacking leucine.

Yeast genetic and biochemical manipulations. Standard conditions and procedures for culture, mating, sporulation, and dissection were used (36, 45). Transformation of *S. cerevisiae* was performed as described by Beggs (5), with spheroplasts generated by Glusulase treatment. Genomic alleles were replaced by transformation of yeast with linear DNA fragments (44) containing mutations made in vitro in the cloned fragments (gene disruptions). This technique makes use of the observation of Orr-Weaver et al. (40) that cut ends of DNA are recombinogenic. Small-scale yeast DNA preparations suitable for restriction enzyme digestion or for recovering plasmid DNA in *E. coli* were made from 5- to 10-ml cultures (2, 45).

Mating type tests were made by replica plating cells to be tested onto lawns of either *MAT*a or *MAT*α tester cells on YEPD (45), which were then incubated for 12 h at 30°C before replica plating to selective medium. Restrictive mating type tests utilized *leu2* tester strains (DC5 and SX50-1C) and selective medium lacking leucine, such that the mating ability of only those cells that had retained a plasmid would be assayed. Nonrestrictive mating type tests used *LEU2* tester strains (DC14 and DC17).

Efficiency-of-mating determinations used tester strains K567 and K566. Fresh overnight cultures were diluted into fresh liquid medium and grown for approximately 4 h, at which time cells to be tested were combined with at least a fivefold excess of tester cells. Cells were lightly pelleted and allowed to incubate at 30°C with no shaking for 6 h. At the time of mixing, the frequency of prototrophs due to reversion and the cell numbers of both strains were determined by

plating appropriate dilutions on selective media. At 6 h, the number of prototrophs due to mating was determined by plating dilutions on selective medium.

Complementation tests for *sir1* mutations were made by crossing strains to either DC59 or IX44-11C and assaying the ability of the resultant diploids to sporulate. Owing to the mutation at *MAT* in the testers, sporulation of the diploid requires expression of the silent cassettes and indicates that the strain being tested contains a mutation unable to complement *sir1-1*. In a similar manner, complementation tests to detect mutations at *SIR2* and *SIR3* in *HMLα MATα HMRα* segregants used tester strains K96 and PC10-7B, respectively.

Bacterial manipulations. Bacterial transformations were performed essentially as previously described (34). Large-scale (1-liter culture) plasmid preparations were prepared by the method of Clewell (9), and small-scale minipreps (5-ml culture) were made by the boiling procedure of Holmes and Quigley (20). Manipulations of M13 bacteriophage are described elsewhere (6).

TABLE 1. *S. cerevisiae* strains

Strain	Genotype	Source
DC5	*HMLα MATa HMRa his3 can1 leu2-3 leu2-112*	This laboratory
SX50-1C	*HMLα MATα HMRa his3 leu2-3 leu2-112 trp1 ura3-52*	This laboratory
DC14	*HMLα MATa HMRa his1*	This laboratory
DC17	*HMLα MATα HMRa his1*	This laboratory
K567	*HMLα MATa HMRa ilv5*	This laboratory
K566	*HMLα MATα HMRa ilv5*	This laboratory
DC59	*HMLα matα2-1 HMRa sir1-1 ade6 arg4-17 his6 lys2*	This laboratory
IX44-11C	*HMLα mata1-1 HMRa sir1-1 ade6 leu2 cry1*	This laboratory
K96	*HMLa mata hmra sir2-1 (mar1-1) his4 leu2 lys1 met13 thr4 trp1*	This laboratory
PC10-7B	*HMLa mata hmra sir3-1 (mar2-1) ade8 leu2 met13 ura3*	This laboratory
DC40	*HMLα matα1-5 HMRa sir1-1 ade6 arg4-17 cry1-3 leu2 lys2 MAL*	This laboratory
YD108-8D	*HMLα MATα HMRa SAD sir1-1 ade6 arg4 his4 leu2 trp1 ura3*	Y. Kassir
SX26-15C	*HMLα mata1-1 HMRa his3-7 leu2-3 leu2-112 trp1 ura3-52*	This laboratory
DC6	*HMLα MATα HMRa his4 can1 leu2-3 leu2-112*	This laboratory
IX44-11C	*HMLα mata1-1 HMRa sir1-1 ade6 cry1 leu2*	This laboratory
K207	*HMLα MATa HMRa sir2-1 (mar1-1) can1 his3 leu2-3 leu2-112 trp1*	This laboratory
K154	*HMLα MATα HMRα sir2-1 (mar1-1) ade8-10 his4 ilv3 leu2 thr4 trp1-1 ura3*	This laboratory
K175	*HMLα MATα HMRa sir3-1 (mar2-1) his3 leu2-3 leu2-112*	This laboratory
K120	*HMLα MATα HMRα sir3-1 (mar2-1) his4 leu2 lys1 lys2*	This laboratory
250	*HMLα MATα HMRa sir4*(Ts) *ade1 leu2 trp1*	K. Nasmyth
IX16-17A	*HMLα MATα HMRa sir4-351*(Oc) *can1 his4 leu2-3 leu2-112 trp1 ura3*	This laboratory
ho27B	*HMLα mata1-1 HMRα ade8 his4 leu2*	This laboratory
XR160-12B	*HMLα mata HMRα ade2 can1 cyh1 leu1 rme ura3*	J. Rine
R7-8	XR160-12B *sir1-78*	J. Rine
R37-3	XR160-12B *sir2-373*	J. Rine
R33-7	XR160-12B *sir3-337*	J. Rine
R18-5	XR160-12B *sir4-185*	J. Rine
A364a	*MATa ade1 ade2 gal1-1 his7 lys2 tyr1 ura1*	L. Hartwell

TABLE 2. Nomenclature of *SIR* genes

Locus	Linkage group	References
SIR1	XI-R	Rine,[a] 22a, 42
SIR2 MAR1	IV-L	Rine,[a] 26
SIR3 MAR2 CMT STE8	XII-R	Rine,[a] 12, 14, 21, 22a, 28
SIR4 STE9	IV-R	Rine,[a] 14, 22a

[a] J. Rine, Ph.D. thesis, University of Oregon, Eugene, 1979.

Biochemical techniques. Commercially prepared restriction endonucleases, DNA polymerases, and ligase were used as recommended by the manufacturer. Restriction endonuclease digestions were analyzed by electrophoresis through 0.8% agarose and transferred to nitrocellulose by the method of Southern (47). ^{32}P-labeled probes were prepared by nick translation (41), and hybridization was done at 65°C for 12 to 24 h in 5× SCP (20× SCP is 2 M NaCl–0.6 M Na_2HPO_4 [pH 6.2]–0.02 M disodium EDTA)–0.8% Sarcosyl–0.1 mg of denatured salmon sperm DNA per ml. Nonspecific binding of probe to nitrocellulose was removed by two or more 20-min washes at 65°C in 2× SCP–0.5% sodium dodecyl sulfate.

Total RNA from yeast was prepared by the method of Carlson and Botstein (8). Poly(A)$^+$ RNA was selected by passing the RNA over oligo(dT)-cellulose (4). RNA was size fractionated by electrophoresis through 1.5% agarose (ME agarose; SeaKem) in the presence of 2.2 M formaldehyde (33). To determine the positions of 25S and 18S rRNAs, which were used as size markers, one extra lane of each gel was stained with ethidium bromide and visualized with UV light. Gels were blotted to a solid support (either nitrocellulose or GeneScreen [New England Nuclear Corp.]) and probed and washed in a manner identical to DNA blots. Strand-specific probes were prepared by the method of Hu and Messing (22), which involves extension of a 13-nucleotide M13 probe primer (Bethesda Research Laboratories, Inc.) and M13 phage single-stranded DNA as template. As a control for the presence of RNA in lanes which hybridized no strand-specific probes, the membrane was washed in 0.05× SCP–10 mM Tris (pH 7.5)–1 mM EDTA–0.1% sodium dodecyl sulfate at 65°C and reprobed with nick-translated YIp5 (52), which hybridizes to the *URA3* transcript.

RESULTS

***sir* mutations allow transcription of *HML* and *HMR*.** Several investigators have isolated *sir* mutations, for which the nomenclature is summarized in Table 2. Tight mutant alleles are known for *SIR2*, *SIR3*, and *SIR4*. In contrast, mutations in *SIR1*, including deletions (this paper), cause only partial expression of the silent cassettes. That transcription of *HML* and *HMR* occurs in Sir$^-$ strains is shown in Fig. 2. Poly(A)$^+$ RNA was prepared from a set of isogenic Sir$^-$ strains provided by Rine (Table 1) and probed for **a** and α transcripts. The parental strain XR160-12B (*HMLα mata1-1 HMRα*) mates as an **a** cell (defective *mata* cells mate as **a**) and expresses only the **a1** and **a2** transcripts. The *sir2*, *sir3*,

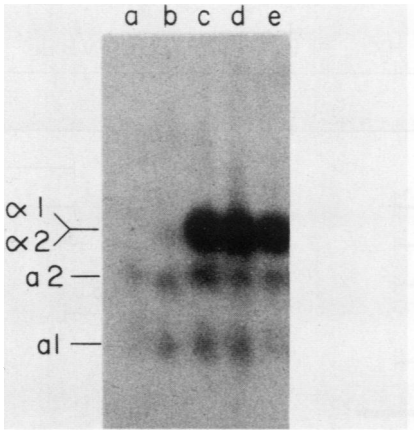


FIG. 2. Blot analysis of mating type transcripts. RNA was prepared from a set of isogenic *SIR* mutant strains. Lanes: a, XR160-12B, *SIR*; b, R7-8, *sir1*; c, R37-3, *sir2*; d, R18-5, *sir4*; e, R33-7, *sir3*. Complete genotypes are given in Table 1. The probe used was p103.1 (Fig. 1) which hybridizes to the **a***1*, **a***2*, α*1*, and α*2* transcripts; the latter two RNAs, however, did not separate on this gel.

and *sir4* mutants express high levels of the α transcripts in addition to the **a***1* and **a***2* transcripts, and because α is dominant to mutant **a** information, these strains mate as α. The *sir1* mutant expresses levels of α transcripts intermediate between those of the parental strain and the other Sir$^-$ strains. Additionally, the *sir1* mutant mates with both **a** and α mating type tester cells. Such bi-mating ability presumably is due to leaky expression of α information, such that only a fraction of the cells express the α phenotype. The leakiness of this *sir1* mutation is similar to that of the original UV-induced *sir1-1* mutation (42).

