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Isolation and Functional Analysis of Sporulation-Induced Transcribed Sequences from *Saccharomyces cerevisiae*

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Strains of the yeast *Saccharomyces cerevisiae* that are heterozygous for the mating-type locus (*MAT*a/*MAT*α) undergo meiosis and spore formation when they are starved for nitrogen and are provided with a nonfermentable carbon source such as potassium acetate. Haploids and diploids homozygous for the mating-type locus (*MAT*α/*MAT*α or *MAT*a/*MAT*a) are asporogenous and undergo neither meiosis nor spore formation when incubated under the same conditions. A small number of genes produce transcripts that appear to be induced specifically in sporulating cells. These transcripts either are not found or are present at much lower levels both in vegetatively growing cells and in cells from asporogenous strains that have been incubated in sporulation medium. Several genes complementary to these *MAT*a/*MAT*α-dependent sporulation-induced transcripts were isolated from a gene-size insert yeast-λ recombinant DNA library, by differential-plaque filter hybridization. An attempt was made to determine the function of three of these genes by mutating them in the yeast genome with in vitro mutagenesis and one-step gene replacement techniques. One gene was extensively disrupted by both a 0.3-kilobase deletion and the insertion of two large DNA sequences at different sites within the gene. Surprisingly, this compound mutation did not appear to affect meiosis or the production of viable ascospores, indicating that this gene was dispensable for differentiation. The other two genes were disrupted by simple insertion mutations at a site where it was possible that they might still possess some gene activity. These mutations also did not appear to affect sporulation. These results suggest that not all sporulation-induced genes are essential for meiosis and the production of viable ascospores under the conditions examined.

Sporulation of the yeast *Saccharomyces cerevisiae* provides an excellent model system for the study of cell differentiation (11, 12, 16). First, the process is inducible and occurs with moderate synchrony. As a result, large amounts of sporulating cells at distinct stages of differentiation can be obtained to facilitate biochemical studies. Second, sporulation is amenable to genetic studies (11, 12). Third, the process occurs in only 10 to 24 h, depending on the strain and medium used (11, 12, 16, 19, 24, 27, 36). Finally, sporulation is associated with meiosis, a process that is virtually universal to eucaryotes. Accordingly, mechanisms involved in differentiation that are associated with yeast meiosis may be applicable to other eucaryotic organisms.

Sporulation is induced when diploid strains that are heterozygous for the mating-type locus (*MAT*a/*MAT*α) are starved for nitrogen and are provided with a nonfermentable carbon source such as potassium acetate. Haploid strains and diploid strains that are homozygous for the mating-type locus (*MAT*a/*MAT*a or *MAT*α/*MAT*α) are asporogenous and undergo neither meiosis nor sporulation when incubated under these same conditions (11, 12, 16, 19).

Recent studies have suggested the existence of a sporulation-specific program of gene expression (22, 26, 28, 46, 48). Many transcripts and peptides exhibited patterns of expression that were unique to sporulating cells. These patterns were not displayed when haploid or homozygous *MAT* diploid cells were incubated in sporulation medium. In addition, hybridization studies indicated that there were approximately 200 *MAT*a/*MAT*α-dependent sporulation-induced transcripts. These transcripts were either absent or barely detectable in both haploid and homozygous or heterozygous *MAT* diploid cells grown vegetatively. They were also missing or barely detectable in asporogenous haploid or homozygous *MAT* diploid cells incubated in sporulation medium (D. Mills, personal communication). Recombinant DNA plasmids and bacteriophages complementary to approximately 20 of these sporulation-induced transcripts have been isolated thus far (6, 33). However, the functions of these genes are not yet known.

Determining the roles of genes that give rise to sporulation-induced transcripts is important for understanding the molecular events leading to sporulation. With only 200 sporulation-induced transcripts, it seemed reasonable to expect that most would be important for differentiation. However, two vegetatively expressed genes not thought required for sporulation, *CDC10* and *GAL10*, produced much more abundant transcripts in sporulating cells than in either vegetative or asporogenous cells incubated in sporulation medium (22). In addition, a sporulation-specific 20S RNA species was found in some but not all sporulation-competent strains (13). These findings suggest that genes that produced sporulation-induced transcripts need not be required for sporulation. Accordingly, it is important to determine whether the genes isolated on the basis of differential expression are essential or important for sporulation.

This report describes the isolation of several additional *MAT*a/*MAT*α-dependent sporulation-induced genes by differential-plaque filter hybridization. It also describes our attempts to determine the function of three of these genes by using one-step gene replacement techniques. Differentially expressed genes were mutated in vitro and inserted in the yeast genome in place of their wild-type (wt) alleles (38). Homozygous mutant diploids were constructed and tested for their ability to sporulate. We expected the mutants to show a sporulation-defective phenotype indicative of the

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TABLE 1. Yeast strains and recombinant plasmids

Strain or plasmid	Genotype or insert	Source (reference)
Strains		
SK1	*MAT***a** *HO* / *MAT*α *HO*	R. Roth (25)
g716-5A[a]	*MAT***a** *ho hom3-10 his1-7 can1*	J. Game (22)
AP1	*MAT*α *ade1 ade2 gal1 tyr1 lys2 his7 ura1 URA3 CAN1 CYH2 LEU1* / *MAT*α *ADE1 ade2 GAL1 TYR1 LYS2 HIS7 URA1 ura3 can1 cyh2 leu1*	A. Hopper (20)
Y185	*MAT***a** *ade2 lys2 his2 HIS8* / *MAT*α *ADE2 LYS2 HIS2 his8*	H. Halvorson (28)
DK337[b]	*MAT***a** *ade2 ADE1 leu2-3,112 his3-11,15 trp1-1 ura3-1 CAN1* / *MAT*α *ADE2 ade1 leu2-3,112 his3-11,15 trp1-1 URA3 can1-100*	This study
DK337Ω*lgn2*[c]	*lgn2::HIS3* / *LGN2*	This study
DK337 Ω*lgn2-4A*	*MAT*α *lgn2::HIS3 ade1 ura3-1 leu2-3,112 his3-11,15 trp1-1*	This study
DK337 Ω*lgn2-2C*	*MAT***a** *lgn2::HIS3 ade2 ura3-1 leu2-3,112 his3-11,15 trp1-1*	This study
DK337 ΩΔ*lgn2-4A*	*MAT*α *lgn2::LEU2-HIS3 ade1 ura3-1 leu2-3,112 his3-11,15 trp1-1*	This study
DK337 ΩΔ*lgn2-2C*	*MAT***a** *lgn2::LEU2-HIS3 ade2 ura3-1 leu2-3,112 his3-11,15 trp1-1*	This study
DK337 Δ*lgn2-4A*	*MAT*α *lgn2::LEU2 ade1 ura3-1 leu2-3,112 his3-11,15 trp1-1*	This study
DK337 Δ*lgn2-2C*	*MAT***a** *lgn2::LEU2 ade2 ura3-1 leu2-3,112 his3-11,15 trp1-1*	This study
DK337 Ω*lgn3*[c]	*lgn3::HIS3* / *LGN3*	This study
DK337 Ω*lgn4*[c]	*lgn4::HIS3* / *LGN4*	This study
Plasmids[d]		
pLF35	*LNG2*	This study
pLF44	*lgn2::HIS3*[e]	This study
pLF46	*lgn2::LEU2*[f]	This study
pLF23	*LGN3*	This study
pLF47	*lgn3::HIS3*[e]	This study
pLF24	*LGN4*	This study
pLF43	*lgn4::HIS3*[e]	This study

[a] g716-5A is a heterothallic haploid strain closely related to SK1. It was isolated from a diploid constructed by first crossing an unrelated heterothallic haploid to an SK1 haploid segregant and then repeatedly backcrossing heterothallic haploid segregants from subsequent crosses with homothallic SK1 haploid segregants (J. Game, personal communication).

[b] DK337 is a cross between W301-18A-1A *MAT***a** (R. Rothstein) and D139-11B *MAT*α (J. Szostak).

[c] Transformant of DK337, having the same genotype plus the noted gene disruption.

[d] All plasmids were constructed with pBR322 as the vector and amplified in *E. coli* SF8.

[e] The source of *HIS3* DNA used in these constructions was pWJ79, courtesy of R. Rothstein.

[f] The source of *LEU2* DNA used in this construction was YEp13(5), courtesy of J. Hicks.

function of the differentially expressed gene. Surprisingly, strains carrying a homozygous mutation in any of the three genes tested appeared to sporulate normally to produce viable ascospores. Thus, these *MAT***a**/*MAT*α-dependent sporulation-induced genes were dispensable for sporulation under the conditions investigated. We discuss reasons why these genes appeared to be dispensable for sporulation.

MATERIALS AND METHODS

Growth and sporulation. *S. cerevisiae* strains used are listed in Table 1. Cells were grown and sporulated as described previously (22).

RNA isolation. Poly(A)$^+$ RNA was isolated and purified by oligo(dT)-cellulose chromatography as described previously (22).

