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The Yeast Gene *ERG6* Is Required for Normal Membrane Function but Is Not Essential for Biosynthesis of the Cell-Cycle-Sparking Sterol

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In *Saccharomyces cerevisiae*, methylation of the principal membrane sterol at C-24 produces the C-28 methyl group specific to ergosterol and represents one of the few structural differences between ergosterol and cholesterol. C-28 in *S. cerevisiae* has been suggested to be essential for the sparking function (W. J. Pinto and W. R. Nes, J. Biol. Chem. 258:4472–4476, 1983), a cell cycle event that may be required to enter G1 (C. Dahl, H.-P. Biemann, and J. Dahl, Proc. Natl. Acad. Sci. USA 84:4012–4016, 1987). The sterol biosynthetic pathway in *S. cerevisiae* was genetically altered to assess the functional role of the C-28 methyl group of ergosterol. *ERG6*, the putative structural gene for S-adenosylmethionine:Δ^{24}-methyltransferase, which catalyzes C-24 methylation, was cloned, and haploid strains containing *erg6* null alleles (*erg6Δ1* and *erg6Δ::LEU2*) were generated. Although *erg6Δ* cells are unable to methylate ergosterol precursors at C-24, they exhibit normal vegetative growth, suggesting that C-28 sterols are not essential in *S. cerevisiae*. However, *erg6Δ* cells exhibit pleiotropic phenotypes that include defective conjugation, hypersensitivity to cycloheximide, resistance to nystatin, a severely diminished capacity for genetic transformation, and defective tryptophan uptake. These phenotypes reflect the role of ergosterol as a regulator of membrane permeability and fluidity. Genetic mapping experiments revealed that *ERG6* is located on chromosome XIII, tightly linked to *sec59*.

Although the structural distinctions between ergosterol, the principal membrane sterol of fungi, and cholesterol, the animal counterpart, are few, they confer critical differences in the function of these molecules in vivo. For example, under certain conditions yeast cells can take up cholesterol and thus decrease their requirement for ergosterol to very low levels. Cholesterol alone, however, cannot completely substitute for ergosterol (7, 22, 24). Thus, at least some of the structural features specific to ergosterol are required for essential cell functions in *Saccharomyces cerevisiae*.

Two distinct roles for ergosterol in *S. cerevisiae* have been postulated. The first, fulfilled by the majority of the sterol in the cell, that of a bulk membrane function affecting membrane fluidity and permeability (3, 15, 23, 24; this report), can probably be provided by cholesterol (7, 22, 23). Ergosterol also appears to play an essential role in the yeast cell cycle. Sterol-starved cells undergo G_1 arrest, the release from which can be mediated by the addition of exogenous ergosterol (7). The sparking function (23, 24) or sterol synergism (22) can be mediated by very low concentrations of ergosterol or ergosterollike sterols.

Given the molecular complexity among different ergosterol precursor molecules and the interconversions that occur among them, it is not surprising that the structural requirements of the putative sparking sterol have been the subject of some controversy. Two ergosterol-specific structural features, the C-28 methyl group and the C-5,6 double bond, have been at the focal point of this dispute. Sterol biosynthesis in *S. cerevisiae* can be inhibited by growing cells anaerobically, through the use of specific inhibitors, or genetically with mutants that block both the ergosterol and heme pathways (Erg^- and Hem^-, respectively). Under any of these conditions, the uptake of exogenous sterols is enhanced, allowing sterol feeding experiments in an attempt to identify which sterols can support growth. Rodriguez and Parks (24) showed that the bulk sterol requirement of Erg^- Hem^- cells could be satisfied by cholestanol (5 μg/ml) if very small amounts of a sparking sterol (10 ng/ml) are included in the growth medium. They reported that only sterols possessing the C-5,6 double bond (e.g., ergosterol) or, similarly, those capable of being desaturated at C-5 fulfill the sparking requirement. On the other hand, Nes and colleagues used anaerobiosis and an inhibitor of squalene cyclase to induce exogenous sterol dependency and suggested that under these conditions growth is dependent on sterols that possess the C-28 methyl group (20, 21).