Strategy for identification of *SIR* gene clones. *SIR* gene clones were isolated from an *S. cerevisiae* gene bank by their ability to complement *sir* mutations in vivo. The bank, constructed by Nasmyth and Tatchell (39), contains partial *Sau*3A genomic fragments inserted at the unique *Bam*HI site of the vector YEp13 (7). This vector contains pBR322 sequences and yeast 2μm sequences, which enable it to replicate in *E. coli* and *S. cerevisiae*, and the yeast *LEU2* gene, which is expressed in both *E. coli* and *S. cerevisiae*. We transformed *sir leu2* yeast strains with the bank and screened *LEU2* transformants for a change in mating ability. Plasmids from those transformants which regained their original mating behavior upon loss of the plasmid were retained for further study. To confirm the identity of each cloned fragment with its corresponding genetic locus, subclones were integrated into the genome by homologous recombination (19). The sites of integration were then meiotically mapped relative to the appropriate *sir* mutations.

Identification of *SIR1*. Plasmids able to complement *sir1-1* were identified in strain DC40 (*HML*α *mat*α*1-5 HMR***a** *sir1-1 leu2-3 leu2-112*). The defective *mat*α*1-5* allele alone confers a nonmating phenotype, but *sir1-1* in combination with *HML*α suppresses the mating defect to yield an α phenotype (42). Since complementation of *sir1-1* should make DC40 a nonmater, we screened *LEU2* transformants for nonmaters and found nine examples.

It was important to distinguish between sequences that actually complemented *sir1-1* and those that merely conferred a nonmating phenotype. It was not possible to assay the plasmids in *MAT***a** *sir1-1* or *MAT*α *sir1-1* strains, because these strains mate normally. We therefore rescreened these plasmids in the yeast strain YD108-8D (*HML*α *MAT*α *SAD HMR***a** *sir1-1*). Kassir et al. (23) and Hicks et al. (18) have shown that *SAD*, a fusion mating type cassette composed of the left half of *HMR* and the right half of *MAT*, is associated with direct tandem duplication of the sequences from *MAT* through *HMR* and contains **a** mating type sequences (Fig. 1B). The level of expression of **a***1* at *SAD* is insufficient to prevent a *MAT*α strain from mating as an α. In the absence of *SIR1* function, however, **a***1* at *SAD* is expressed at a level sufficient to make a *MAT*α strain a nonmater. Eight of the nine putative *SIR1* plasmids caused YD108-8D to mate efficiently with the *MAT***a** tester strain, indicating that these eight complement *sir1-1*. Restriction enzyme digests of these eight plasmids showed that they are identical.

A restriction map of the *SIR1* region is presented in Fig. 3A. To localize *SIR1* function within this region, we subcloned fragments and tested them for *SIR1* activity in vivo. Initial subcloning used the several *Hin*dIII sites of the plasmid pJH570 (Fig. 3A). This plasmid was digested with *Hin*dIII, the mixture of restriction fragments was ligated, and yeast strain DC40 was transformed with the ligation mixture. Digestion by *Hin*dIII does not destroy the vector (YEp13) and leaves 1.6 kilobases (kb) of yeast sequence from the right end of the insert attached to the vector. Plasmid pJI14.61 (Fig. 3A), which lacked *SIR1* activity, contained only those yeast sequences. Another, pJI14.71 (Fig. 3A), which possessed *SIR1* activity, had, in addition, the 2.6-kb *Hin*dIII fragment. This *Hin*dIII fragment alone in the construction pJI20.1 (Fig. 3A) had *SIR1* activity when tested in strain YD108-8D, thus confirming that *SIR1* activity is encoded by the 2.6-kb *Hin*dIII fragment. The left half of the *Hin*dIII fragment alone (pJI15.1; Fig. 3A) was insufficient to provide *SIR1* activity.

Attempts to map the *sir1-1*-complementing cloned fragment to the *SIR1* genetic locus by the technique used to map *SIR2*, *SIR3*, and *SIR4* (see below) were unsuccessful. Therefore, we mapped a genetically marked null mutation relative to *sir1-1*. Plasmid pJI6 (the original insert of pJH570 transferred to pBR322) was opened with *Bam*HI, and into this site was inserted a 3-kb *Bgl*II *LEU2* fragment (construction pJI21.4; Fig. 3A). With this construction, the genomic *SIR1* locus of two strains, DC5/DC6 (*MAT***a**/*MAT*α *leu2*/*leu2*) and SX26-15C (*HML*α *mat***a***1-1 HMR***a** *leu2*), was replaced (see Materials and Methods). Two stable Leu$^+$ DC5/DC6 transformants, when sporulated and dissected, segregated the leucine requirement $2^+:2^-$. Although the mating ability of the Leu$^+$ segregants was unaffected, complementation tests indicated that all Leu$^+$ segregants of four complete tetrads carried the *sir1* mutation. The one stable Leu$^+$ SX26-15C transformant obtained exhibited bi-mating ability and was capable of sporulation. The ability to grow on medium lacking leucine segregated $4^+:0^-$ in six complete tetrads, and all meiotic segregants were bi-maters. This mating behavior is the same as that which we have observed for a *mat***a***1-1 sir1-1* strain (J. Ivy, unpublished data) and results in mating frequently enough that zygotes may be observed within colonies. The bi-mating behavior of *mat***a***1-1 sir1*::*LEU2-21.4* accounts for the sporulation ability and 4Leu$^+$:0Leu$^-$ meiotic segregation of the original transformant if mating occurred within the transformant colony. Complementation tests indicated that all four meiotic segregants of one tetrad were defective for *sir1* function. Other crosses indicated that *sir1*::*LEU2-21.4* is recessive. Analysis of genomic DNA by blot hybridization (Fig. 4A) indicated the presence of the *sir1*

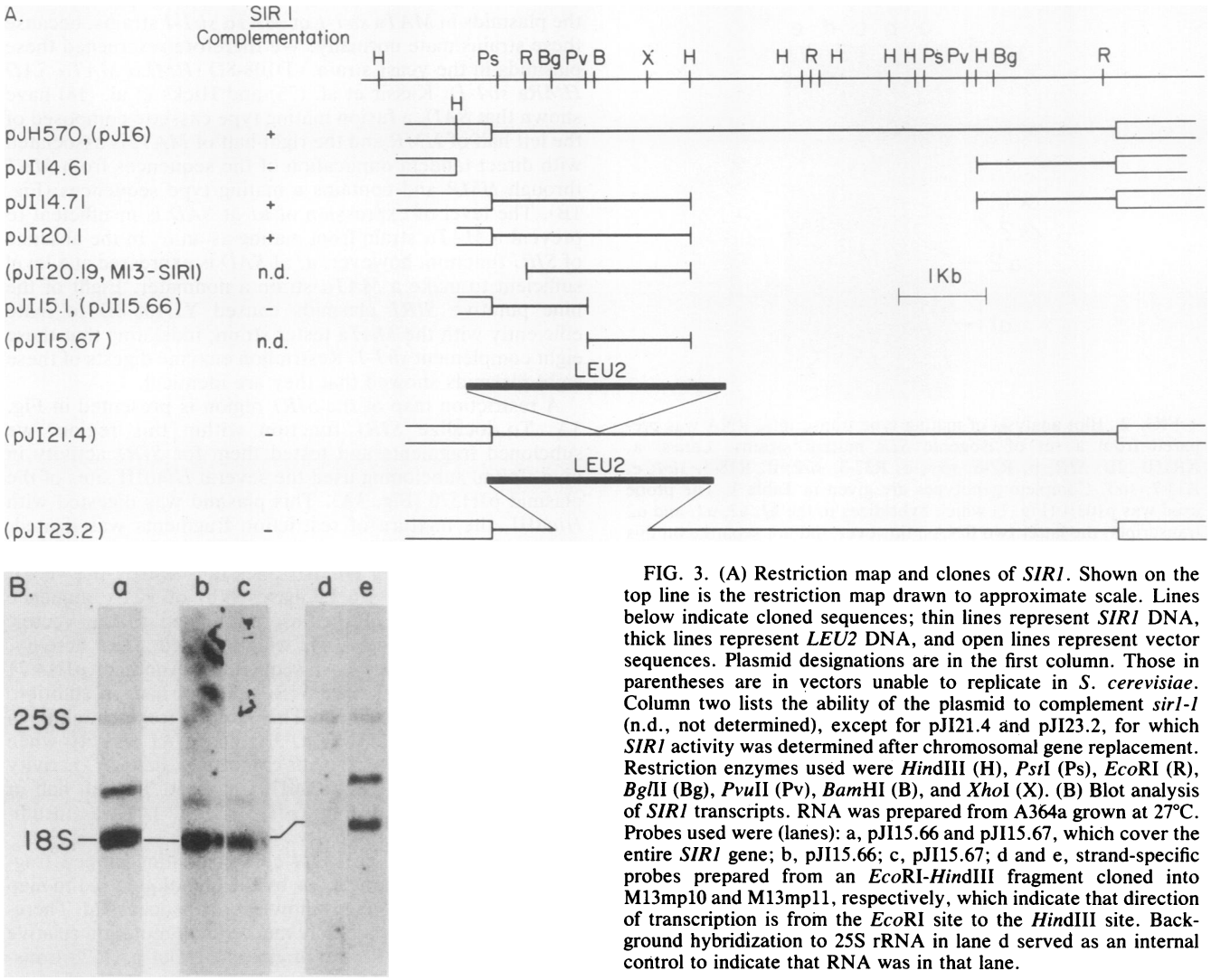


FIG. 3. (A) Restriction map and clones of *SIR1*. Shown on the top line is the restriction map drawn to approximate scale. Lines below indicate cloned sequences; thin lines represent *SIR1* DNA, thick lines represent *LEU2* DNA, and open lines represent vector sequences. Plasmid designations are in the first column. Those in parentheses are in vectors unable to replicate in *S. cerevisiae*. Column two lists the ability of the plasmid to complement *sir1-1* (n.d., not determined), except for pJI21.4 and pJI23.2, for which *SIR1* activity was determined after chromosomal gene replacement. Restriction enzymes used were *Hin*dIII (H), *Pst*I (Ps), *Eco*RI (R), *Bgl*II (Bg), *Pvu*II (Pv), *Bam*HI (B), and *Xho*I (X). (B) Blot analysis of *SIR1* transcripts. RNA was prepared from A364a grown at 27°C. Probes used were (lanes): a, pJI15.66 and pJI15.67, which cover the entire *SIR1* gene; b, pJI15.66; c, pJI15.67; d and e, strand-specific probes prepared from an *Eco*RI-*Hin*dIII fragment cloned into M13mp10 and M13mp11, respectively, which indicate that direction of transcription is from the *Eco*RI site to the *Hin*dIII site. Background hybridization to 25S rRNA in lane d served as an internal control to indicate that RNA was in that lane.

disruption only in segregants with the Leu$^+$ Sir1$^-$ phenotype.