Recombinant DNA techniques. DNA was digested with restriction endonucleases according to the specifications of the supplier. Restriction fragments were purified by electroelution as described by either Maniatis et al. (29) or Tabak and Flavell (44). Ligations were carried out with T4 DNA ligase (New England BioLabs, Inc.) as described by Maniatis et al. (29). DNA was labeled by nick translation by the method of Rigby et al. (37), using α-^{32}P-labeled deoxynucleoside triphosphates (Amersham Corp.). Plasmids listed in Table 1 were amplified in *Escherichia coli* SF8 and isolated as described by Maniatis et al. (29). All other recombinant DNA techniques were performed as described by Maniatis et al. (29). Bacteriophage λ607 (courtesy of

Noreen Murray) (31) and its recombinant derivatives were grown by using either *E. coli* SF8 or C600 as the host. Phage stocks were prepared and DNA was isolated as described by Davis et al. (9).

Construction of a small insert *S. cerevisiae*-λ recombinant DNA library. To facilitate the isolation of differentially expressed sequences, a library containing gene-size [1 to 5 kilobases (kb)] yeast DNA inserts was constructed. The library was necessary because *S. cerevisiae* contains, on the average, one transcript for every 2 to 3 kb of DNA (15, 21; K. Coleman, H. Y. Steensma, J. Pringle, and D. B. Kaback, submitted for publication), and 95% of the transcripts made during vegetative growth are also made during sporulation (D. Mills, personal communication). Using libraries which contain 7- to 22-kb inserts gives a high probability that the hybridization signal from a sporulation-specific induced transcript would be obscured by hybridization from transcripts present at both stages of the life cycle. A library containing gene-size inserts should minimize this problem and might facilitate the isolation of DNA from which even nonabundant RNA is differentially expressed.

S. cerevisiae Y185 DNA was isolated from nuclei prepared by the method of Gorner and Phillips (14), using a modification (Coleman et al., submitted for publication) of a previously published procedure (27). DNA was randomly sheared by sonication to 0.5 to 10 kb and centrifuged on a 5 to 30% (wt/vol) sucrose gradient [25,000 rpm, 22 h at 4°C, Beckman SW28 rotor (Beckman Instruments Inc.)]. The fractions containing 1 to 5 kb of DNA were pooled and ethanol precipitated, and the product was suspended in 10 mM Tris (pH 7.4)–1.0 mM EDTA. Single-stranded ends were "filled in" with avian myeloblastosis virus reverse transcriptase and "trimmed" with S1 nuclease. DNA (25 μg) was incubated in a 50-μl reverse transcriptase reaction (50 mM Tris hydrochloride [pH 7.5], 50 mM KCl, 10 mM $MgCl_2$, 5.0 μM each deoxynucleoside triphosphate) at 37°C for 30 min with 1.0 μl (23 U) of reverse transcriptase (Life Sciences, Inc.) (29). The DNA was then sequentially extracted twice with phenol and five times with ether and ethanol precipitated. The product was suspended in 125 μl of S1 nuclease buffer (0.5 M NaCl, 50 mM sodium acetate [pH 4.5], 2.0 mM $ZnSO_4$) and was incubated at 37°C for 1 h with 1.0 μl (25 U) of S1 nuclease (Bethesda Research Laboratories, Inc.).

Synthetic decameric *Eco*RI linkers (Collaborative Research, Inc.) (300 pmol) phosphorylated in 500 μl of 55 mM Tris hydrochloride (pH 8.0)–10 mM $MgCl_2$–10 mM dithiothreitol–6.0 μM [γ-^{32}P]ATP (30 Ci/mmol) at 37°C for 1 h with 1 μl (5 U) of T4 polynucleotide kinase (New England BioLabs). Linkers were separated from unincorporated ATP on a water-equilibrated Sephadex G-50 column and concentrated in a Speed-Vac (Savant Instruments, Inc.).

A 10-fold molar excess of phosphorylated *Eco*RI linkers was ligated to 20 μg of filled-in and trimmed 1- to 5-kb *S. cerevisiae* DNA in a 20-μl T4 DNA ligase reaction performed as described by Maniatis et al. (29). Unligated linkers were removed by passage of the mixture through a Sepharose 2B column equilibrated with 10 mM Tris hydrochloride (pH 7.4)–1 mM EDTA. Excluded fractions containing *S. cerevisiae* DNA were pooled and digested to completion with *Eco*RI. These steps ensure that all DNA contains synthetic or natural *Eco*RI cohesive ends.

The *S. cerevisiae* DNA was then ligated to *Eco*RI-cleaved λ607 DNA that had been treated with bacterial alkaline phosphatase according to the instructions of the manufacturer (New England BioLabs) to prevent its religation without inserts. λ607 DNA (12.5 μg) was incubated in 100 μl of T4 ligation buffer (29) for 15 min at 50°C to allow annealing of cohesive ends. *S. cerevisiae* DNA (1.5 μg, 10 μl) and 20 U of T4 DNA ligase were added, and the reaction mixture was incubated for 16 h at 15°C. The ligation mixture was packaged in vitro according to the method of Hohn and Murray (18) and was used to transfect *E. coli* to give a total of 1.6×10^6 plaques. A total of 21% of the plaques were clear. λ607 gives a clear plaque when it contains a DNA insert due to disruption of its *c*I gene (31). The library was harvested and amplified once on solid medium. Amplification gave 90% clear plaques. The increase in the percentage of clear plaques was probably due to the increased yield of infective phage particles in the clear plaques from the first plating. The library contained approximately 3×10^5 independent yeast-λ recombinant DNA molecules.

Isolation of *MAT*a/*MAT*α-dependent sporulation-induced transcribed sequences. The small insert yeast:λ607 recombinant DNA library was screened by differential-plaque filter-hybridization for sequences that were induced in sporulating cells. [^{32}P]cDNA was synthesized from poly(A)$^+$ RNA, using avian myeloblastosis virus reverse transcriptase (Life Sciences) essentially as described by Maniatis et al. (29). Approximately 50 replica filters, each carrying 100 insert-containing, clear plaques, were first screened with [^{32}P] cDNA synthesized from poly(A)$^+$ RNA either from glucose-grown vegetative SK1 cells (VEG) or from a mixture of sporulating SK1 cells (SPOR) harvested at 1-h intervals between 1 and 12 h (22). Twelve plaques that clearly hybridized the SPOR probe but not the VEG probe were selected, plaque purified, and rescreened. Rescreening was performed with the SPOR probe, the VEG probe, and two additional [^{32}P]cDNA probes synthesized from poly(A)$^+$ RNA from vegetative SK1 cells grown on potassium acetate as the sole carbon source (VEG acetate) or from a mixture of asporogenous g716-5A *MAT*a haploid cells harvested at 1-h intervals from 1 to 12 h after being suspended in sporulation medium (non-SPOR haploid). The VEG acetate probe was included to distinguish yeast sequences that were induced by growth on acetate. The non-SPOR haploid probe was used to show sequences that were induced by starvation and were not induced specifically by sporulation.

For rescreening, the replica filters contained control recombinant λ bacteriophages λBKV, λ*CDC10*, λ*GAL*, λCONST, and λVEG1. λBKV contains human BK virus DNA and serves as a heterologous DNA control for nonspecific background hybridization (M. Pater, personal communication). λ*GAL* (λSc481 of T. St. John and R. W. Davis) (42) contains the *GAL7,10,1* gene cluster. λ*CDC10* contains the 0.9-kb *Eco*RI fragment from the *CDC10* gene subcloned in λ607 (22). The *CDC10* and *GAL7,10,1* transcripts are more abundant during sporulation than during vegetative growth on either glucose or acetate as the sole carbon source (Fig. 1). In sporulating cells, *GAL* transcripts are expressed at low abundance, while *CDC10* is expressed at moderate abundance (22). λ*GAL* and λ*CDC10* were included to determine the lower limit of our ability to detect differential hybridization signals. λ*GAL* plaques showed hybridization to the sporulation probe only after long autoradiographic exposures. This hybridization signal is not visible in Fig. 1. In contrast, λ*CDC10* showed a clear differential signal. We conclude that the *GAL* genes are differentially expressed below the practical limit of detection, while *CDC10* is expressed within our ability to detect a differential hybridization signal. λCONST (constitutive) was isolated because it hybridized both the vegetative and the sporulation probe in

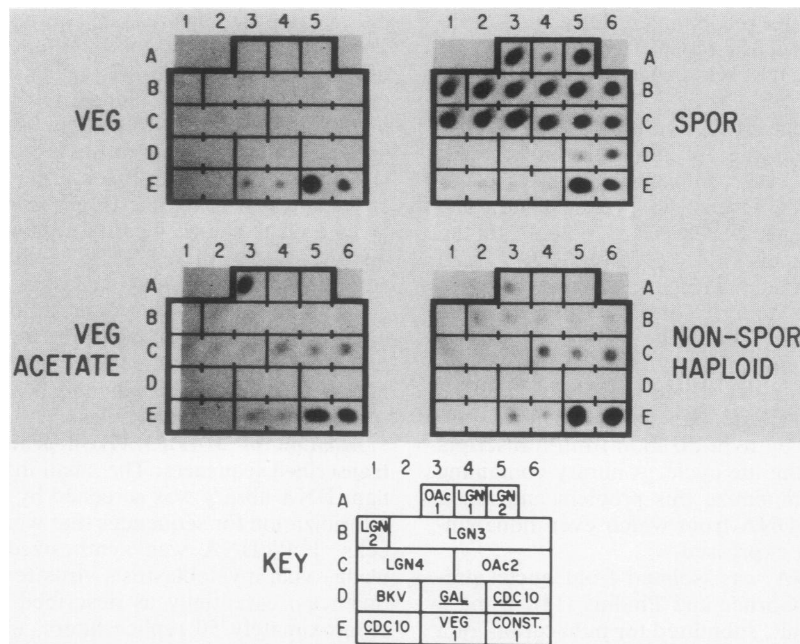

FIG. 1. Differential-plaque filter hybridization of *MATa/MATα* dependent sporulation-induced transcribed sequences. A 1- to 5-kb insert *S. cerevisiae*-λ recombinant DNA bacteriophage library was screened for transcribed sequences that were preferentially expressed during sporulation using differential-plaque filter hybridization as described in the text. Bacteriophages containing putative sporulation-induced sequences were plaque-purified and rescreened as shown above. Details for the isolation of RNA for each [^{32}P]cDNA probe have been described previously (22). Individual plaques are denoted by the key. LGN1 to LGN4 denotes bacteriophages that preferentially hybridized only the SPOR probe and contain *MATa/MATα*-dependent sporulation-induced sequences. OAc1 and OAc2 denote bacteriophages that contain sequences induced by potassium acetate-containing medium. The other plaques, BKV, *GAL*, *CDC10*, VEG1, and CONST, contain recombinant λ bacteriophages that were included on the replica filters as positive and negative controls as described in the text.