The enzymatic modifications of sterol that give rise to ergosterol-specific structural features are excellent targets for mutational studies. However, in part because of the unknown molecular nature of mutations used thus far, genetic studies using strains that contain mutations in the ergosterol biosynthetic pathway have not yet provided a clear understanding of the specific structural requirements of the sparking sterol(s) (2, 17, 18). The enzyme that catalyzes sterol methylation at C-24 in *S. cerevisiae* is S-adenosylmethionine:Δ^{24}-sterol-C-methyltransferase (SMT; EC 2.1.1.41). Thus, SMT, presumed to be encoded by the *ERG6* gene (4, 17, 19), imparts one of the key structural differences between ergosterol and cholesterol. McCammon et al. (17) described *erg6-5* mutant cells in which they failed to detect C-24-methylated membrane sterols. Although their data suggest that the structural requirements of the sparking sterol do not include methylation at C-24, the nature of the *erg6-5* allele remained unknown. A leaky *erg6* allele might still satisfy the sparking function if very low concentrations of the sterol are sufficient.

To examine the effect of a null allele of *ERG6*, we cloned the *ERG6* gene, disrupted it in vitro, and through gene

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TABLE 1. Strains used in this work

Strain	Genotype	Source or reference
JR5	α *erg6-5*	17
RSY27	α *sec59-1 his4-593 ura3-52 suc2-432*	R. Schekman
P3	**a** *ura3-52 erg6-5*	This study
R757	α *his4-15 ura3-52 lys9*	9
R1579	α *his4-15 ura3-52 lys9* [pRG461-2]	This study
R1739	**a** *ura3-52 erg6-5*	This study
R1838	**a** *ura3-52 trp1Δ1*	This study
R1842	**a** *his4-15 ura3-52 lys9*	This study
CG378/CG379	**a**/α *ura3-52/ura3-52 leu2-3,112/leu2-3,112 his5/+ trp1-289/trp1-289*	C. Giroux
CG378/CG379-T1	CG378/CG379 *erg6Δ::LEU2/ERG6*	This study
CG378/CG379-T2	CG378/CG379 *erg6Δ::LEU2/ERG6*	This study
R1799	α *leu2-3,112 ura3-52 trp1-289 his5 erg6Δ::LEU2*	This study
BKY36-2A	**a** *his4-15 ura3-52 lys9 leu2-3,112 trp1Δ1*	This study
BKY36-2A-T4	**a** *his4-15 ura3-52 lys9 leu2-3,112 trp1Δ1 erg6Δ::LEU2*	This study
BKY36-3-6B	**a** *his4-15 ura3-52 leu2-3,112*	This study
BKY36-3-6B-T1	**a** *his4-15 ura3-52 leu2-3,112 erg6Δ::LEU2*	This study
BKY36-2-8A	α *lys9 ura3-52 leu2-3,112*	This study
BKY36-2-8A-T3	α *lys9 ura3-52 leu2-3,112 erg6Δ::LEU2*	This study
BKY48	**a**/α *his4-15/+ leu2-3,112/+ ura3-52/ura3-52 lys9/+ trp1-289/+ erg6Δ::LEU2/+*	This study
BKY48-3A	α *his4-15 erg6Δ::LEU2*	This study
BKY48-3B	**a** *his4-15 erg6Δ::LEU2*	This study
BKY48-5C	α *leu2-3 ura3-52 erg6Δ::LEU2*	This study
BKY51	**a**/α *his4-15/his4-15 erg6Δ::LEU2/erg6Δ::LEU2*	This study
BKY135-1C	**a** *his4-15 erg6Δ::LEU2 sec59*	This study
A121-3D	α *leu2-3 pet8 met14 ade5 ura3 his7 lys1*	8
R1930	α *his4-15 ura3-52 lys9 ERG6*::pRG471::*erg6Δ1*	This study
R1931	α *his4-15 ura3-52 lys9 erg6Δ1*	This study
R1932	α *his4-15 ura3-52 lys9 erg6Δ1*::*pRG471*::*erg6Δ*	This study

replacement techniques generated haploid strains containing deletions within *ERG6*. *ERG6* is not required for normal vegetative growth, meiosis, or sporulation, strongly suggesting that C-24 methylation is not an essential feature of the sparking sterol in *S. cerevisiae*. However, *erg6Δ* mutations confer a variety of phenotypes consistent with alterations in membrane permeability and fluidity (3, 13, 15; this report). One of these phenotypes, decreased tryptophan uptake, may explain why *erg6* was previously thought to be linked to the *trp1* locus on chromosome IV (17).