That the gene disruption is within *SIR1* or a tightly linked locus was demonstrated by crossing SX26-15C (*mat***a***1-1 sir1*::*LEU2*) to IX44-11C (*mat***a***1-1 sir1-1 leu2*). Mating type determination of ascosporal colonies derived from this diploid showed that all members of 26 complete tetrads were bi-maters (Fig. 4B). This is the result expected if there was no recombination between *sir1-1* and *sir1*::*LEU2-21.4*.

The transcripts encoded by *SIR1* were determined by blot hybridization of poly(A)$^+$ RNA. Two contiguous subclones from the region (pJI15.66 and pJI15.67; Fig. 3A) both hybridized to two transcripts of approximate size 1.5 and 2.0 kb (Fig. 3B). Strand-specific probes were prepared from the 1.9-kb *Hin*dIII-*Eco*RI fragment cloned in the M13 phage vectors M13mp10 and M13mp11 (Fig. 3A). The M13mp11 probe was homologous to both transcripts, while the M13mp10 probe was homologous to neither (Fig. 3B). Thus, both transcripts encoded by *SIR1* cross the unique *Pvu*II site (Fig. 3A), and both are transcribed in the direction from the *Eco*RI site to the *Hin*dIII site.

Identification of *SIR2*. Eight plasmids capable of complementing a *sir2* mutation were identified in strain K207 [*HML*α *MAT***a** *HMR***a** *sir2-1* (*mar1-1*)] by their ability to transform K207 from a nonmater to an **a**. Comparison of these eight by restriction endonuclease digestion indicated that they represent four different constructions and that they share a 4.5-kb *Hin*dIII fragment. A subclone of this *Hin*dIII fragment (pJH81.13; Fig. 5A) retained the ability to complement *sir2-1*, while a 450-bp *Bam*HI deletion internal to this fragment (pJH84.3; Fig. 5A) destroyed *SIR2* activity. Three additional subclones, the *Hin*dIII-*Bam*HI, the *Bam*HI, and the *Bam*HI-*Hin*dIII fragments (pSIR2L, pSIR2M, and pSIR2R; Fig. 5A), all failed to complement *sir2-1*. Thus, *sir2*-complementing activity crosses at least one, if not both, of the *Bam*HI sites contained within the *Hin*dIII fragment of pJH81.13.

To establish that the cloned sequence contains *SIR2*, we examined the meiotic segregation of a chromosomally integrated plasmid relative to *sir2-1*. For this purpose, a fragment derived from pJH16 (one of the eight YEp13-*SIR2* plasmids) was subcloned into pBR322, to which the *Sal*I-*Xho*I yeast *LEU2* gene fragment was added at the pBR322 *Sal*I site (pLSA3.2; Fig. 5A). The *SIR* wild-type strain DC5 (*MAT***a** *leu2-3 leu2-112*) was transformed with pLSA3.2 which had been digested with *Bam*HI to direct integration

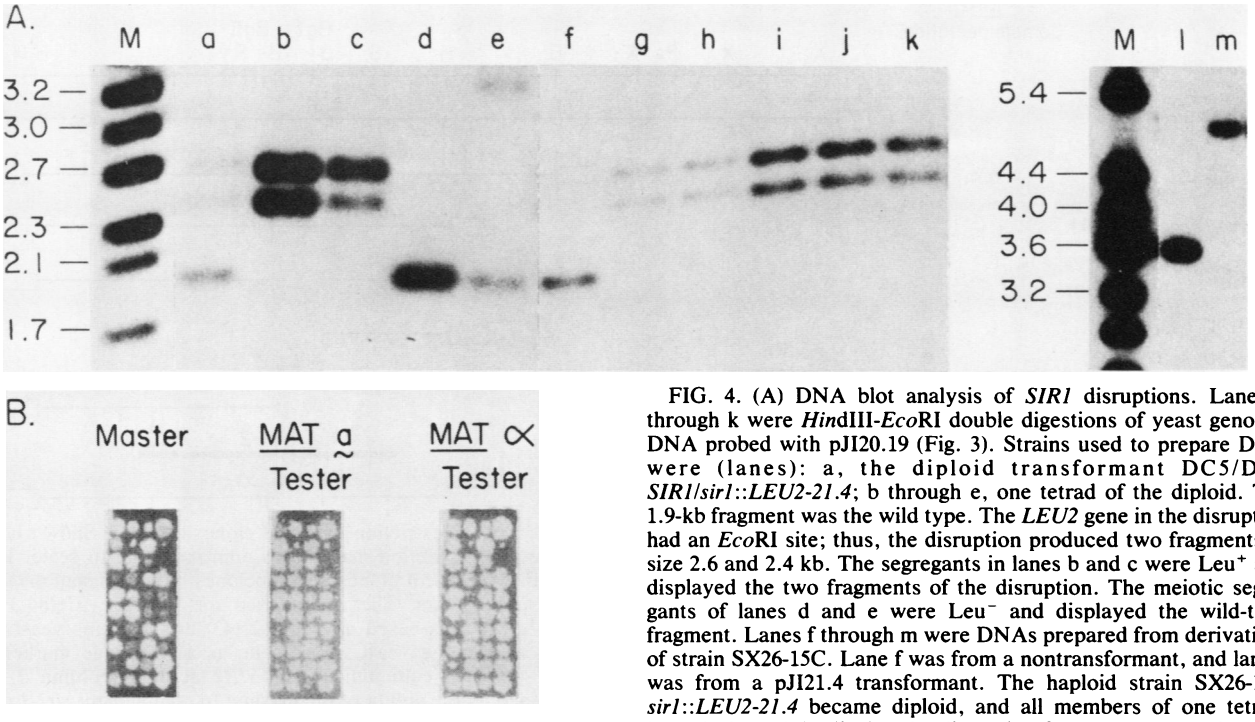


FIG. 4. (A) DNA blot analysis of *SIR1* disruptions. Lanes a through k were *Hin*dIII-*Eco*RI double digestions of yeast genomic DNA probed with pJI20.19 (Fig. 3). Strains used to prepare DNA were (lanes): a, the diploid transformant DC5/DC6 *SIR1/sir1::LEU2-21.4*; b through e, one tetrad of the diploid. The 1.9-kb fragment was the wild type. The *LEU2* gene in the disruption had an *Eco*RI site; thus, the disruption produced two fragments of size 2.6 and 2.4 kb. The segregants in lanes b and c were Leu^+ and displayed the two fragments of the disruption. The meiotic segregants of lanes d and e were Leu^- and displayed the wild-type fragment. Lanes f through m were DNAs prepared from derivatives of strain SX26-15C. Lane f was from a nontransformant, and lane g was from a pJI21.4 transformant. The haploid strain SX26-15C *sir1::LEU2-21.4* became diploid, and all members of one tetrad, lanes h through k, display the disruption fragments. Lanes l and m were *Hin*dIII digestions probed with pJI20.19. Lane l was from the wild type, and lane m was from a transformant containing the *sir1::LEU2-23.2* disruption. The wild-type fragment was missing and was replaced by a fragment of size 5.0 kb. Markers were in lanes labeled M. Fragment sizes in kilobases are indicated in the margins. (B) Mating type tests for meiotic segregants of the diploid SX26-15C/IX44-11C *mata1-1/mata1-1 sir1::LEU2-21.4/sir1-1*. Shown are 10 tetrads, all of which were complete except for the third from the top. The faint appearance of some colonies on the master was due to their failure to regrow as much as others after replica plating. Any segregant that showed a weak ability to mate with one tester (e.g., the spore colony in the fifth row, second column mated weakly with the *MAT*α tester) was retested and shown to possess mating ability.

within sequences homologous to the insert (40). Crosses demonstrated that the plasmid had integrated at a single site. The integrated plasmid, which generates a duplication separated by vector sequences including *LEU2*, will be designated *SIR2::LEU2*.

DC5 *SIR2::LEU2* was then crossed to K154 (*HML*α *MAT*α *HMR*α *sir2-1 leu2*), and the diploid was sporulated and dissected. Since *HML*α *MAT*α *HMR*α *sir2-1* segregants cannot be scored for *sir2-1*, we determined the *SIR2* genotype of all α segregants by complementation tests (see Materials and Methods). If pLSA3.2 had integrated at *SIR2*, then only parental ditypes for the markers *LEU2* and *SIR2* (*LEU2 SIR2* and *leu2 sir2-1*) were expected. The DC5/K154 *SIR2::LEU2/sir2-1* diploid gave 17 parental ditypes, no nonparental ditypes, and no tetratypes, indicating that pJH16 contains *SIR2*.