our first screen. This sequence subsequently appeared to be expressed during all four conditions and was included as a positive control to ensure that probes hybridized to each replica filter. λVEG1 was isolated in the first screen as a sequence that was preferentially expressed in vegetative cells and expressed at lower levels in sporulating cells. In the rescreening experiments, we found that it was also expressed in asporogenous haploid cells. This sequence was included as a second positive hybridization control for the vegetative probe.

Rescreening the initial 12 plaques showed that 4 hybridized only the sporulation probe. These were classified as *MATa/MATα*-dependent sporulation-induced transcribed sequences and named *LGN1* to *LGN4* (Fig. 1). (These sequences were recently renamed *SIT1* to *SIT4* for sporulation-induced transcript genes. However, throughout the present report, with the exception of the discussion, they are referred to by their old *LGN* designations.) Three of them, *LGN2*, *LGN3*, and *LGN4*, were characterized as described below. The eight other initial plaques also hybridized the VEG acetate and non-SPOR haploid probes. These were classified as acetate-induced sequences (*OAc1* to *OAc8*) and were not investigated further. Two of these, *OAc1* and *OAc2*, are shown in Fig. 1.

Northern blot hybridizations. RNA was electrophoresed on denaturing agarose gels and analyzed on diazobenzyloxymethyl-paper (Transabind; Schleicher and Schuell, Inc.) (2) as described previously (22).

Genomic blot hybridizations. *S. cerevisiae* DNA was prepared according to the method of Davis et al. (10), digested with the noted restriction enzyme, electrophoresed on 0.7% agarose gels, blotted to nitrocellulose paper (BA85, Schleicher and Schuell; or HAWP, Millipore Corp.), using 20× SSC (3.0 M NaCl, 0.3 M sodium citrate [pH 7.0]) and hybridized to nick-translation-labeled [^{32}P]DNA probes by the method of Southern (41). To show that genes were single-copy genomic sequences, blots containing *Eco*RI-, *Bam*HI-, or *Hpa*I-digested genomic DNA samples were probed with ^{32}P-labeled DNA from cloned sporulation-induced sequences.

Yeast transformations and genetic techniques. Yeast transformations were carried out by using the LiCl procedure of Ito et al. (20) with 0.3 M LiCl. Genetic procedures were performed as described by Sherman et al. (40).

R-loop hybridization and electron microscopy. Preparation and electron microscopy of R-loop-containing DNA were performed as described previously (21). Quantitation of transcript abundance by electron microscopy of R-loop-containing hybrids was carried out as described previously (23).

RESULTS

Isolation and characterization of *MATa/MATα*-dependent sporulation-induced transcribed regions. A gene-size insert yeast-λ607 recombinant library was constructed and screened by using differential-plaque filter hybridization (42) as described above. Four bacteriophages containing *MATa/MATα*-dependent sporulation-induced transcribed sequences were isolated from the library. These bacteriophages produced the plaques that most clearly hybridized cDNA made from poly(A)$^+$ RNA from sporulating cells (SPOR) but showed little or no hybridization to cDNA probes from either glucose-grown vegetative (VEG) acetate-grown vegetative (VEG acetate) diploid cells or from asporogenous haploid (non-SPOR) cells incubated in sporulation

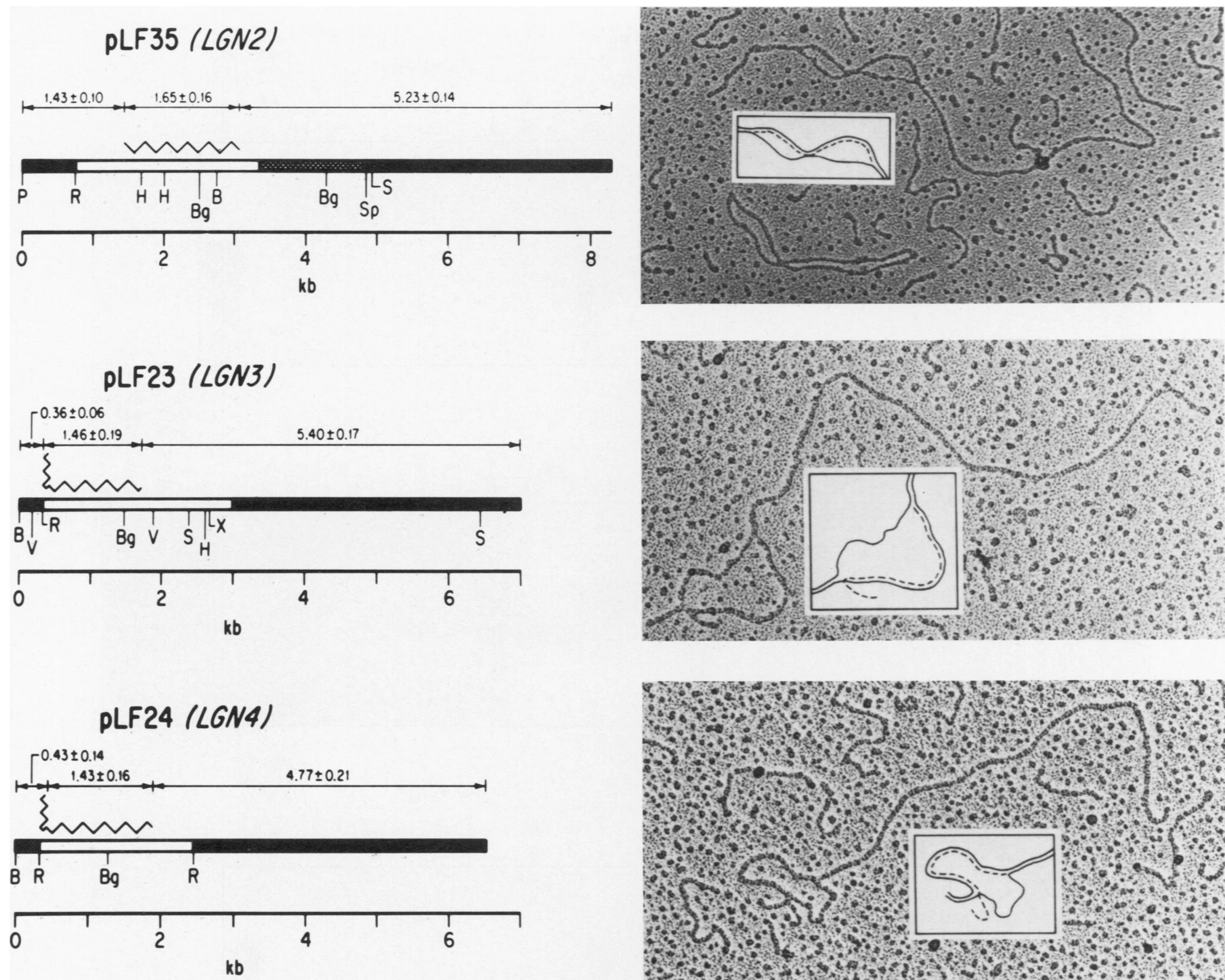

FIG. 2. Physical maps of plasmids containing sporulation-induced transcribed DNA. Electron microscopy of R-loop-containing DNA was used to map transcribed regions on the plasmid inserts. Plasmids were linearized using the noted restriction endonuclease and lightly cross-linked with Trioxsalen and UV light. Plasmid DNA (250 to 300 ng) was combined with 2.5 μg of poly(A)$^+$ RNA from time (T) = 7-h sporulating cells under conditions that permit the formation of R-loops {0.1 M PIPES [piperazine-*N*,*N'*-bis(2-ethanesulfonic acid)] [pH 7.2], 0.5 M sodium chloride, 0.01 M EDTA, 70% [vol/vol] recrystallized formamide}. The volume of the hybridization reaction was 50 μl, and incubations were carried out at 49.5°C for 14 to 18 h. R-loops were stabilized by treatment with 1.0 M glyoxal (12°C, 2 h) and prepared for electron microscopy as previously described (21). ϕX174 double-stranded DNA served as a length standard (5,385 bp) and was added to the R-loop mixtures before they were spread on copper grids. R-loop-containing molecules (20 to 50) were photographed and measured. The measured lengths ± standard deviation (in kilobases) are shown above the restriction maps. A representative R-loop structure (photograph) containing the sporulation-induced transcript is shown to the right of each restriction map. Restriction maps were generated by standard techniques (29). In addition, the *LGN2* insert contained no *Pvu*II sites; the *LGN3* insert contained no *Sma*I, *Xba*I, *Cla*I, or *Pst*I sites; and the *LGN4* insert contained no *Sph*I, *Pst*I, *Sal*I, *Hin*dIII, *Pvu*II, *Nae*I, *Nru*I, *Cla*I, *Eco*RV, *Sma*I, *Kpn*I, *Sac*I, *Sac*II, *Bst*EII, *Bcl*I, *Mlu*I, *Hpa*I, *Xho*I, *Stu*I, *Xba*I, *Apa*I, or *Nco*I sites. Symbols: ▭, yeast insert; ▨, λ DNA; ■, pBR322 DNA; zigzag line, sporulation-induced transcribed region defined by R-loop electron microscopy; P, *Pst*I; R, *Eco*RI; H, *Hpa*I; Bg, *Bgl*II; B, *Bam*HI, Sp, *Sph*I; S, *Sal*I; X, *Xho*I; V, *Eco*RV.

medium. The sporulation-induced transcribed sequences were designated *LGN1* to *LGN4* (Fig. 1).