MATERIALS AND METHODS

Media, strains, and plasmids. Strains of *S. cerevisiae* containing the *erg6-5* mutation were constructed through genetic crosses with strain JR5 (kindly supplied by L. W. Parks). Strains containing the *sec59-1* mutation were generated from crosses with strain RSY27 (kindly supplied by R. Schekman). YPD and YNB media and routine genetic techniques are described by Sherman et al. (28). YNB was supplemented with amino acids (YNB+AA) and, when required, with 0.05 μg of cycloheximide per ml or 2 mg of nystatin per ml. The strains and plasmids used are described in Table 1. Plasmids were selected and propagated in *Escherichia coli* HB101 (16).

Yeast transformation. Transformation of wild-type (*ERG6*) yeast strains was performed by the method of Ito et al. (12). The transformation frequency of *erg6-5* strains was severely impaired and required the following modifications. Cultures of *erg6-5* cells were grown to mid-log phase, diluted back to 2×10^6 to 3×10^6 cells per ml in YPD, and grown to 6×10^6 to 7×10^6 cells per ml on an orbital shaker at 30°C. A 50-ml sample of culture was harvested, washed twice in 0.1 M lithium acetate–10 mM Tris (pH 8.0)–1 mM EDTA, and suspended in a final volume of 200 μl of 0.1 M lithium acetate–10 mM Tris (pH 8.0)–1 mM EDTA. Immediately afterward, 20 μg of plasmid DNA and 100 μg of sheared calf thymus DNA were added, and the cell suspension was incubated on a roller at 30°C for 1.5 h. Aliquots (100 μl) of cell suspension were then transferred to microcentrifuge tubes to which 0.7 ml of 36% polyethylene glycol–0.1 M lithium acetate–1× TE was added. The cells were vortexed thoroughly, incubated at room temperature for 1 h, and heat-shocked at 42°C for 2 min before being plated out on selective medium (YNB+AA without uracil). To clone the wild-type *ERG6* gene, a library of wild-type yeast DNA fragments constructed in the shuttle vector YCp50 (25) was used.

Quantitative mass-mating assays. Quantitative mass-mating assays were performed by the method of Dutcher (S. Dutcher, Ph.D. thesis, University of Oregon, Eugene) essentially as described by Trueheart et al. (30). Mid-log-phase *MAT***a** and *MAT*α cells, containing appropriate auxotrophic markers, were mixed together and concentrated onto a 0.45-μm-pore-size nitrocellulose filter (Millipore Corp., Bedford, Mass.) at a density of 3×10^6 cells per parent. The filter was then transferred aseptically to a YPD plate and incubated for 3 h at 30°C to allow mating. The cells were then removed from filters by vigorous agitation (vortexing) in sterile water. The cell suspensions were diluted and plated on YPD plates to determine the titer of total cells and on YNB plates to determine the titer of prototrophic diploids. The ratio of these two numbers was defined as the mating frequency.

Plasmid constructions. Frameshift mutations were introduced into the cloned DNA fragment containing the *ERG6* gene by treating pRG458 with *Sal*I, *Xba*I, or *Asp*718 (*Kpn*I) restriction endonucleases, followed by treatment with the *E. coli* DNA polymerase I large fragment (Klenow). The blunt