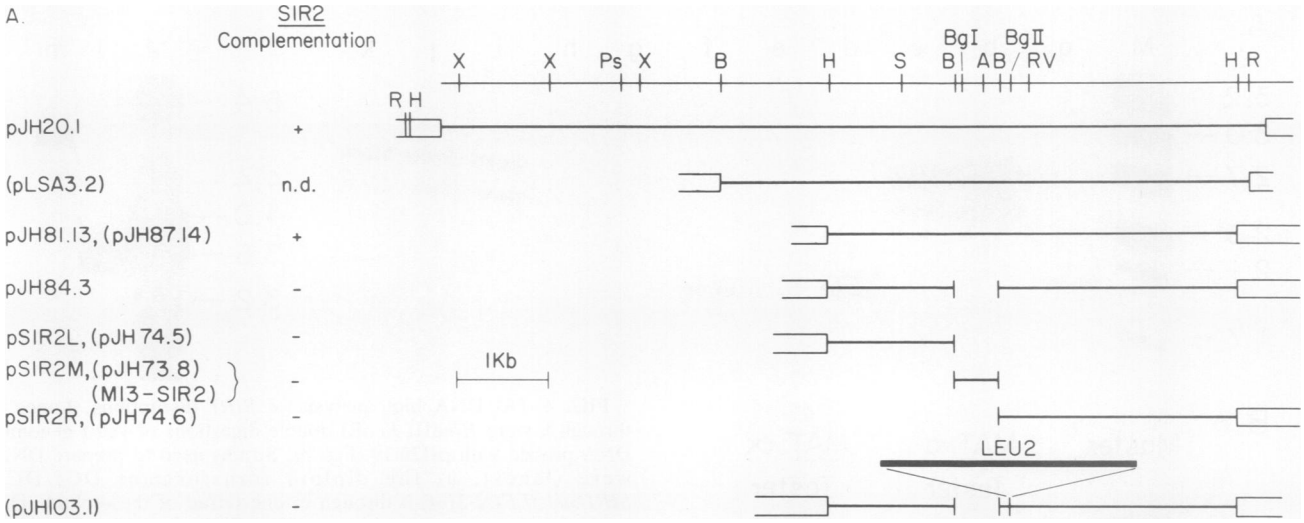
The transcript corresponding to *SIR2* was identified with subclones of the *SIR2* *Hin*dIII fragment as probes for blots of poly(A)$^+$ RNA. Subclones pJH74.5, pJH73.8, and pJH74.6 (Fig. 5A) hybridized to one transcript in common (Fig. 5B). Since deletion of the *Bam*HI fragment from the plasmid sequence (pJH84.3) destroys *SIR2* activity, we conclude that this 1.8-kb transcript corresponds to *SIR2*.

Direction of *SIR2* transcription was determined with strand-specific probes prepared from the 450-bp *Bam*HI fragment cloned in M13mp11 in both orientations. Use of the probes (Fig. 5B) indicated that *SIR2* is transcribed in the direction of the *Bgl*I site to the *Bgl*II site (Fig. 5A).

Identification of *SIR3*. Plasmid pKAN63, identified by its ability to complement the temperature-sensitive *sir3*(Ts) (*ste8*) mutation, was provided by K. Nasmyth; at 36°C, a nonmating *sir3*(Ts) strain became a mater after transformation with pKAN63. We subsequently showed that this plasmid also complements *sir3-1* (*mar2-1*) in strain K175 (*HML*α *MAT*α *HMR***a**). Fragments encoding *SIR3* were determined by subcloning restriction enzyme fragments into YEp13 and assaying for *SIR3* activity by transformation of strain K175. We found that two *Hin*dIII fragments together (pSIR3L+R; Fig. 6A) provided *SIR3* function, whereas neither fragment did alone (pSIR3L and pSIR3R; Fig. 6A).

The correspondence of pKAN63 to *SIR3* was demonstrated in a manner analogous to that used for *SIR2*. A derivative of pKAN63, pLSA4.3 (Fig. 6A), was integrated into the genome of strain DC5 (*MAT***a** *leu2-3 leu2-112*). The strain DC5 *SIR3::LEU2* was crossed to K120 (*HML*α *MAT*α *HMR*α *sir3-1 leu2*), and the diploid was sporulated and dissected. The *SIR3* genotypes of all α-mating spore colonies were determined by complementation tests to detect *HML*α *MAT*α *HMR*α *sir3-1* segregants (see Materials and Methods). For the markers *LEU2* and *SIR3*, 18 parental ditypes, no nonparental ditypes, and 1 tetratype were obtained. Because the subcloned fragment used in this mapping study does not include *SIR3*, integration of the plasmid created a duplication of sequences flanking *SIR3*. An exchange between homologs involving the member of the duplication proximal to *SIR3* should produce the observed tetratype. We conclude, therefore, that pKAN63 does contain *SIR3*.

Identification of the *SIR3* transcript was based upon the requirement for both *Hin*dIII fragments to supply *SIR3* activity and the observation that the two individual *Hin*dIII fragments (pJH72.3 and pJH72.6; Fig. 6A) hybridized to a



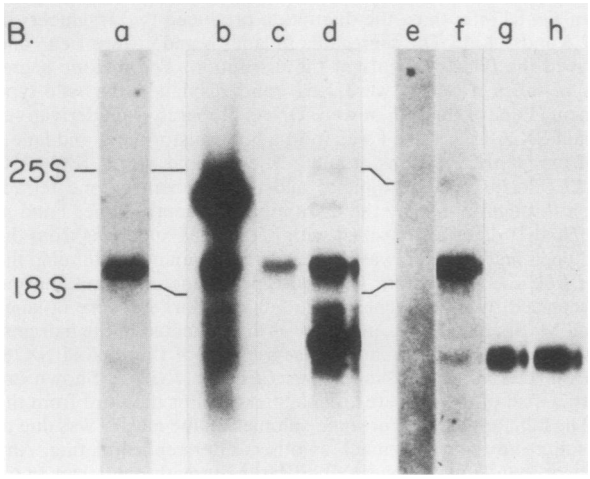


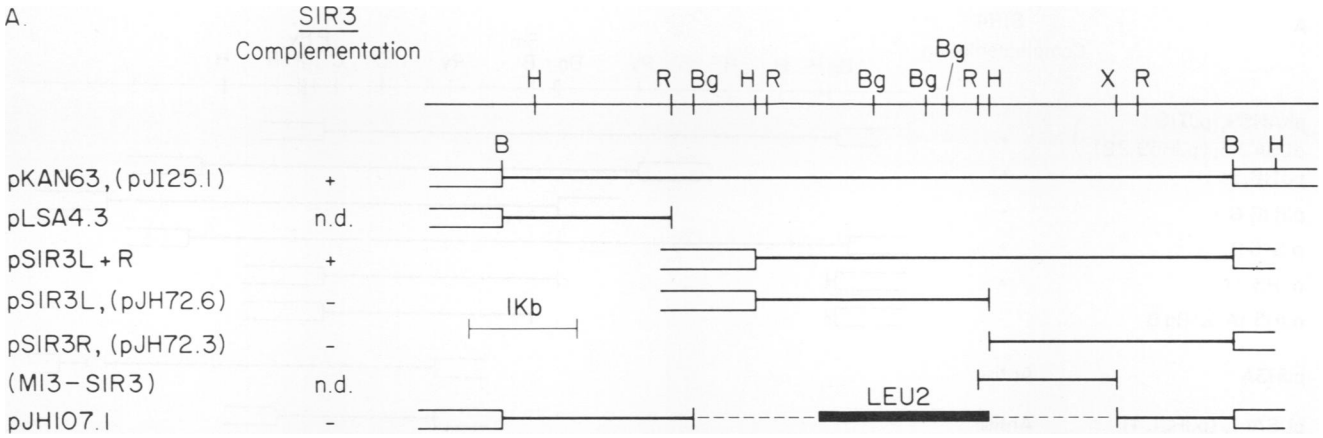
FIG. 5. (A) Restriction map and clones of *SIR2*. Shown on the top line is a restriction map drawn approximately to scale. Lines below that represent clones and subclones with designations given in the first column. The vector used for subclone pJH84.3 was pDB262, which is based on pTR262 (43) and contains yeast 2μm sequences and the yeast *LEU2* gene as a selectable marker (D. Beach, personal communication). *SIR2* activity (column 2) was determined by the ability of the plasmid to complement *sir2-1* or, in the case of pJH103.1, by the mating phenotype of a strain after gene replacement. Conventions are the same as in Fig. 3, with the addition of *Sph*I (S), *Bgl*I (BgI), *Ava*I (A), *Bgl*II (BgII), and *Eco*RV (RV). (Note: pJH103.1 is not the same as p103.1 in Fig. 1.) (B) Blot analysis of *SIR2* transcripts. RNA was prepared from A364a grown at 27°C. Lane a was probed with pJH73.8. Lanes b, c, and d were from the same gel and were probed with pJH74.5, pJH73.8, and pJH74.6, respectively. Lanes e and f were probed with strand-specific probe prepared from the *Bam*HI fragment cloned into M13mp11 in both directions (see the text) and establish the direction of transcription as being from the *Bgl*I site to the *Bgl*II site. Lanes g and h were lanes e and f washed and reprobed with YIp5.

single transcript of approximate size 3 kb (Fig. 6B). This RNA is transcribed in the direction from the *Hin*dIII site to the *Xho*I site as determined with strand-specific probes prepared from subclones in the phage M13. The *Eco*RI to *Xho*I fragment (Fig. 6A) in vector M13mp10, but not in vector M13mp11, hybridized to the 3-kb transcript (Fig. 6B).

Identification of *SIR4*. Plasmid pKAN59, identified by its ability to complement the temperature-sensitive *sir4*(Ts) (*ste9*) mutation at high temperature, was also provided by K. Nasmyth. Subsequently, we demonstrated the ability of pKAN59 to complement *sir4-351*(Oc) (the ochre-suppressible allele *sir35-1*; Rine, Ph.D. thesis) as well. This mutation of *SIR4* was originally assigned to the *SIR1* complementation group, owing to lack of complementation between *sir1-1* and *sir4-351*(Oc) (Rine, Ph.D. thesis). That *sir4*(Oc) is not an allele of *SIR1* was suggested originally by the inability of the cloned *SIR1* gene to complement this mutation. We found complementation between *sir4*(Oc) and *sir1-1* and failure of complementation between *sir4*(Ts) and *sir4*(Oc). Furthermore, pLSA2.3, a derivative of pKAN59 (Fig. 7A), genetically maps to the locus defined by both *sir4*(Ts) and *sir4*(Oc) (data below).

To map the chromosomal location of the cloned sequence, pLSA2.3 (Fig. 7A), constructed in a manner analogous to pLSA3.2 (*SIR2*) and pLSA4.3 (*SIR3*), was integrated by homologous recombination in yeast strain DC5 (*MAT***a** *leu2-3 leu2-112*). Both strains 250 (*HMLα MATα HMR***a** *sir4*(Ts) *leu2*) and IX16-17A (*HMLα MATα HMR***a** *sir4*(Oc) *leu2*) were crossed to DC5 *SIR4*::*LEU2*. Strain 250 was crossed at 30°C, at which it is capable of mating, and meiotic segregants were assayed for mating at 36°C. To cross IX16-17A, we used a pKAN59 transformant which is mating proficient, and the resultant diploid was grown nonselectively to allow loss of pKAN59. For the markers *LEU2* and *SIR4*, DC5/250 *SIR4*::*LEU2*/*sir4*(Ts) gave 8 parental ditypes in 8 tetrads, and DC5/IX16-17A *SIR4*::*LEU2*/*sir4*(Oc) gave 42 parental ditypes in 42 tetrads. Thus, pKAN59 does contain *SIR4*.