Three of these sequences, *LGN2*, *LGN3*, and *LGN4*, were excised from their bacteriophage vector and subcloned in plasmid pBR322 to give pLF35, pLF23, and pLF24, respectively (Table 1; Fig. 2). For pLF35, the fragment containing the yeast insert also contained approximately 1.5 kb of adjacent DNA from the large arm of the λ607 vector (31). RNA blot hybridization experiments (Fig. 3) showed that each of the three plasmids was complementary to a sporulation-induced transcript with a length of either 1.7 or 1.9 kb. These transcripts had similar but not identical patterns of expression. All were detected between 5 and 16 h after transfer of the cells to sporulation medium, but each appeared most abundant at a different time between 6 and 8 h. The three transcripts were not detected in either glucose-grown or acetate grown vegetative cells or in non-SPOR haploid (g716-5A) and diploid AP1 (*MAT*α/*MAT*α) cells that were incubated in sporulation medium. Plasmids pLF23 (*LGN3*) and pLF24 (*LGN4*) were each complementary to

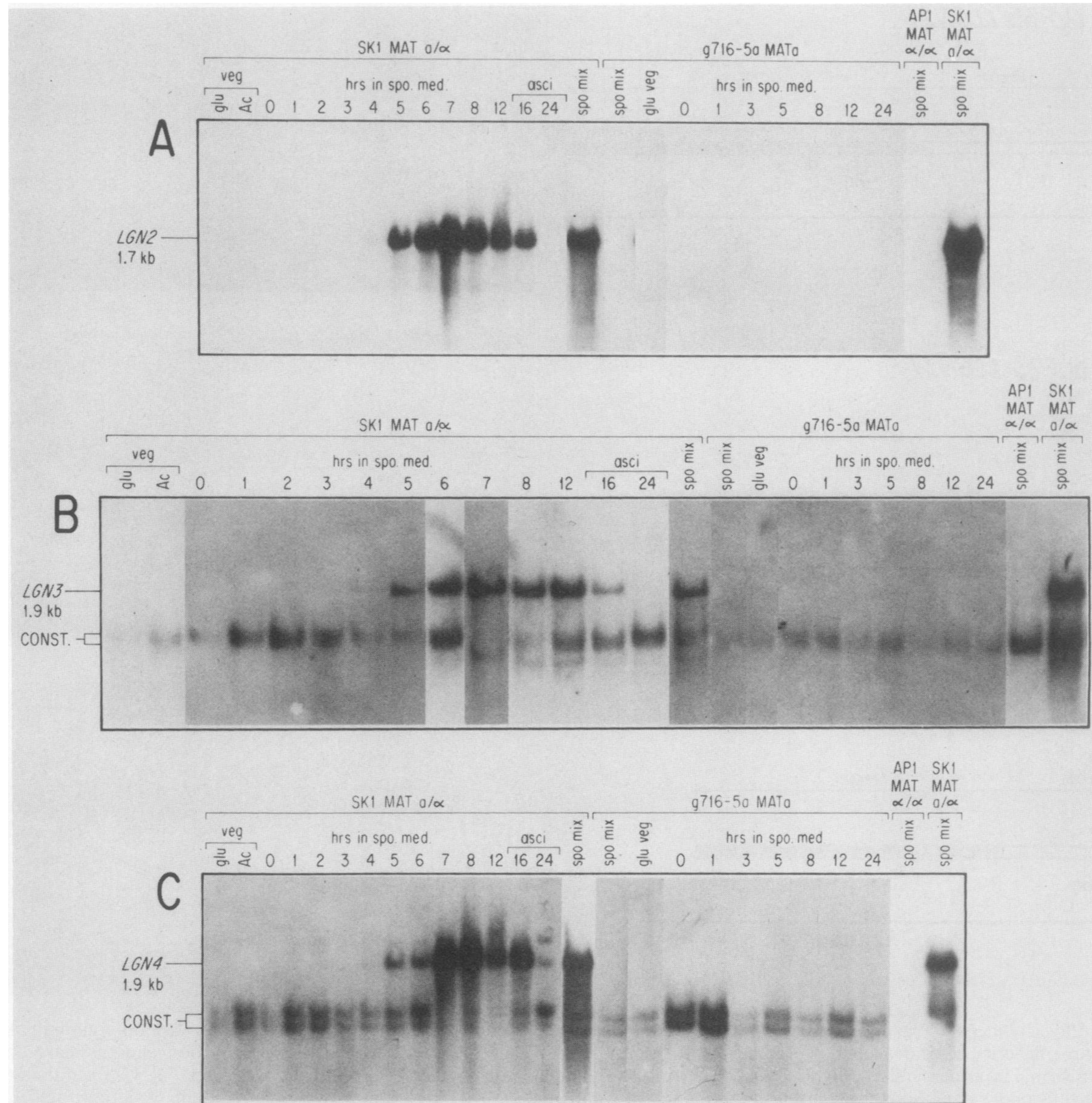

FIG. 3. Appearance of sporulation-induced transcripts. Poly(A)$^+$ RNA was prepared as previously described (22) from sporulating SK1 and asporogenous haploid g716-5A cells harvested from sporulation medium at the noted times (hrs in spo. med.), from mixtures of cells harvested at 1-h intervals from 1 to 12 h from sporulation medium from strain SK1, g716-5A, or AP1 (*MAT*α/*MAT*α) (spo. mix), and from glucose-grown (glu veg) or acetate-grown (Ac veg) vegetative cells. Poly(A)$^+$ RNA (2.0 μg) from each sample was electrophoresed on 5.0 mM CH_3HgOH, 1.0% (wt/vol) agarose gels, blotted to diazobenzyloxymethyl-paper, and hybridized to ^{32}P-labeled plasmid DNA as previously described (2, 22). Ethidium bromide staining of the gels both before and after blotting indicated that each lane contained approximately the same amount of essentially undegraded RNA, and transfer from the gel was virtually complete. Prior and subsequent hybridization experiments with these blots indicated that each lane maintained the same relative amount of bound hybridizable RNA (22). Hybridization experiments for each gene were usually performed in parallel, allowing for a direct comparison of the relative transcript abundance in each RNA sample. For A and B, the intensity of the hybridization signal appears to be increased in the blots containing the SK1 and AP1 (*MAT*α/*MAT*α) spo. mix samples in adjacent lanes. These samples were not processed in parallel with the others. Therefore, this change was due either to increased autoradiographic exposure times or to hybridization probes with higher specific activities.

two additional constitutively expressed RNA species. These transcripts hybridized to restriction fragments that were adjacent to the sporulation-induced sequence (data not shown). The constitutive transcripts complementary to pLF24 (*LGN4*) were detected at lower levels in AP1 (*MAT*α/*MAT*α) that were difficult to visualize in the figure shown. In addition to the 1.9-kb sporulation-induced transcripts, pLF23 was complementary to a less abundant, lower-molecular-weight transcript and pLF24 was complementary to a less abundant, higher-molecular-weight transcript. These transcripts were detected in sporulating cells only. However, owing to their low abundance, the nature of these transcripts was not investigated further.

Restriction maps of the three plasmids were generated, and the location of each sporulation-induced transcribed region was determined by using R-loop electron microscopy (Fig. 2). For each plasmid, poly(A)$^+$ RNA from 7-h sporulating cells gave a single R-loop at a position where

TABLE 2. Abundance of sporulation-induced transcripts

Gene	Transcript size (kb)	Fraction of plasmids containing R-loops[a]	Amt of complementary plasmid DNA (ng)[b]	Weight-fraction of transcripts[c]	Approx no. of transcripts/cell at 7 h[d]
LGN2	1.70	0.40	30	0.0047	17–27
LGN3	1.85	0.33	34	0.0056	18–30
LGN4	1.95	0.35	20	0.0056	17–28

[a] R-loops were formed as described in the legend to Fig. 2, using 250 to 300 ng of linearized plasmid DNA and 2.5 μg of poly(A)⁺ RNA from 7-h sporulating cells. The fraction of plasmids containing the sporulation-induced R-loop was determined by examining at least 200 plasmids in the electron microscope.