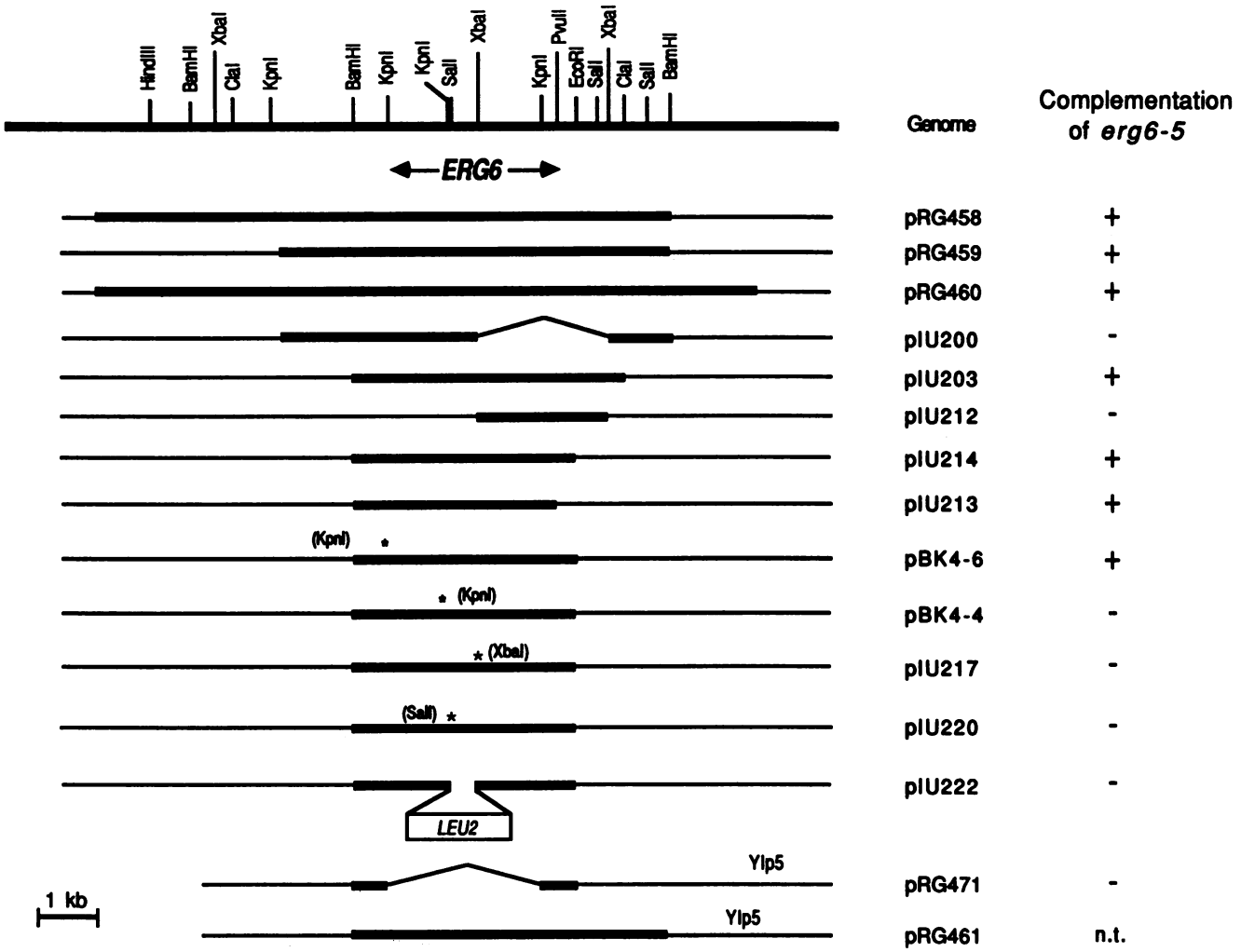

FIG. 1. Restriction endonuclease sites of the yeast *ERG6* gene and construction of a deletion-substitution mutation within the *ERG6* gene. Genomic clones are pRG458, pRG459, and pRG460. Subclones pIU200, pIU203, pIU212, pIU214, pIU217, pIU220, and pIU222 are in YCp50; pRG461 is in YIp5. Plasmid constructions are described in Materials and Methods. The ability of each plasmid to confer an Erg^+ phenotype after introduction into an *erg6-5* recipient is indicated at the right of each clone. Additional *Eco*RI restriction sites present outside the functional boundaries of *ERG6* are not presented. Disruption of the *Xba*I, *Sal*I, and *Asp*718 sites was performed as described in Materials and Methods. n.t., Not tested.

ends were then ligated to recircularize the plasmid. Plasmid pIU200 is pRG459 with the 2.4-kilobase-pair (kb) *Xba*I fragment deleted. Plasmids pIU203, pIU212, pIU213, and pIU214 represent subclones that contain the 4.95-kb *Bam*HI-*Cla*I, 2.4-kb *Xba*I, 3.7-kb *Bam*HI-*Pvu*II, and 4.1-kb *Bam*HI-*Eco*RI fragments from pRG458, respectively (Fig. 1). The subclones were constructed by ligation of the gel-purified DNA fragments into appropriately digested YCp50 (25). Plasmid pRG461 was constructed by subcloning the 3-kb *Bam*HI fragment from pRG458 into *Bam*HI-digested YIp5 (5). pIU217 and pIU220 are plasmid pIU214 with frameshift mutations generated at the *Xba*I and *Sal*I sites, respectively, by cleavage and subsequent treatment with Klenow prior to ligation. Plasmids pBK4-6 and pBK4-4 are plasmid pIU214 in which frameshift mutations were generated at the *Bam*HI-proximal *Kpn*I site and the middle *Kpn*I site, respectively, by cleavage with *Asp*718 and subsequent treatment with Klenow prior to ligation.