Additional *SIR4*-containing plasmids were obtained by screening the YEp13 gene bank (39) for sequences able to complement *sir4*(Oc) in vivo. Among the 13 plasmids obtained from this screen were 3 distinct plasmids which contained overlapping fragments that did not correspond to any of the *SIR* genes. These are still under investigation and will be reported elsewhere. The other 10 *sir4*(Oc)-complementing plasmids represent at least four different constructions. When representatives of these four classes were restriction enzyme mapped in detail, we found that pJI16.1 is identical to pKAN59 and that pJI16.3, pJI16.6, and pJI16.9 define an overlap with pKAN59 of approximately 2.6 kb, which must encode *sir4*-complementing activity (Fig. 7A). Two deletions made in pLSA2.3 are in agreement with



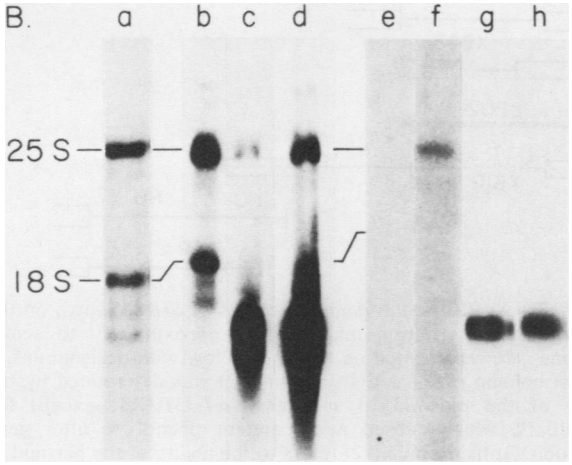


FIG. 6. (A) Restriction map and clones of *SIR3*. The top line is a restriction map drawn approximately to scale. Lines below represent clones and subclones with designations in the first column. *SIR3* activity (column 2) was assayed by the ability of the plasmid to suppress *sir3-1* or, in the case of pJH107.1, by mating ability after gene replacement. pLSA4.3 was able to replicate in *S. cerevisiae*, indicating that the insert fragment contained an *ars*. Conventions are the same as in Fig. 3. (B) Blot analysis of *SIR3* transcripts. RNA was prepared from A364a grown at 27°C. Lane a (from the same gel as lanes a in Fig. 3 and 5) was probed with pJH72.6. Lanes b and c were from the same gel and were probed with pJH72.6 and pJH72.3, respectively. Lane d was a longer exposure of lane c. Lanes e and f were probed with strand-specific probes prepared from the *Eco*RI-*Xho*I fragment cloned into M13mp11 and M13mp10, respectively, which establish the direction of transcription as being from the *Hin*dIII site to the *Xho*I site. Lanes g and h represent lanes e and f washed and reprobed with YIp5.

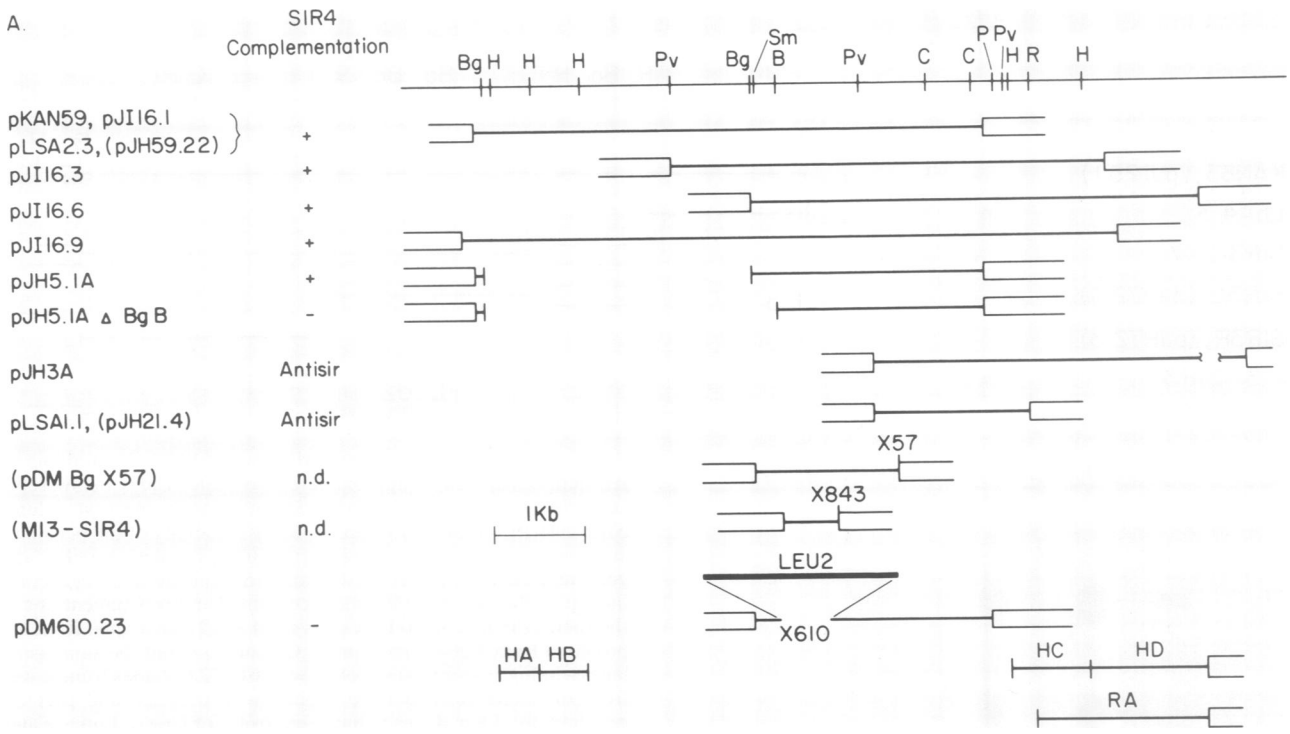
this; pJH5.1A (Fig. 7A), lacking a *Bgl*II fragment, retained *SIR4* activity, whereas pJH5.1AΔBgB (Fig. 7A), containing a *Bgl*II to *Bam*HI deletion, did not. Therefore, cloned fragments beginning at the *Bgl*II site defined by the left end of the pJI16.6 insert and extending rightward are sufficient to provide *SIR4* activity.

Use of pJH59.22 (the insert of pKAN59 transferred to pBR322; Fig. 7A) to probe RNA blots identified three transcripts of approximate size 0.8, 2.1, and 4.4 kb (Fig. 7B) that are encoded within or overlap this cloned fragment. Gel-purified fragments used as probes mapped the 0.8-kb transcript to the left end of the map and the 2.1-kb transcript to the right end of the map. The *Hin*dIII fragment, HA (Fig. 7A), hybridized to the 800-nucleotide transcript exclusively (C. Stephens, personal communication; M. Marshall, personal communication), while the two *Hin*dIII fragments, HC and HD, and the one *Eco*RI fragment, RA (Fig. 7A), hybridized to the 2.1-kb transcript (data not shown). A probe (pDMBgX57; Fig. 7A) internal to the region identified as encoding *SIR4* activity hybridized only to the 4.4-kb transcript (Fig. 7B). One *Hin*dIII fragment, HB (Fig. 7A), also hybridized to the 4.4-kb transcript (M. Marshall, personal communication). Direction of transcription of the *SIR4* RNA was determined with the *Bam*HI to *Xho*I linker no. 843 (J. R. Broach and J. B. Hicks, unpublished data; Fig. 7A) fragment subcloned into M13mp11 and M13mp18. Only strand-specific probe prepared from the M13mp11 subclone was homologous to the 4.4-kb RNA (Fig. 7B), indicating that transcription occurs in the direction of the *Bam*HI site to the *Xho*I site.

DNA blot analysis of the *SIR* genes. Analysis of DNA blots (Fig. 4A; see Fig. 11) demonstrated that genomic restriction fragments correspond in size to those contained in the cloned sequences, thus indicating that no rearrangement of sequences had occurred during cloning. Furthermore, none of the *SIR* gene probes showed any evidence of cross-homology with other *SIR* gene fragments under the conditions used. Finally, all of the clones, with the exception of *SIR3*, appear to contain unique sequences. When DNA blots were washed at a stringency lower than normal, hybridization of the *SIR3* probe to several additional restriction fragments was apparent. These fragments do not correspond to other *SIR* gene fragments and indicate that the cloned *SIR3* gene contains repeated elements. One such band of approximate size 6.9 kb can still be seen in Fig. 11, lane a, even though this gel was washed at normal stringency.

Complementation patterns of cloned *SIR* genes. The cloned *SIR* genes were tested for complementation of mutations in the other *SIR* loci. Plasmids pJH570 (*SIR1*), pJH16 (*SIR2*), pKAN63 (*SIR3*), pKAN59 (*SIR4*), or their equivalents were introduced into yeast strains YD108-8D (*sir1-1*), K207 (*sir2-1*), K175 (*sir3-1*), and IX16-17A (*sir4-351*(Oc)), and the transformants were tested for mating ability. Qualitative mating type tests are shown in Fig. 8, and the data are summarized in Table 3. The most striking result of this test is that pKAN63 suppressed the *sir4*(Oc) mutation, although not as well as did pKAN59. pKAN63 also suppressed *sir4*(Ts), indicating that the suppression is not allele specific.