[b] The amount of complementary DNA that was added to the hybridization reaction is the amount of plasmid DNA added to the reaction × [length of the transcribed region/(2 × length of the plasmid)]. When the entire transcribed region was not contained on the plasmid, the value for the amount of complementary DNA that was added to the reaction was corrected by multiplying it by the reciprocal of the gene fraction contained on the plasmid.

[c] The weight-fraction of each transcript is (the fraction of plasmids containing R-loops × amount of complementary DNA added to the reaction)/amount of poly(A) RNA added to the reaction.

[d] The number of transcripts per cell is: [the weight-fraction of transcript × 6×10^6 to 10×10^6 nucleotides of poly(A)⁺ RNA per cell]/nucleotides per transcript. This estimate is approximate because the amount of poly(A)⁺ RNA per cell at this stage of sporulation has not been accurately determined. Our estimate is based on our observations that 7-h sporulating cells yield on average of one-third to one-fifth as much poly(A)⁺ RNA as do vegetative cells (22) and that vegetative cells contain approximately 20,000 poly(A)⁺ RNA molecules of an average size of 1.5 kb (15). Accordingly, 7-h cells should contain 4,000 to 7,000 poly(A)⁺ RNA molecules of the same assumed average size (1.5 kb) equal to approximately 6×10^6 to 10×10^6 nucleotides of poly(A)⁺ RNA per cell.

R-loops were not observed using RNA from glucose-grown vegetative cells. Plasmid pLF35 appeared to contain an intact sporulation-induced transcribed region; pLF23 and pLF24 contained only about 75% of the length of their respective sporulation-induced transcribed regions. These sequences were truncated at the plasmid-vector junction. The remaining fragments of these genes were not isolated. For pLF23 (*LGN3*) and pLF24 (*LGN4*), vegetative RNA gave a few R-loops in the region adjacent to the sporulation-induced transcribed regions, results consistent with the RNA blot hybridization experiments. Although these transcribed regions were not mapped, their position clearly never overlapped the sporulation-induced region. No R-loops were observed in these regions by using 7-h sporulation RNA. However, the RNA blots (Fig. 3) indicated that these transcripts were present at a sufficiently low level at this time to make detection of R-loops difficult.

R-loop hybridization reactions were carried out in plasmid DNA sequence-excess; conditions that enable an estimation of the relative transcript abundance (weight-fraction) from the fraction of plasmids containing R-loops (23). Based on these estimates, these three sporulation-induced transcripts were present at 17 to 30 copies per cell at 7 h (Table 2).

Genomic blot hybridization experiments were carried out as described above, with DNA from the following strains: SK1, the source of the sporulation RNA; Y185, the source of the library DNA; DK337, the strain that was used in subsequent DNA transformation experiments. Results showed that each sporulation-induced sequence was complementary to single-copy genomic DNA (data not shown; also see Fig. 5 and 7). In addition, these analyses showed no evidence for restriction-enzyme-fragment-length polymorphisms.

Gene disruption of a *MATa/MATα*-dependent sporulation-induced transcribed sequence. Linear DNA introduced by transformation into *S. cerevisiae* will undergo homologous recombination with chromosomal DNA (17). Mutant genes constructed in vitro will replace wt sequences by a double-crossover mechanism (38). The procedure, called one-step gene replacement, provides a means to assess in vivo functions of genes and chromosomal elements (4, 7, 25).

To determine the function of *LGN2* in sporulating cells, we disrupted its genomic copy with a series of insertion and insertion-deletion mutations. First, a 1.7-kb *Bam*HI fragment containing the *S. cerevisiae HIS3* gene (43) was inserted into the *Bam*HI site of the sporulation-induced transcribed region (Fig. 4). Yeast DNA transformations utilized a *his3/his3 leu2/leu2* diploid strain, DK337, to ensure the survival of recessive lethal mutations. His⁺ putative transformant colonies were selected and screened by using genomic blot hybridizations (41) to identify those containing the gene disruption (*lgn2::HIS3*). DNA from untransformed His⁻ controls contained a single 7.8-kb DNA *Eco*RI fragment complementary to the *LGN2* sequence. In contrast, most of the His⁺ colonies contained a new 9.5-kb *Eco*RI fragment in addition to the resident 7.8-kb fragment (Fig. 5, lanes 1 and 2). The predicted size for the insertion of the 1.7-kb *HIS3* fragment into the wt 7.8-kb *LGN2 Eco*RI fragment is 9.5 kb. These colonies (DK337 Ω*lgn2*) were heterozygous for the gene disruption. A few His⁺ colonies did not have the 9.5-kb fragment. The colonies were presumably due to gene conversion of the *his3* gene to *HIS3* (17).

Several bona fide transformants containing the 9.5-kb fragment were sporulated, and asci were dissected. All showed excellent spore viability. Thus, the *lgn2::HIS3* mutation did not cause lethality. This result suggests the *LGN2* gene is dispensable for vegetative growth on rich yeast extract-peptone-dextrose medium. A Southern blot of DNA from four haploid spores from one tetrad showed that the His⁺ spores contained only the new 9.5-kb *Eco*RI fragment, while the His⁻ spores contained only the wt 7.8-kb fragment (Fig. 5, lanes 3 to 6). In addition, genetic analysis of a His⁺ (*lgn2::HIS3*) haploid crossed to an unrelated *HIS3* haploid, DK71-4C, gave some 3:1 and 2:2; *HIS3*:*his3* tetrads. These tetrads indicated that the transforming *HIS3* gene integrated at a new position, leaving a resident *his3* gene in the His⁺ transformant (17). Taken together, these results demonstrate that the *lgn2::HIS3* gene disruption replaced the wt *LGN2* sequence in the genome.

Several *lgn2::HIS3/lgn2::HIS3* mutant homozygotes, *lgn2::HIS3/LGN2* heterozygotes, and wt *LGN2/LGN2* diploids were constructed by pairwise matings of haploid segregants from DK337 Ω*lgn2*. These strains were tested for their ability to sporulate on solid sporulation medium (40). Surprisingly, the homozygous mutant strains sporulated to about the same extent as the heterozygous and homozygous wt controls. Furthermore, the mutants were able to undergo meiotic recombination and most asci contained four viable spores (Table 3).

To avoid the possibility that the simple insertion did not functionally inactivate *LGN2*, the gene was disrupted further (Fig. 4). A 0.3-kb *Hpa*I fragment from the middle of the transcribed region was deleted from pLF35 and a 2.1-kb *Hpa*I fragment containing the *S. cerevisiae LEU2* gene (5, 35) was inserted in its place to give pLF46 (*lgn2::LEU2*). The insert was released from the vector with the combination of *Eco*RI and either *Bgl*II or *Sph*I to give two kinds of linear fragments. Each fragment was used to transform to Leu⁺ related *leu2* haploid strains of both mating types that carried the *lgn2::HIS3* gene disruption (DK337 Ω*lgn2-4A* and DK337 Ω*lgn2-2C*). In most cases we obtained more than 20