Construction of the *erg6* deletion and deletion-substitution mutations. Two mutations at *ERG6*, a deletion-substitution and a larger deletion, were constructed to generate null alleles. In the first case, yeast strain CG378/CG379-T1, a diploid homozygous for the *leu2-3,112* mutation and heterozygous for a deletion-substitution of the *ERG6* gene, was generated by the one-step gene disruption method of Rothstein (26) in the following manner. Plasmid pIU214 was digested with *Xba*I, followed by treatment with DNA polymerase I large fragment (Klenow). This linear, blunt-ended molecule was then treated with *Sal*I to generate a 400-base-pair deletion in *ERG6* (see Results). The 1.9-kb *Hpa*I-*Sal*I fragment from YEP13 (5), containing the *LEU2* gene, was then inserted into the *erg6* deletion, yielding plasmid pIU222. Digestion of pIU222 plasmid DNA with *Bam*HI and *Pvu*II released a 5.6-kb fragment containing the *LEU2* gene that was used to transform strain CG378/CG379 to leucine prototrophy.

A larger deletion mutation that removed at least 90% of the *ERG6* coding region was constructed in the following manner. The minimal DNA fragment containing the functional *ERG6* gene is the 2.9-kb region between the leftmost *Kpn*I site and the *Pvu*II site carried by plasmid pIU213 (Fig. 1). A haploid yeast strain containing a 2.6-kb deletion within

this region was constructed by digesting plasmid pRG461 to completion with *Kpn*I followed by ligation to circularize the DNA. The resulting plasmid, pRG471, containing the *erg6*Δ mutation designated *erg6*Δ*1*, was used to transform the *ura3-52 ERG6* strain R757 to a uracil prototrophy. Prior to transformation, the plasmid DNA was cleaved at the remaining *Xba*I site that lay outside of the deletion in order to enhance the frequency of integration at the *ERG6* locus. Ura^+ transformants presumably containing a duplication at the *ERG6* locus were colony purified and plated out onto YPD medium to which nystatin had been added. Nystatin-resistant colonies that arose were colony purified and tested for growth on YNB+AA without uracil to determine whether or not the cells had lost the integrated plasmid sequences and, concomitantly, the wild-type *ERG6* gene. Ura^- Nys^r and Ura^+ Nys^r cells were picked for Southern blot analysis to confirm loss of the wild-type *ERG6* gene and retention of the *erg6*Δ*1* mutation. Ura^- Nys^r cells retained only the *erg6*Δ*1* mutation, and Ura^+ Nys^r cells appeared to be *erg6*Δ*1*/*erg6*Δ*1* homozygous gene convertants that retained the integrated plasmid (*URA3*) sequences (see Results for details).

Construction of *ERG6* and *erg6*Δ::*LEU2* isogenic strains. Wild-type (*ERG6*) strains BKY36-2A, BKY36-2-8A, and BKY36-3-6B were transformed to leucine independence with the 5.6-kb *Bam*HI-*Pvu*II fragment described above to generate isogenic haploid *erg6*Δ::*LEU2* derivatives.

DNA manipulations. Rapid plasmid DNA isolation was done by the method of Holmes and Quigley (11); restriction analysis, gel electrophoresis, and Southern analysis were performed as described by Maniatis et al. (16). Hybridization probes were made by the method of random DNA hexamer priming according to the manufacturer's specifications (Pharmacia). *ERG6*-specific probes were prepared by gel purification of either the 4.1-kb *Bam*HI-*Eco*RI fragment or the 1.7- and 1.1-kb *Kpn*I fragments from pRG458. Filters containing the hybridized probe were washed four times for 15 min in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 65°C (high stringency) or in 6× SSC–0.1% sodium dodecyl sulfate at 55°C (low stringency) before autoradiography.