One other, although less dramatic, example of suppression



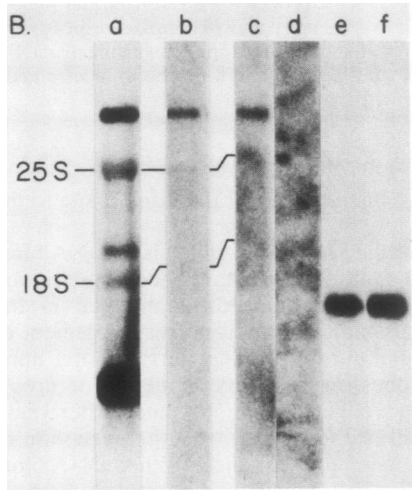


FIG. 7. (A) Restriction map and clones of *SIR4*. Shown on the top line is a restriction map drawn approximately to scale. Subclones are represented on the lines below with designations in the first column. *SIR4* activity (column 2) was determined by the ability of the plasmid to suppress *sir4-351*(Oc), except for pDM610.23, which caused a Sir mutant phenotype after gene disruption. Antisir (column 2) refers to the ability of the plasmid to disrupt Sir regulation. pLSA1.1, derived from pJH21.4 by the addition of the *Sal*I-*Xho*I *LEU2* fragment to the pBR322 *Sal*I site, was the smallest subclone with Antisir activity. pLSA2.3 and pLSA1.1 were capable of replicating in *S. cerevisiae*, indicating that the inserts contained *ars* activity. Conventions are the same as in Fig. 3, with the addition of *Sma*I (Sm), PstI (P), and *Cla*I (C). The labels HA, HB, HC, HD, and RA represent gel-purified restriction enzyme fragments used as probes (see the text).Three *Xho*I linker mutations (J. R. Broach and J. B. Hicks, unpublished data) used are labeled X57, X843, and X610. (B) Blot analysis of *SIR4* transcripts. RNA was prepared from A364a grown at 37°C. The probes used were (lanes): a, pJH59.22, which hybridized to three transcripts; b, pDMBgX57, which contained a fragment internal to the overlap defined by clones possessing *SIR4* activity; c and d, strand-specific probes prepared from the *Bam*HI-*Xho*I no. 843 fragment cloned into M13mp11 and M13mp18, respectively. Hybridization by only the former indicated that *SIR4* was transcribed from the *Bam*HI site to the *Xho*I no. 843 site. Lanes e and f represent lanes c and d washed and reprobed with YIp5.

was the ability of the transformant YD108-8D (*sir1-1*) pKAN59 (*SIR4*) to mate weakly as an α (Fig. 8). Because the nonmating phenotype of YD108-8D requires both the *sir1-1* mutation and the hybrid mating type *SAD* locus (described above), high-copy, *SIR4*-directed loss of *SAD* might be one mechanism to account for this example of suppression.

We also tested the multi-copy *SIR* gene clones in the wild-type *SIR* strain ho27B (*HML*α *mat*a*1-1 HMR*α *SIR*) to determine whether possible *SIR* gene overexpression might adversely affect the regulation of *HML* and *HMR*. Expression of the silent cassettes changes the mating ability of ho27B from **a** to α. Qualitative mating tests on petri plates (Fig. 8) showed that pKAN59 causes ho27B to display bi-mating ability. Such partial expression of the *HM* loci was observed for transformants containing other *SIR4* plasmids, namely, pJI16.3 and pJI16.9 but not pJI16.6. None of the other *SIR* plasmids, however, produced an observable effect on the mating ability of ho27B.

Disruption of Sir regulation associated with a truncated *SIR4* gene. Another unexpected finding was the ability of a DNA segment consisting of the 3′ end of the functionally defined *SIR4* gene, when carried by a plasmid, to cause complete loss of Sir control. Two identical plasmids, pJH3A and pJH15A, were found to convert strain ho27B (*HML*α *mat*a*1-1 HMR*α *SIR*) from the **a** mating type to α (Fig. 8). Comparison of restriction maps and DNA blotting data (not shown) indicated that the inserts in these plasmids partially overlap the functional *SIR4* DNA sequences (Fig. 7A).

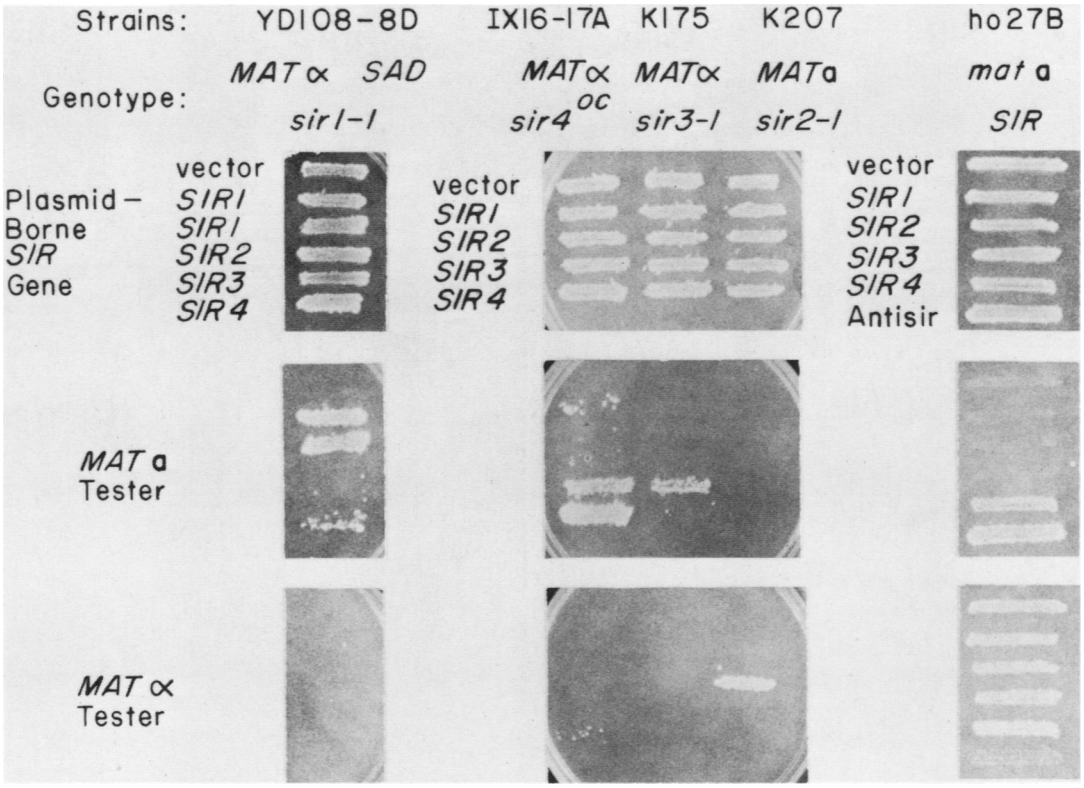


FIG. 8. Mating type tests of transformants. Listed across the top are strains used and their partial genotypes (see Table 1 for complete genotypes). Except for strain ho27B, which has the *HMLα* and *HMRα* alleles, all the strains have the normal *HMLα* and *HMRa* alleles. The *SAD* locus contains **a** mating type information. The upper row of photographs shows growth of the transformants on synthetic medium lacking leucine. The plasmid-borne *SIR* gene contained by the transformants is indicated in the margins. The vector YEp13 served as a negative control. Mating type tests (see Materials and Methods) used *LEU2* testers for Sir$^-$ strains and *leu2* testers for ho27B.

Progressive deletion from the right side of the insert indicated that the anti-Sir activity of pJH3A resided on the DNA segment that shared homology with the *SIR4* gene. The smallest active subclone obtained, pLSA1.1 (Fig. 7A), overlaps the last 400 bp of the *SIR4* transcription unit as well as the 3′ 300 to 400 bp of the neighboring 2.1-kb transcript. We presume that the anti-Sir activity is a property of the truncated *SIR4* gene rather than the unidentified 2.1-kb transcript, because deletions cutting various distances into the 2.1-kb transcript had no effect on anti-Sir activity. This suggests the possibility that the 3′ end of the *SIR4* gene is expressed from an unidentified YEp13 promoter and that the truncated gene product interferes with normal *SIR4* function.

Lack of transcriptional regulation among *SIR* genes. One possibility to account for the suppression of *sir4* mutations by the cloned *SIR3* gene is that *SIR3* transcription normally is *SIR4* dependent but that the cloned *SIR3* gene is transcribed independently of *SIR4* expression. To test this possibility, we examined the level of *SIR3* transcript in a *sir4* mutant strain. Poly(A)$^+$ RNA was prepared from a set of isogenic Sir$^-$ strains provided by Rine (Table 1), size fractionated on an agarose gel, and blotted to nitrocellulose. Hybridization with probes for each *SIR* gene showed each

TABLE 3. Mating types of transformants

Strain	Genotype[a]	Plasmid-borne *SIR* gene					
		Vector	*SIR1*	*SIR2*	*SIR3*	*SIR4*	Anti-Sir
YD108-8D	α α **a** *SADa sir1-1*	Nm[b]	α	Nm	Nm	Nm	Nm
K207	α **a a** *sir2-1* (*mar1-1*)	Nm	Nm	**a**	Nm	Nm	Nm
K175	α α **a** *sir3-1* (*mar2-1*)	Nm	Nm	Nm	α	Nm	Nm
250[c]	α α **a** *sir4*(Ts)	Nm	Nm	Nm	weak α	α	Nm
IV16	α α **a** *sir4-351*(Oc)	Nm	Nm	Nm	weak α	α	ND[d]
DC5	α **a a** *SIR*	**a**	ND	ND	ND	**a**	Nm
ho27B	α **a**$^-$ α *SIR*	**a**	**a**	**a**	**a**	Bi[e]	α

[a] Mating type cassettes are given in the order *HML MAT HMR*.
[b] Nm, Nonmating.
[c] Mating type test was performed at 36°C.
[d] ND, Not determined.
[e] Bi, Bi-mater, i.e., both mating phenotypes expressed.