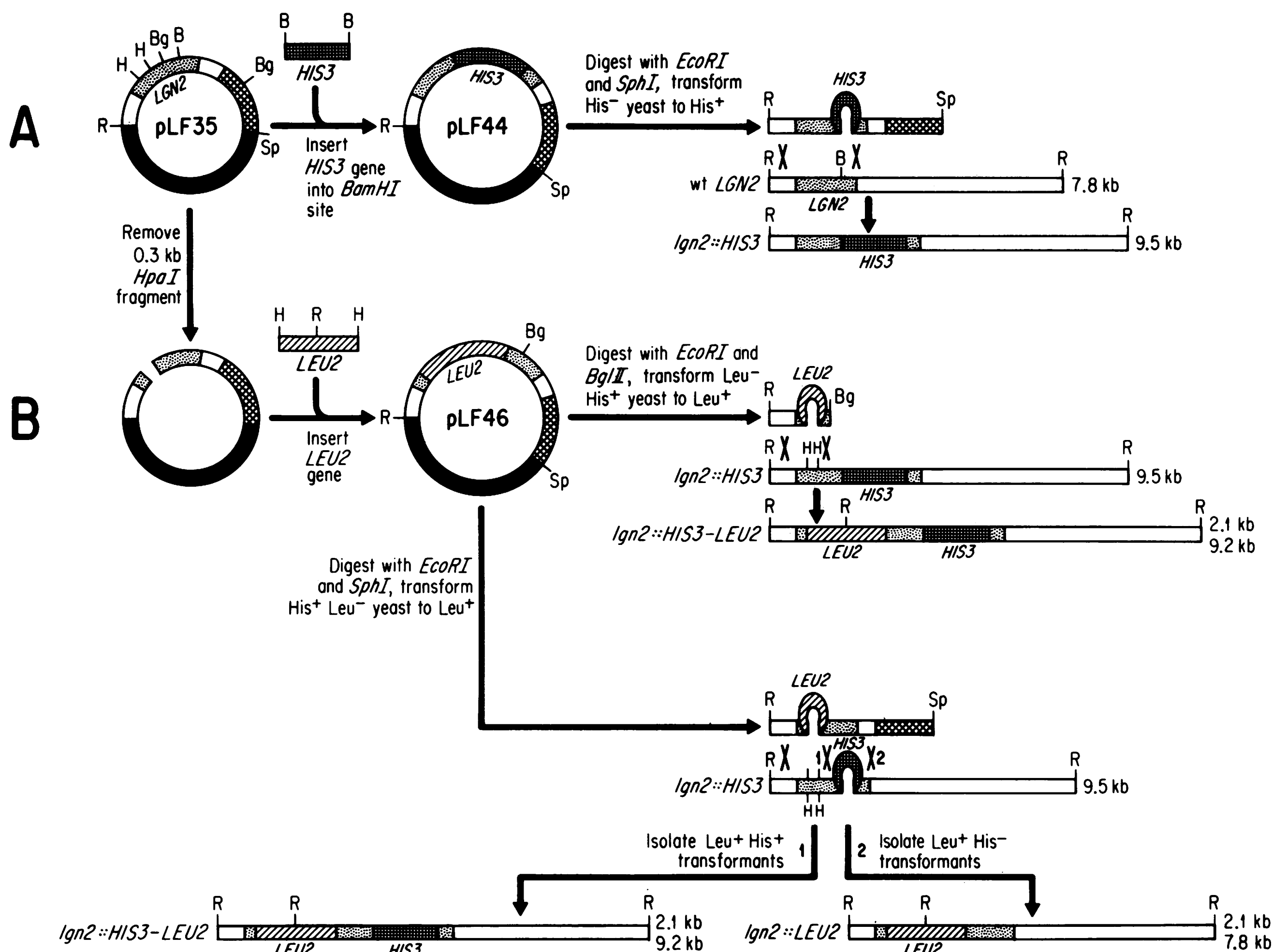

FIG. 4. Gene disruption of the sporulation-induced transcribed region, *LGN2*. (A) The *LGN2* sequence was first mutated in vitro by inserting the 1.7-kb *Bam*HI fragment containing the *S. cerevisiae HIS3* gene into the *Bam*HI site of the sporulation-specific transcribed region contained in pLF35. The resultant plasmid pLF44 was cleaved with the combination of *Eco*RI and *Sph*I and transformed into DK337. (B) The *LGN2* sequence was mutated further by constructing a *LEU2* insertion-deletion mutation. The 2.2-kb *Hpa*I fragment containing the *S. cerevisiae LEU2* gene was inserted in place of the 0.3-kb *Hpa*I fragment in the sporulation-specific transcribed region of pLF35 to give pLF46. Compound mutants containing both the *HIS3* insertion and the *LEU2* insertion-deletion mutation were constructed by transforming haploid strains containing the *HIS3* insertion mutation in *LGN2* with *Eco*RI-*Bgl*II-treated pLF46 DNA and selecting Leu⁺ transformants that were still His⁺. Single *LEU2* insertion-deletion mutations were introduced by transforming these same His⁺ strains to Leu⁺ with *Eco*RI-*Sph*I-digested pLF46 and selecting transformants that were now His⁻. The *Eco*RI-*Sph*I digest also gave Leu⁺ His⁺ transformants that sometimes contained the compound mutation.

transformants for each mating type and DNA utilized. DNA prepared from 4 to 12 transformants was analyzed by using Southern blots to identify those with the proper gene disruption.

The *Eco*RI-*Bgl*II fragment which did not overlap the site of the *HIS*3 insertion gave compound mutations containing both the *LEU2* insertion-deletion and the *HIS3* insertion (*lgn2*::*HIS3-LEU2*). Both *MAT*α and *MAT***a** transformants were isolated where the 9.5-kb *Eco*RI fragment from the *lgn2*::*HIS3* insertion strains (Fig. 5, lane 8) was replaced by the expected two new bands of 2.1 and 9.2 kb (DK337 Ω Δ*lgn2-4A* and DK337 ΩΔ*lgn2-2C*). Homozygous diploids were constructed by mating two correctly transformed haploid strains. A Southern blot containing DNA from one of these homozygotes is shown in Fig. 5, lane 9. In addition, genetic analysis of the transformants confirmed that the *LEU2* gene was now very tightly linked to the *HIS3* gene (101:0:0; parental ditype-nonparental ditype-tetratype asci).

The *Eco*RI-*Sph*I fragment which overlapped the *HIS3* insertion mutation (*lgn2*::*HIS3*) gave both compound *lgn2*::*HIS3-LEU2* and simple *LEU2* insertion-deletion (*lgn2*::*LEU2*) mutations. Southern blots indicated that some of the His⁺ Leu⁺ transformants contained the *lgn2*::*HIS3-LEU2* compound mutation, while the others contained gene conversions to *LEU2* at the *leu2* gene (data not shown). Southern blots of DNA from the Leu⁺ His⁻ colonies showed that they contained the predicted new 2.1- and 7.8-kb fragments replacing the 9.5-kb fragment. *MAT*α (DK337 Δ*lgn2-4A*) and *MAT***a** (DK337 Δ*lgn2-2C*) transformants were isolated and mated to give homozygous diploids. A Southern blot of DNA from one of these diploids is shown (Fig. 5, lane 10).

TABLE 3. Sporulation of strains containing mutations in sporulation-induced transcribed sequences

Genotype	% Sporulation[a]	% Spore viability[b]	Meiotic recombination[c]
LGN2 (wt) / *LGN2*	55 ± 25 (5)	97	+
lgn2::HIS3 / *LGN2*	67 ± 13 (8)	89	+
lgn2::HIS3 / *lgn2::HIS2*	54 ± 18 (6)	80	+
lgn2::LEU2 / *lgn2::LEU2*	+++[d] (4)	ND[e]	ND
lgn2::HIS3-LEU2 / *lgn2::HIS3-LEU2*	+++[d] (10)	89	+
LGN3 (wt) / *LGN3*	57 ± 11 (4)	ND	ND
lgn3::HIS3 / *LGN3*	46 ± 8 (3)	94	+
lgn3::HIS3 / *lgn3::HIS3*	44 ± 19 (2)	99	+
LGN4 (wt) / *LGN4*	55 ± 5 (2)	100	ND
lgn4::HIS3 / *LGN4*	55 ± 12 (8)	85	+
lgn4::HIS3 / *lgn4::HIS3*	57 ± 21 (7)	100	+

[a] Haploid segregants that were either wt or contained the noted gene disruption were obtained by dissecting heterozygous DK337 transformants. Related homozygous mutant, heterozygous, or wt diploids were constructed by mating these haploids in pairwise combinations. Purified diploids were streaked onto sporulation plates, incubated at 30°C for 4 days, and examined microscopically. The percentage of sporulation was determined by examining 200 to 500 cells from several different diploids. The mean percentage of sporulation ± the standard deviation is shown. The number in the parentheses is the number of different diploids containing the noted genotype that was examined. Similar results were obtained using minimal sporulation plates (1.0% [wt/vol] potassium acetate pH 7.0, 2.0% [wt/vol] agar), except the overall percentages of sporulation were reduced from what is shown.

[b] Spore viability was obtained by randomly selecting one or two diploids, dissecting 10 or more four-spored asci onto rich yeast extract-peptone-dextrose medium, and determining the fraction of ascospores that gave rise to colonies.

[c] The ability to undergo meiotic recombination was judged by examining the above noted dissections for the appearance of tetratype asci for the gene pairs *ADE2-ADE1*, *MAT-ADE1*, and in some cases *URA3-ADE1*, *URA3-ADE2*, and *URA3-MAT*. +, Meiotic recombination was observed. (Also see Table 4.)

[d] For these gene disruptions, homozygous mutants were constructed by pair-wise crosses between several independently obtained DK337 ΩΔ*lgn2-4A* and DK337 ΩΔ*lgn2-2C* transformants. The results were not accurately quantitated. However, in each diploid the sporulation was approximately 60%. This is about the same percentage found both for the wt/wt controls obtained by crossing wt haploid segregants from the original heterozygous *lgn2::HIS3/LGN2* transformant and for the parent diploid DK337.

[e] ND, Not determined.

Homozygous diploid strains containing the simple *lgn2::LEU2* mutation or the compound *lgn2::HIS3-LEU2* mutation were screened for the ability to sporulate on solid sporulation medium. As before, all diploids sporulated equally well. The kinetics and extent of sporulation in liquid medium (34) were also measured for the compound *lgn2::HIS3-LEU2* mutant. Again, no differences were found comparing homozygous mutants with related wt diploid

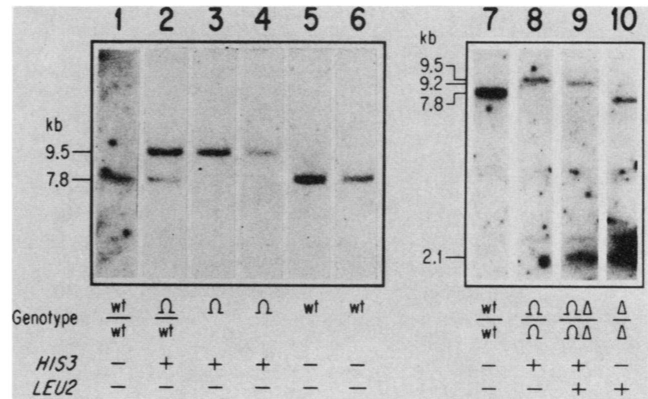

FIG. 5. Blot hybridization of DNA from transformants containing the *LGN2* gene disruption. DNA was digested with *Eco*RI, electrophoresed on 0.7% agarose gels, transferred to nitrocellulose, and hybridized to nick-translation-labeled ^{32}P-pLF35 DNA by the method of Southern (41). Genotypes of each strain tested are shown below the autoradiograph: wt, wild type; Ω, *lgn2::HIS3* insertion mutation; Δ, *lgn2::LEU2* deletion-substitution mutation; ΩΔ, *lgn2::HIS3-LEU2* compound mutation. Lanes: 1, untransformed DK337; 2, heterozygous transformant; 3 to 6, haploid segregants from the heterozygous transformant; 7, untransformed DK337; 8, homozygous *lgn2::HIS3/lgn2::HIS3* mutant; 9, homozygous *lgn2::HIS3-LEU2/lgn2::HIS3-LEU2* mutant; 10, homozygous *lgn2::LEU2/lgn2::LEU2* mutant. The minor bands in lanes 7 through 10 at approximately 2.3 and 4 kb are due to cross hybridization of bacterial DNA sequences to 2μm DNA sequences.

controls. Mutants and wt strains both sporulated to about 40% with the same kinetics (data not shown). In addition, spore viability was excellent for all compound and simple mutants tested (Table 3), confirming the previous observation that *LGN2* is dispensable for germination and growth on rich medium. Finally, recombination frequencies were nearly equal in compound-mutant and wt strains (Table 4). Differences were shown to be insignificant using the chi-square test. Thus, neither recombination, meiosis, nor the formation of viable ascospores appeared significantly affected by the mutations in the *LGN2* gene.