Tryptophan transport assays. The ability of strains to take up tryptophan from the growth medium was measured in the following manner. Cultures of *ERG6 trp1*Δ*1* (BKY36-2A) and *erg6*Δ::*LEU2 trp1*Δ*1* (BKY36-2A-4) cells were grown to saturation at 37°C in YPD. Cells were harvested and washed twice in sterile double-distilled water prior to a 1:10 dilution into YNB+AA (without tryptophan). The cultures were starved for tryptophan in this medium for 12 h at 23°C while being shaken. Tryptophan-starved cells were harvested, washed twice in cold double-distilled water, and suspended at a final concentration of 6,000 Klett units in YNB plus 0.2 mM tryptophan. [^{14}C]tryptophan (51.8 mCi/mmol; Dupont, NEN Research Products) was added to the cell suspension at a final concentration of 1.9 μCi/mmol. Following the addition of glucose (2%), 150-μl samples were taken at indicated time points, diluted into 5 ml of cold YNB+AA medium, filtered, and washed three times with cold YNB+AA medium. Radioactivity was measured with a Beckman LS 7000 scintillation counter.

Sterol analyses. Nonsaponifiable sterols were extracted from stationary-phase yeast cells into *n*-heptane as previously described (18). Extracts were scanned between 200 and 300 nm for absorption maxima. Gas chromatography-mass spectroscopy (GC-MS) analyses were carried out on a Finnegan MAT model 4500. GC separation was carried out on a Durabond DB-5 column (15 m by 0.323 mm) with an 0.25-μm film thickness as previously described (14). Alternatively, GC-MS analysis was carried out on a model HP5988A by using an HP-1 column (10 m by 0.53 mm) with a 2.65-μm film thickness. Column temperature was programmed from 200 to 300°C at 5°C/min, and the carrier gas was helium at a velocity of 25 cm/s. The MS was operated at an ionizing potential of 100 eV, and the ion source was at 300°C.

RESULTS

Genetic transformation of *erg6-5* strains. All *erg6-5* strains generated from crosses with parental strain JR5 exhibited very low frequencies of genetic transformation. Using the centromeric plasmid YCp50 and the transformation method of Ito et al. (12), we obtained, on average, less than 1 transformant per 100 μg of plasmid DNA. This phenotype segregated with the *erg6-5* allele in crosses between *erg6* and *ERG6* cells (data not shown). The frequency of transformation in *erg6-5* strains was increased markedly upon lowering the concentration of polyethylene glycol to 36% and changing the period of incubation in lithium acetate (see Materials and Methods). These modifications resulted in a transformation frequency of approximately 200 transformants per μg of YCp50. The decreased transformation frequency of *erg6-5* cells is not surprising given the pleiotropic membrane-related effects of this mutation (3, 15, 17; this report).

Isolation of the *ERG6* gene. *erg6* strains exhibit pleiotropic recessive phenotypes, including nystatin resistance (2, 18) and cycloheximide sensitivity (M. Bard, unpublished observations). Three plasmids containing the *ERG6* gene were isolated from a library of wild-type yeast DNA fragments on the basis of their ability to suppress the cycloheximide sensitivity in the *erg6-5* recipient strain R1739. R1739 cells failed to grow on YNB medium containing 0.05 μg of cycloheximide per ml, whereas transformants containing plasmids pRG458, pRG459, or pRG460 exhibited wild-type growth on this medium. All three plasmids were recovered in *E. coli* and, following transformation back into *S. cerevisiae*, conferred a Ura^+ Erg^+ cycloheximide-resistant (Cyh^r) phenotype to R1739. Restriction site cleavage analysis of the plasmids revealed that they share a large region of overlap (Fig. 1).

Since a number of yeast genes that confer cycloheximide resistance might be cloned by using this strategy, we tested our yeast transformants for other *erg6*-related recessive phenotypes. *erg6-5* cells (R1739) containing pRG458 or YCp50 were tested for nystatin sensitivity and analyzed for sterol composition. R1739/YCp50 cells were resistant to 2 μg of nystatin per ml, whereas pRG458 suppressed the nystatin resistance due to the *erg6-5* mutation. UV absorption spectra from R1739/YCp50 cells exhibited an absorption maximum at 235 nm typical of ergosterolless (*erg6-5*) cells, whereas R1739/pRG458 cells exhibited absorption spectra lacking this peak, consistent with the presence of ergosterol within their membranes (18; data not shown). GC-MS analysis of sterols present in R1739 and R1739/pRG458 cells also suggested that pRG458 contained the *ERG6* gene. R1739 cells contained sterols with molecular weights of 384 (zymosterol), 382 (cholestatrien-3b-ol), and 380 (cholestatetraen-3b-ol), indicative of the failure of these cells to methylate zymosterol at C-24. The sterols present in R1739/pRG458 cells were those found in wild-type yeast cells (14), including zymosterol, ergosterol (M_r, 396), and two ergostadienols (M_r, 398), each with a double bond both in the ring