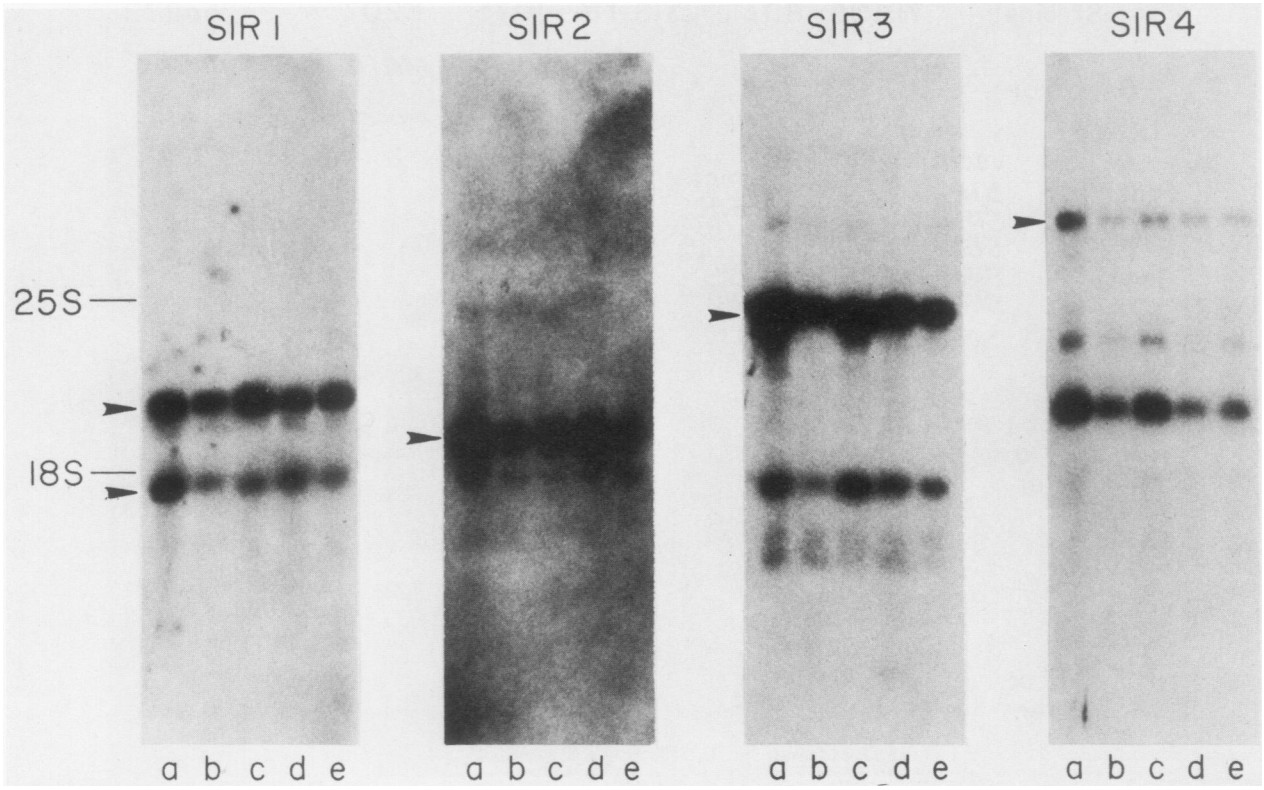


FIG. 9. Blot analysis of *SIR* transcripts in Sir⁻ mutant strains. RNA was prepared from a set of isogenic strains of Rine (Table 1). *SIR* genotypes of strains used to prepare RNA were (lanes): a, *SIR*; b, *sir1-78*; c, *sir2-373*; d, *sir4-185*; e, *sir3-337*. The probes used for the four panels were, respectively, pJI20.19, pJH73.8, pJH72.6, and pJH21.4. The amount of RNA loaded from a given genotype was the same for each panel. *SIR* transcripts are indicated by arrowheads. Transcripts from flanking genes hybridized by the *SIR3* and *SIR4* probes served as internal controls. Although there were no internal controls for *SIR1* and *SIR2*, it was evident that these two RNAs were present in all genotypes. One novel transcript of approximate size 2.7 kb is apparent in the *SIR4* panel. Its occurrence was not reproducible, and its origin remains unknown.

SIR transcript to be unaltered in each mutant strain (Fig. 9). We conclude that *SIR4* does not transcriptionally regulate *SIR3*, nor does any *SIR* gene transcriptionally regulate another *SIR* gene.

Approximate steady-state levels of the *SIR* gene transcripts were determined by probing an RNA blot with *SIR* gene fragments cloned in the vector YIp5 (52; Fig. 10). Hybridization of each probe to the *URA3* transcript served as an internal control. From scanning densitometry tracings of the lanes in Fig. 10, we estimated the steady-state levels of the *SIR1*, *SIR2*, *SIR3*, and *SIR4* transcripts to be approximately 1:8:1:1, respectively. Furthermore, the *SIR1*, *SIR3*, and *SIR4* transcripts were present in very low abundance, less than 4% of that observed for *URA3*.

***SIR* gene disruptions.** To examine whether the *SIR* genes encode essential functions, we produced null mutations by creating disruptions in the cloned sequences in vitro with which the genomic alleles were replaced. These gene disruptions all involved deletion of DNA and insertion of a selectable marker (*LEU2*).

Insertional disruption of *SIR1* is described in a preceding section. A deletion within *SIR1* was made by replacing a 1.1-kb *Bgl*II-*Xho*I *SIR1* fragment with the *Bgl*II-*Xho*I *LEU2* fragment (pJI23.2; Fig. 3A). Following gene replacement with the deletion construction, strain SX26-15C (*HML*α *mat***a***1-1 HMR***a**) exhibited a bi-mating phenotype. DNA blots (Fig. 4A) indicated that the wild-type *SIR1* sequence is no longer present in the haploid. These observations indicate that a *sir1* null allele is viable and that there remains partial regulation of the silent cassettes.

Disruption of *SIR2* employed the deletion of a *Bam*HI fragment required for *SIR2* function plus the insertion of a *Bgl*II *LEU2* fragment at an adjacent *Bgl*II site (pJH103.1; Fig. 5A). Similarly, *SIR3* was disrupted by inserting the *Bgl*II-*Xho*I *LEU2* fragment into a *Bgl*II-*Xho*I deletion within *SIR3* (pJH107.1; Fig. 6A), and *SIR4* was disrupted by inserting the *Sal*I-*Xho*I *LEU2* fragment into a deletion of approximately 500 bp (pDM610.23; Fig. 7A) associated with *Xho*I linker mutation no. 610 (Broach and Hicks, unpublished data). Following transformation of strain DC5 (*MAT***a**), the diploid DC5/DC6 (*MAT***a**/*MAT*α), or both with the disruption-containing DNA fragments, DNA blot analysis of transformants indicated that they contained the desired constructions (Fig. 11). Furthermore, strains containing the disrupted *SIR* genes and no corresponding wild-type allele were viable. That the disruptions were in the *SIR* loci was evident from the Sir⁻ phenotype of every disruption made. When tested for mating ability, DC5 *sir2*::*LEU2-103.1*, DC5 *sir3*::*LEU2-107.1*, and DC5 *sir4*::*LEU2-610.23* were nonmaters. Efficiency of mating tests indicated that all three mated at least 6 orders of magnitude less well than DC5. We conclude that the *SIR* genes do not encode essential functions in *S. cerevisiae*.

The earlier finding that the cloned *SIR3* gene suppresses both the *sir4*(Oc) and the *sir4*(Ts) mutations suggests that this suppression is not allele specific. If this is the case, then

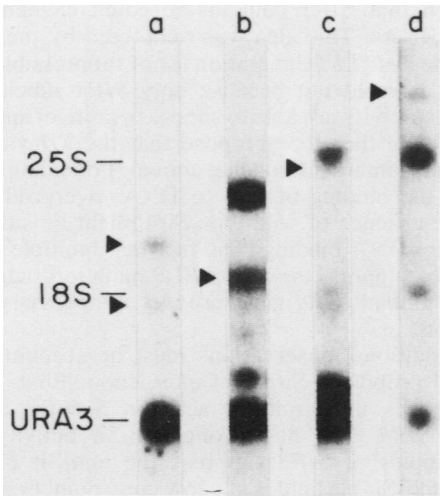


FIG. 10. Quantitation of *SIR* gene transcripts. Approximate levels of the *SIR* gene transcripts were determined by probing blots of poly(A)$^+$ RNA with *SIR* gene fragments cloned in YIp5 (52). The *URA3* gene contained in the vector hybridized to the *URA3* transcript which served as an internal control. The probes used were (lanes): a, *SIR1*, the *Hin*dIII-*Eco*RI fragment of pJI20.19 (Fig. 3) transferred to YIp5; b, *SIR2*, the *Hin*dIII fragment of pJH87.14 (Fig. 5) transferred to YIp5; c, *SIR3*, the *Bam*HI fragment of pJI25.1 (Fig. 6) transferred to YIp5; d, *SIR4*, the *Hin*dIII-*Bgl*II fragment of pJH5.1A (Fig. 7) transferred to YIp5. Arrowheads indicate the positions of the *SIR* gene transcripts. The *SIR3* transcript appears as a faint band below nonspecific binding of the probe to 25S rRNA.

the *SIR3* plasmid should also be able to suppress *sir4*::*LEU2*. To this end, strains with the *sir4*::*LEU2* mutation in a *ura3-52* mutant background were transformed with YEp24-*SIR3* and YEp24-*SIR4* plasmids (pJR104 and pJR106, respectively; J. Rine, personal communication). Both a *MAT***a** and a *MAT*α strain with the *sir4* null mutation remained nonmaters when transformed with pJR104, but both strains acquired mating ability, albeit weak, when transformed with pJR106. This result suggests that suppression of *sir4* mutations by the *SIR3* plasmid is allele specific. That the suppression of *sir4*(Ts) and *sir4*(Oc) is not due to translational suppression by a tRNA gene carried on the YEp13-*SIR3* plasmid is suggested by the inability of subclones lacking *SIR3* activity to effect this suppression. For example, pSIR3L+R (Fig. 6) suppresses *sir4*(Oc) and *sir4*(Ts), but rearrangement of the two *Hin*dIII insert fragments of this plasmid inactivates *sir4*-suppressing activity concomitant with inactivating *sir3*-complementing activity. Thus, it is the *SIR3* activity provided by the plasmid that suppresses *sir4*(Oc) and *sir4*(Ts).

DISCUSSION

We cloned *SIR* genes by their ability to complement *SIR* mutations in vivo, and we demonstrated that the cloned fragments correspond to the *SIR* genes. The latter conclusion was established by showing that *sir* mutations segregate from the corresponding cloned fragments when integrated at sites of homology. Restriction mapping of the cloned fragments combined with genomic DNA blot analysis suggested that the *SIR* genes share no DNA sequence homology. We created null mutations in each *SIR* gene by replacing the genomic alleles with deletion-insertion mutations created in vitro. These null mutant strains exhibited a Sir$^-$ phenotype and were viable, suggesting that the *SIR* genes are not involved in essential functions.