LGN2 transcript appearance in mutant and wt cells. Since sporulation did not appear to be affected by the *lgn2::HIS3-LEU2* mutation, a homozygous mutant diploid was examined for *LGN2* RNA as a further control for gene disruption. Neither sporulating nor vegetative mutant cells appeared to accumulate the 1.7-kb *LGN2* transcript (Fig. 6, lanes 3 and 4). Long autoradiographic exposures revealed traces of a sporulation-induced 0.85-kb transcript in the mutant that hybridized the *LGN2* probe. This transcript was not visible in Fig. 6. The 0.85-kb transcript was not found in mutant vegetative cells or in the two wt control strains. A *HIS3* probe (but not a *LEU2* probe) also hybridized to a sporulation transcript of this size in the mutant. The 0.85-kb transcript was noticeably larger than the 0.6-kb constitutively expressed wt *HIS3* transcript (43). We believe the 0.85-kb transcript results from the fusion of *HIS3* sequences to part of the *LGN2* transcript. Intensities of hybridization signals indicated that the fusion-transcript was composed primarily of *HIS3* sequence and contained little *LGN2* sequence. Therefore, the likelihood of this transcript-producing *LGN2* activity is considered extremely small.

Control experiments confirmed that the wt parent, DK337, also produced a sporulation-induced *LGN2* transcript at levels comparable to sporulating SK1 cells while vegetative

TABLE 4. Recombination frequencies in strains containing disruptions in sporulation-induced transcribed regions[a]

Sporulation-induced gene	Gene pair	Ω/Ω[b]			Ω/wt[c]			wt/wt[d]		
		No. of asci		Combined % recombination[e]	No. of asci		Combined % recombination[e]	No. of asci		Combined % recombination[e]
		Ditype	Tetratype		Ditype	Tetratype		Ditype	Tetratype	
LGN2[f]	*URA3-MAT*	21	39	33				18	37	34
	ADE1-MAT	24	36	30				16	39	35
	URA3-ADE1	49	11	9				48	12	10
	URA3-ADE2	11	49	41				19	41	34
	ADE1-ADE2	12	48	40				19	41	34
LGN3[g]	*URA3-MAT*	14	22	31	5	6	16			
	ADE1-MAT	11	25	35	12	16	29			
	URA3-ADE1	24	12	17	8	3	14			
	URA3-ADE2	9	23	36	1	10	45			
	ADE1-ADE2	5	31	43	8	20	36			
LGN4[g]	*ADE1-MAT*	24	47	33	20	24	27			
	ADE1-ADE2	21	50	35						

[a] Recombination frequencies were examined by determining the number of tetratype asci for the noted markers.
[b] Homozygous gene disruption mutant diploids.
[c] Heterozygous gene disruption/wt diploids.
[d] DK337, parent wt diploid.
[e] The combined percent recombination between the noted markers and their respective centromeres is 100 × no. of tetratype asci/(2 × total no. of tetrads).
[f] To determine the effect of mutating the *LGN2* sequence, we compared a related homozygous *lgn2::LEU2-HIS3* mutant with the wt diploid strain DK337. The mutant homozygote was obtained by crossing meiotic products from a heterozygous *lgn2::HIS3-LEU2* diploid. The heterozygote was from a second backcross of a diploid made by crossing DK337 Δ Ω*lgn2-4A* to a haploid segregant from DK337, genotype *MAT***a** *ade2 trp1-1 leu2-3, 112 his3-11,15*.
[g] To determine the effect of mutating either the *LGN3* or the *LGN4* sequence, we used heterozygous and homozygous strains containing either *lgn3::HIS3* or *lgn4::HIS3* gene disruptions. These strains were made by crossing appropriately marked haploid segregants from either DK337 Ω*lgn3* or DK337 Ω*lgn4*.

DK337 cells contained no detectable *LGN2* transcript (Fig. 6, lanes 1, 2, and 5).

Gene disruption of *LGN3* and *LGN4*. The two other *MAT***a**/*MAT*α-dependent sporulation-induced transcribed regions were mutated by inserting a DNA fragment containing the *S. cerevisiae HIS3* gene into the *Bgl*II site contained in each gene (Fig. 7). For *LGN3*, two *HIS3* genes were inserted fortuitously in tandem. The disrupted *LGN3* and *LGN4* sequences were excised from their respective vectors and used to transform strain DK337. His$^+$ putative transformant colonies were screened by using genomic Southern blots for ones that contained heterozygous gene disruption mutants. For the *lgn3::HIS3/LGN3* heterozygote (DK337 Ω*lgn3*), the new *Eco*RI fragment was increased by 3.4 kb due to the two 1.7-kb *HIS3* inserts. For the *lgn4::HIS3/LGN4* heterozygote (DK337 Ω*lgn4*), the new fragment was increased by the expected 1.7 kb because of insertion of a single *HIS3* gene. Heterozygous transformants containing the wt and the expected altered restriction fragments were sporulated. The His$^+$ phenotype cosegregated with the new restriction fragment (Fig. 7). Spore viability was again almost 100% for both *lgn3::HIS3* and *lgn4::HIS3*, indicating that neither gene disruption resulted in lethality (data not shown). For each gene disruption, His$^+$ and His$^-$ segregants were picked and mated in several pairwise combinations to give wt, homozygous mutant, and heterozygous diploids for either *LGN3* or *LGN4*. These strains were tested for their ability to sporulate. Again, both the *lgn3::HIS3* and *lgn4::HIS3* homozygotes sporulated at frequencies comparable to wt and heterozygous controls, spore viability was high, and the frequency of meiotic recombination was not significantly affected (Tables 3 and 4). Both homozygous mutants gave slightly higher levels of meiotic recombination than the heterozygous controls; however, chi-square analyses indicated the differences were insignificant. In addition, almost no differences were observed when the mutants were compared with the wt homozygote used as a control for the *lgn2* mutants. These results show that intact copies of *LGN3* and *LGN4* are not required for meiotic recombination, meiosis or sporulation.

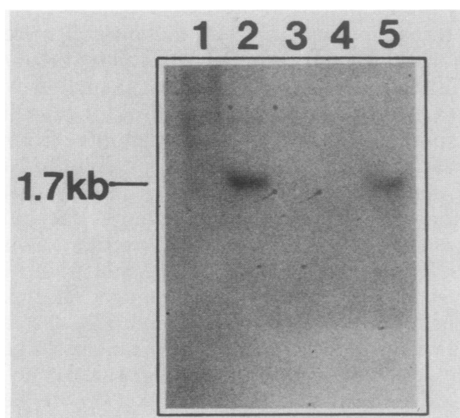

FIG. 6. Sporulation-induced *LGN2* transcript is not expressed in a homozygous *lgn2::HIS3-LEU2* mutant strain. Total RNA prepared from either glucose-grown vegetative cells or 1-h time point mixtures of sporulating cells (see legend to Fig. 1) was electrophoresed on CH_3HgOH-agarose gels, blotted to diazobenzyloxymethyl-paper, and probed with ^{32}P-labeled *LGN2* DNA. Lanes: 1, wt DK337 vegetative cells; 2, wt DK337 sporulating cells; 3, mutant *lgn2::HIS3-LEU2* vegetative cells; 4, mutant *lgn2::HIS3-LEU2* sporulating cells; 5, SK1 sporulating cells.