and in the side chain (data not shown). Thus, the cloned region of yeast DNA in pRG458 suppresses all of the recessive phenotypes exhibited by *erg6-5* cells.

We performed a directed-integration experiment to determine whether the cloned DNA fragments encode the *ERG6* gene. A 6.4-kb *Bam*HI fragment from pRG458 was subcloned into the integrative vector YIp5, resulting in the recombinant plasmid pRG461 (Fig. 1). pRG461 was linearized by digestion with *Xba*I to enhance the frequency of integration into the chromosomal site homologous to the subcloned DNA fragment. A *ura3-52 ERG6* strain (R757) was transformed to a Ura$^+$ phenotype with this DNA, and, as expected, all transformants were found to remain Erg$^+$ (Cyh^r). One of the Ura$^+$ Erg$^+$ transformants (R1579) was crossed with the *ura3-52 erg6-5* strain P3, and after sporulation of the resulting diploid, asci were dissected onto YPD agar medium. Each of 10 tetrads showed a 2 Ura$^+$ Erg$^+$:2 Ura$^-$ Erg$^-$ (Cyh^s) segregation pattern, demonstrating complete genetic linkage between the integrated Ura$^+$ plasmid sequences and the Erg$^+$ (Cyh^r) phenotype. Integration of the plasmid at the *ERG6* locus confirmed that the cloned DNA fragments in plasmids pRG458, pRG459, and pRG460 carry the authentic *ERG6* gene.

Frameshift mutagenesis of *ERG6*. The approximate location of the *ERG6* gene on plasmid pRG458 was determined through in vitro mutagenesis of selected restriction endonuclease sites located within the cloned DNA fragment. Frameshift mutations were introduced at *Sal*I, *Xba*I, and *Kpn*I (*Asp*718) sites (see Materials and Methods). The effect of each of these mutations was examined by transforming *ura3-52 erg6-5* cells (strain R1739) with each of the mutant plasmids and testing for their ability to complement the Erg$^-$ (Cyh^s) phenotype. The results of these experiments suggest that one of the *Sal*I sites, one of the *Kpn*I sites, and one of the *Xba*I sites (indicated in Fig. 1) lie within the functional *ERG6* gene. Consistent with this interpretation, a subclone containing the 4.1-kb *Bam*HI-*Eco*RI fragment encompassing these sites conferred a Cyh^r phenotype to the *erg6-5* strain R1739 and thus contains the functional *ERG6* gene.

Disruption of the *ERG6* gene. Reports by Parks and colleagues suggested that the sterol products of SMT may be absent in the *erg6-5* strains they examined (17). Thus, *ERG6* may not be an essential gene and the regulatory sterol that provides the putative sparking function in *S. cerevisiae* may not require the SMT-mediated C-28 methyl group. To test this hypothesis, we examined the effect of two *erg6* deletion mutations.

The first *erg6* deletion mutation, designated *erg6*Δ::*LEU2*, contains a deletion within the functional *ERG6* gene and therefore avoids the potential disruption of adjacent genes. The *erg6*Δ::*LEU2* mutation was generated by replacing the *ERG6*-internal *Sal*I-*Xba*I fragment with the yeast *LEU2* gene (described in Materials and Methods; Fig. 1). A 5.2-kb *Bam*HI-*Pvu*II fragment encompassing the *erg6*Δ::*LEU2* allele was used to transform a *leu2-3,-112/leu2-3,-112 ERG6/ERG6* homozygous diploid (strain CG378/CG379) to leucine prototrophy. The Leu$^+$ transformants that arose were presumed to involve the replacement of the wild-type *ERG6* allele by the *erg6*Δ::*LEU2* allele (26). Southern blot analysis confirmed this interpretation (Fig. 2; described below).