Identification of functional subclones enabled us to identify *SIR* transcripts. *SIR1* encodes two transcripts of sizes 2 and 1.5 kb from the same strand, while *SIR2*, *SIR3*, and *SIR4* each encode one major transcript 1.8, 3, and 4.4 kb in size, respectively. *SIR2* and *SIR3* transcript sizes and direction of transcription were in agreement with those predicted from the DNA sequence and S1 nuclease protection data of Shore et al. (46). Those data predict two approximately 1.85-kb transcripts for *SIR2* (which would not be separable on an agarose gel) and a 3.09-kb transcript for *SIR3*. RNA blot analysis also indicates approximate ratios of the *SIR1*, *SIR2*, *SIR3*, and *SIR4* transcripts of 1:8:1:1, respectively. *SIR2*, the most abundant of the four *SIR* transcripts, was present at only about 40% of the amount of *URA3*. Not knowing relative translational efficiencies or protein stabilities, however, it is difficult to predict steady-state stoichiometries of the various *SIR* gene products.

One interesting feature of these data is that several of the *sir4*-complementing plasmids contain incomplete *SIR4* genes. Specifically, pJI16.3, pJI16.6, and pJH5.1A are missing 1.5 to 2.5 kb of DNA from the 5′ half of the gene. Presumably, these partial *SIR4* cloned fragments produce truncated *SIR4* gene products which still possess *sir4*-complementing activity. Whether all cloned fragments with deletions 5′ of the *Bgl*II site will provide *SIR4* activity has not been addressed, because pJI16.3 and pJI16.6 were screened for providing *SIR4* activity.

A second feature of the *SIR4* cloned fragments was their association with anti-Sir activity; that is, an activity that disrupts regulation of the silent cassettes. In total, three distinct classes of *SIR4* plasmids were observed: (i) plasmids that had *SIR4* and slight anti-Sir activity, (ii) plasmids that

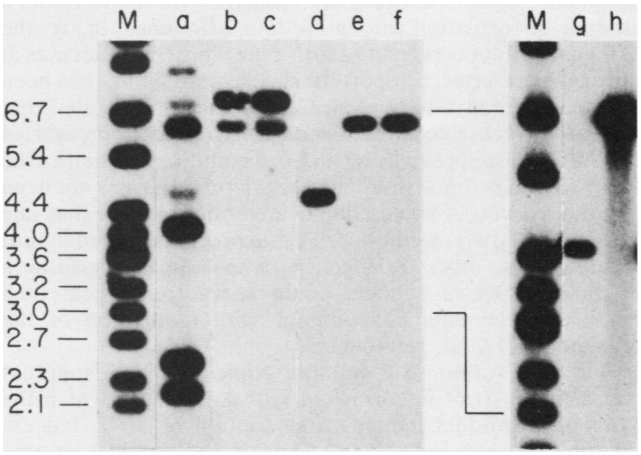


FIG. 11. Blot analysis of *SIR* gene disruptions. Genomic DNA was digested with *Hin*dIII and probed with (lanes): a through c, pJI25.1 (*SIR3*); d through f, pJH59.22 (*SIR4*); g and h, pJH87.14 (*SIR2*). Sources of DNA were (lanes): a, d, and g, DC5; b and c, DC5 *sir3*::*LEU2-107.1*; e and f, DC5 *sir4*::*LEU2-610.23*; h, DC5 *sir2*::*LEU2-103.1*. Markers were in lanes M, with sizes in kilobases in the left margin. As can be seen, wild-type fragment sizes were absent from the disruptions. The fragment of size 6.9 kb in lane a present at less than molar amount was probably due to repeated genomic sequences homologous to pJI25.1. The *SIR3* disruption fragment in lanes b and c ran coincident with this repeat fragment.

had *SIR4* activity only, and (iii) plasmids that had no *SIR4* activity and strong anti-Sir activity. The initial *SIR4* plasmid, pKAN59, which contains the entire *SIR4* gene, is an example of class one. pJI16.3, which has an approximately 1.5-kb 5′ deletion, also is a member of class one. pJI16.6, which has a more extensive 5′ deletion, is the only member of class two. Its lack of any detectable anti-Sir activity may be due to the activity of its particular truncated gene product or, if slight anti-Sir is due to excess Sir4, a lower relative amount of its truncated gene product. Plasmids of class three, those exhibiting strong anti-Sir activity, have long deletions of the 5′ end of the gene. From the nucleotide sequence of *SIR4* (M. Marshall, personal communication), it can be determined that pJH3A contains only 462 nucleotides from the carboxy-terminal end of the *SIR4* open reading frame. That anti-Sir activity requires the synthesis of the carboxy-terminal *SIR4* fragment is deduced from the galactose-inducible anti-Sir activity of a fusion between the *GAL10* gene and a *SIR4* 3′ fragment (J. B. Hicks, unpublished data).

Any model for the regulation of the *HM* loci must account for the fact that regulation is at the level of transcription and involves four *trans*-acting *SIR* genes and the *cis*-acting E and I sites, both of which lie greater than 1,000 bp from the centrally located promoter. It seems likely that at least some of the *SIR* gene products are DNA-binding proteins that recognize E and I. Because the relevant DNA sequences at E and I identified by sequence homology are very small (two 11-bp sequences; 2, 10), perhaps only a subset of the *SIR* gene products recognize E and I, whereas the others transmit the regulatory signal to the promoter. Such transmission could be the basis for the change in chromatin structure of the *HM* loci (38) or for the change in the negative superhelical density of *HM*-containing plasmids (1) observed between Sir^+ and Sir^- strains.

Several models can be proposed to account for the involvement of four *SIR* loci in regulating *HML* and *HMR*. For example: (i) the four *SIR* gene products act independently at the silent cassettes; (ii) the four *SIR* gene products act as a multimeric protein at the silent cassettes; (iii) there exists a cascade of regulation among the four *SIR* genes; or (iv) the *SIR* gene products regulate a fifth gene whose product acts at the silent cassettes. Support for this last possibility has been provided recently. Klar et al. (27a) have demonstrated that *sum1-1*, a recessive mutation obtained as a suppressor of *mar1-1*, also suppresses *sir3* and *sir4* mutations. Whether the four *SIR* genes transcriptionally regulate *SUM1* has not been determined yet. A more complicated possibility is that one *SIR* gene product monitors cell cycle progression and in turn regulates the other *SIR* genes, whose products form a multimer. Such a scheme could serve to duplicate the regulatory elements concomitant with replication of the regulated DNA (cf. reference 35).

Our observation that multiple copies of *SIR3* suppress mutations in *SIR4* is consistent with a model in which the *SIR4* gene product induces transcription of *SIR3*, but expression of the cloned *SIR3* gene is independent of that induction. However, since all *SIR* transcripts are present in strains containing a mutation in any of the four *SIR* genes, this model can be ruled out. These results extend the observation which Shore et al. (46) made for the *SIR2* and *SIR3* transcripts and indicate the absence of a cascade of transcriptional regulation among the *SIR* genes (model 3, above). This does not, however, rule out the possibility of posttranscriptional regulation.

The trivial possibility that multiple copies of *SIR3* translationally suppress *sir4* mutations was suggested by the observation that *SIR3* contains repeated elements which could be tRNAs. This idea was reinforced by the observation that the *sir4*::*LEU2* mutation is not suppressible, but the possibility is ruled out because only *SIR3* subclones that retain *SIR3* activity are able to suppress *sir4*(Oc) and *sir4*(Ts) mutations. We therefore propose that the *SIR3* and *SIR4* gene products interact (model 2, above). For example, if Sir4 facilitates the binding of Sir3 to DNA, overproduction of Sir3 in the absence of wild-type Sir4 might be sufficient to achieve Sir3-DNA binding. The fact that multiple copies of *SIR3* do not suppress the *sir4*::*LEU2* mutation suggests that at least a mutant *SIR4* gene product is necessary for this suppression.

Three additional observations must be accounted for in any model proposing Sir3-Sir4 interaction. First, a cloned *SIR4* gene has slight anti-Sir activity. Second, a cloned, truncated *SIR4* gene has strong anti-Sir activity. Third, multiple copies of *SIR3* suppress the anti-Sir phenotype when the anti-Sir plasmid is at a low copy number integrated in the chromosome (C. Stephens, personal communication). The existence of two functional Sir4 domains, one for Sir3 interaction and the other for DNA binding, could explain these effects. If the anti-Sir product lacks the DNA binding domain, it could form Sir3–anti-Sir dimers unable to bind to DNA. Suppression of the anti-Sir phenotype by multiple copies of *SIR3* in the presence of a genomic wild-type *SIR4* locus could be achieved by providing enough Sir3 to complex with both the anti-Sir product and Sir4, the latter dimer achieving proper regulation of *HML* and *HMR*. Partial disruption of Sir regulation by an excess of wild-type Sir4 might occur when unpaired Sir4 occupies the binding site(s) in the DNA, thus preventing the Sir3-Sir4 dimer from binding.

The role of *SIR1* in repression of the silent mating type cassettes remains an enigma. Even null mutations in *SIR1* retain partial regulation of the *HM* loci. The bi-mating phenotype displayed by *mat***a** *sir1*::*LEU2* strains suggests that sufficient *HML*α to produce the α phenotype is expressed in only a fraction of the cells. That *HML*α and *HMR***a** can be simultaneously expressed in *sir1* mutants was indicated by the ability of a *mat***a***1-1*/*mat***a***1-1 sir1-1*/*sir1*::*LEU2-21.4* diploid to sporulate. The similarity of *sir1* and I-site mutations has suggested the possibility that Sir1 might function exclusively at I (18). If so, no difference in phenotype is expected for the *SAD SIR1* and *SAD sir1* genotypes. Hicks et al. (18) tested this hypothesis by asking whether mutation in *SIR1* increased expression of the *SAD* cassette. They in fact found that *SAD* expression increases when *SIR1* is mutant, suggesting that Sir1 does not act exclusively at the I site. A role for Sir1 might be not in the establishment of a regulatory chromatin structure, but in its maintenance. Such a structure formed in the absence of Sir1 could be unstable, leading to loss of the structure and expression of the *HM* loci during a fraction of the cell cycle or in a fraction of the cells.

In summary, the model of a cascade of transcriptional regulation among the *SIR* genes was ruled out, and our data support the idea that the *SIR3* and *SIR4* gene products interact. *SIR1* appears to provide a peripheral function, and as yet we have no evidence bearing on the role of the *SIR2* gene product. If mRNA levels are a measure of the relative amounts of the corresponding gene products, then the *SIR* gene products must represent a very small percentage of the total cellular protein. Further investigation of these models will require biochemical tools to investigate *SIR* gene product structure and function.

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