Genetic mapping of *LGN4*. During the above genetic studies we noticed that *lgn4::HIS3* was tightly linked to *URA3*

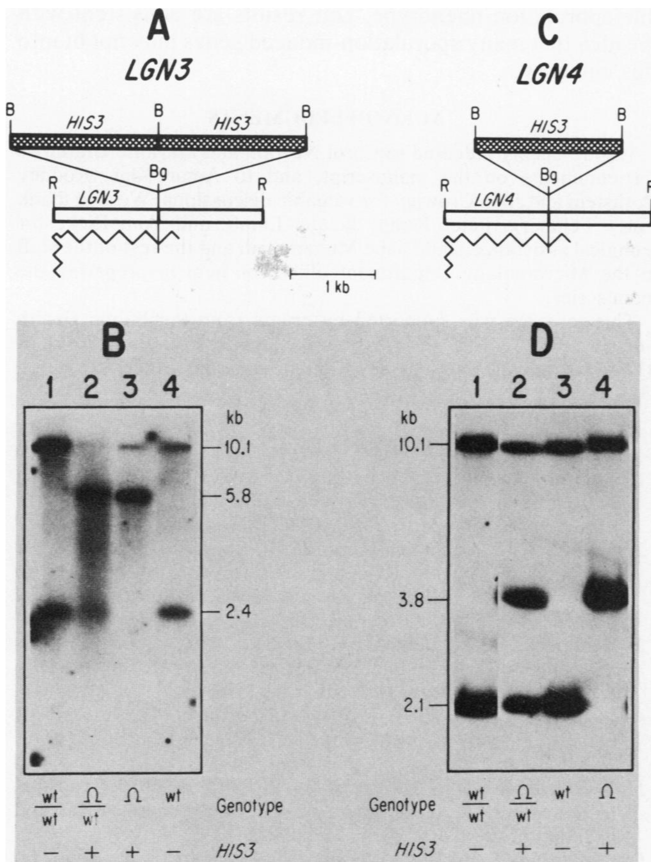

FIG. 7. Gene disruption of sporulation-induced transcribed regions, *LGN3* and *LGN4*. Plasmids pLF23 and pLF24 were linearized with *Bgl*II and ligated to the 1.7-kb *Bam*HI fragment containing the *S. cerevisiae HIS3* gene. Two tandem *HIS3* sequences disrupted *LGN3* (pLF47), while a single *HIS3* sequence disrupted *LGN4* (pLF43). Yeast DNA inserts were excised from each plasmid with *Eco*RI and used to transform DK337 to His$^+$. Heterozygous diploids containing the gene disruption were obtained by screening *Eco*RI-digested DNA from putative transformants by Southern blot hybridization (41). (A) Gene disruption of *LGN3*. (B) Southern blot probed with pLF47 DNA (*lgn3*::*HIS3*). Lanes: 1, untransformed DK337; 2, DK337 Ω*lgn3*, heterozygous transformant; 3 and 4, His$^+$ and His$^-$ haploid segregants from DK337 Ω*lgn3*. (C) Gene disruption of *LGN4*. (D) Southern blot probed with pLF43 DNA (*lgn4*::*HIS3*). Lanes: 1, untransformed DK337; 2, DK337 Ω*lgn4* heterozygous transformant; 3 and 4, His$^-$ and His$^+$ haploid segregants from DK337 Ω*lgn4*. The 10.1-kb band present in both autoradiograms is due to the resident *his3* gene hybridizing to the *HIS3* DNA present in the two probes.

(35 parental ditype:0 nonparental ditype:1 tetratype asci). Therefore, *LGN4* maps very close to the chromosome V centromere (30). This analysis did not allow us to order the *LGN4* gene with respect to the centromere and *URA3*.

DISCUSSION

Differentiating cells of both higher and lower organisms almost always express new sets of genes. Many of these differentiation-specific genes have been cloned and characterized (3, 8, 39, 47, 50). However, these studies were often performed without regard to the function of the differentiation-specific genes because methods for determining function were either difficult or unavailable. Obviously, determining the function of the differentiation-specific expressed genes would seem important for understanding the molecular events that lead to the particular differentiation in which the gene is expressed. We believed the yeast *S. cerevisiae* offered a unique opportunity for studying the function of cloned genes that are specifically expressed during sporulation, a model system for cellular differentiation. In vitro mutagenesis and one-step gene replacement techniques using the differentially expressed, cloned genes make it possible to create mutants that might exhibit phenotypes indicative of the mutated gene's function. Furthermore, *S. cerevisiae* sporulation provided a particularly good system for this approach because there are only about 200 *MAT***a**/*MAT*α-dependent sporulation-induced transcripts (D. Mills, personal communication). In contrast, other model systems show much greater increases in the number of differentiation-specific transcripts. *Dictyostelium discoidium* has 2,000 to 3,000 differentiation-specific transcripts (3); mammalian brain cells show more than 50,000 differentiation-specific transcripts (47). The small number of new genes expressed in sporulating *S. cerevisiae* originally led us to predict that the majority of them would be important for differentiation and lead to an easily identifiable sporulation phenotype when mutated.

In this report we describe the isolation of several genes which give rise to *MAT***a**/*MAT*α-dependent sporulation-induced transcripts. Three of these genes were then examined for function by using gene disruption and replacement. Surprisingly, diploid strains containing homozygous mutations in any of the three genes showed no obvious sporulation-defective phenotype. All mutants underwent meiosis and gave rise to four apparently normal, viable ascospores, suggesting that these three genes were dispensable for sporulation and spore germination.

We now propose to call these *MAT***a**/*MAT*α-dependent sporulation-induced transcribed sequences *SIT1* to *SIT4*, corresponding to *LGN1* to *LGN4*.

The first gene examined, *SIT2* (*LGN2*), was disrupted by a series of mutations: a simple insertion of the *S. cerevisiae HIS3* gene in one part of the gene followed by the insertion of the *S. cerevisiae LEU2* gene into the site of a 0.3-kb deletion in another part of the gene. Based on the sites for gene disruption, it would have been impossible for the mutant to produce a transcript containing much more than one-third of the wt sequences in a contiguous run. Our studies on the actual transcript produced in this mutant suggested that an even smaller part of the gene (probably less than 300 bases long) was expressed on a 0.85-kb fusion transcript composed mostly of *HIS3* sequences. This fusion transcript was much less abundant than wt *SIT2* RNA. Despite the expression of the fusion transcript, we consider it extremely unlikely that this small fraction of the gene would be capable of encoding any *SIT2* activity.

The lack of mutant phenotypes for the other two genes, *SIT3* (*LGN3*) and *SIT4* (*LGN4*), could have been due to residual function of the disrupted genes. These genes were interrupted by simple insertions using the *S. cerevisiae HIS3* gene. Normally, this type of mutation destroys gene function (32; Coleman et al., submitted for publication). However, in at least one case, a simple insertion mutation did not completely eliminate gene activity (1). Since sequences were not deleted from *SIT3* and *SIT4*, it is possible that the mutated genes still carry out their function. Unfortunately, owing to a lack of restriction enzyme cleavage sites (see the legend to Fig. 2), complete deletions that did not affect some of the downstream sequences were difficult to construct

without cloning additional flanking sequences. Accordingly, our conclusions about the dispensability of these two genes must be considered preliminary.

While we considered the lack of an easily detectable phenotype for all three mutants surprising, our results can be explained in several ways. First, the sporulation-induced genes could perform functions that are not essential to complete a single round of differentiation but are evolutionarily advantageous to the organism. Second, these genes could be important or essential under sporulation conditions that are different from those encountered in the laboratory. In each of these cases, it would have been impossible to discern any mutant phenotype with the limited testing that was done. Third, these genes may have been important for sporulation, but were functionally duplicated so both genes would have had to have been mutated to create a noticeable phenotype. Genomic Southern blot experiments done under both stringent (2× SSC, 65°C; Fig. 5 and 7) and nonstringent (2× SSC, 45°C) hybridization conditions (S. Wang, J. Lamb, and D. Kaback, unpublished data) indicated that these sequences were not duplicated. Thus, if duplicated, the copies would have had to evolve sufficiently to no longer cross-hybridize. Alternatively, two structurally unrelated genes could have overlapping functions or the requirement for any of these genes could have been bypassed entirely by alternative biochemical or developmental pathways.

The fourth possibility for the lack of a phenotype is that the *SIT* genes were fortuitously expressed and have no function in sporulating cells. The vegetatively expressed *CDC10* gene, thought to be dispensable for sporulation, produced severalfold more abundant transcripts in sporulating cells than in vegetative cells. We previously suggested that the sporulation-associated expression of *CDC10* also could have been fortuitous (22). However, unlike *CDC10*, the *SIT* genes described here never appeared to be expressed in either glucose- or acetate-grown vegetative cells or in asporogenous haploid or diploid cells incubated in sporulation medium. The *SIT* transcripts were *MAT*a/*MAT*α-dependent and were found only in sporulating cells. This specificity made it seem more likely that they would have had some function, albeit dispensable for sporulation. Therefore, we do not favor the idea of fortuitous expression for the *SIT* genes. However, at present we can only speculate on the potential involvement of these genes in some aspect of sporulation.

The results presented show the difficulties that can be expected in attempting to determine the function of a gene by simply mutating it and expecting the mutant to show a phenotype. This problem has been encountered previously. First, when the gene encoding a unique small-nuclear-RNA species was deleted, the mutant exhibited no phenotype (45). Second, gene-disruption mutants of the heat-shock genes, *HSP26* and *HSP90*, had no phenotype that could be related to the heat-shock response (L. Petko and S. Lindquist, personal communication; D. Finkelstein, personal communication). Finally, disruption of the *S. cerevisiae* sporulation-induced amyloglucosidase gene while preventing sporulation-associated glycogen breakdown, did not prevent meiosis or the production of viable ascospores (49). Thus, the absence of observable phenotypes prevents the definitive assignment of a biological function to even genes encoding known enzymes.

In conclusion, in the types of experiments presented here, only sporulation-induced genes that are both functionally unique and essential or important for meiosis, sporulation, or germination would be expected to give an easily discernible sporulation phenotype. Our results are consistent with the idea that many sporulation-induced genes may not fit into this category.

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