Tetrad analysis of meiotic spore colonies derived from the heterozygous *ERG6/erg6*::*LEU2*-transformed diploids was performed to reveal the effect of the *erg6*Δ::*LEU2* mutation in haploid cells. Germination of the meiotic spores at 22 or 30°C on YPD medium gave rise to a 2 viable:2 nonviable segregation pattern. Only Leu$^-$ spores developed into colonies, suggesting that the *erg6*Δ::*LEU2* mutation resulted in nonviable or subviable cells. However, when spores were dissected from the same Leu$^+$ diploid transformant and incubated on YPD medium at 37°C, viability was approximately 100%; 19 of 20 asci dissected gave rise to 4 viable spores. All 19 tetrads in this experiment exhibited a 2 Leu$^+$:2 Leu$^-$ segregation pattern. All Leu$^+$ spore colonies were Cyh^s, indicating functional disruption of the *ERG6* gene. Southern blot analysis of genomic DNA prepared from the Leu$^+$ Erg$^-$ and Leu$^-$ Erg$^+$ haploids confirmed that the former harbored the *erg6*Δ::*LEU2* mutation (Fig. 2). The tetrads that grew on YPD at 37°C were replica plated to fresh YPD medium and tested for growth at different temperatures. All of the *erg6*Δ::*LEU2* mutants failed to grow at 22 or 30°C but grew normally at 37°C. Thus, the *erg6*Δ::*LEU2* mutation appeared to confer cold sensitivity for growth.

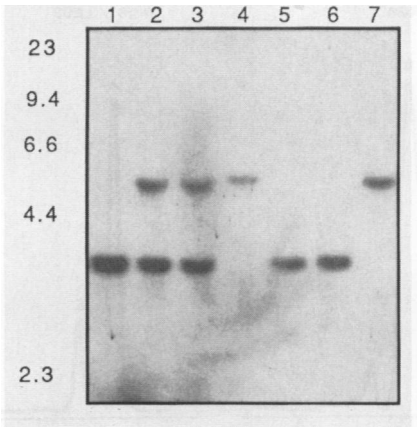

FIG. 2. Southern blot analysis of *ERG6* and *erg6*Δ::*LEU2* genomic DNA. DNA from *ERG6* and *erg6*Δ::*LEU2* strains was digested with *Pvu*II and *Bam*HI prior to electrophoresis and blotting to the filter. The 1.7- and 1.1-kb *Kpn*I fragments from pRG458 were used as the probe. Stringent conditions used to remove nonspecifically bound ^{32}P-labeled probe are detailed in Materials and Methods. Lane 1, homozygous *ERG6/ERG6* diploid strain CG378/CG379; lanes 2 and 3, heterozygous *erg6*Δ::*LEU2/ERG6* (Leu$^+$) transformants of CG378/CG379; lanes 4 and 7, *erg6*Δ::*LEU2* (Leu$^+$ Erg$^-$) haploid progeny; lanes 5 and 6, *ERG6* (Leu$^-$ Erg$^+$) haploid progeny. Lanes 4 to 7 contain all progeny of a single meiotic tetrad obtained from the diploid in lane 3.

Since the *ERG6/erg6*Δ::*LEU2* diploid transformant was homozygous for the *trp1-289* mutation, one of the *erg6*Δ::*LEU2 trp1-289* progeny (R1799) was crossed with a wild-type *ERG6 TRP1* strain (R1842) to examine the phenotype of *erg6*Δ::*LEU2 TRP1* recombinants. After sporulation of the resulting diploid (strain BKY48), asci were dissected onto YPD medium and incubated at 37°C. Of 20 asci dissected, 19 gave rise to 4 viable spore colonies. The spore colonies were replica plated to the appropriate media to test for the segregation of *erg6*Δ::*LEU2* and *trp1-289*. The results of these tests led to two unexpected observations. (i) The *ERG6* and *TRP1* loci, previously reported to be linked (17), showed a segregation pattern indicating nonlinkage: 14 parental ditype:3 tetratype: 6 nonparental ditype. (ii) Only *erg6*Δ::*LEU2 trp1-289* spores exhibited the cold-sensitive phenotype described above; *erg6*Δ::*LEU2 TRP1* recombinants grew normally at all temperatures tested. Thus, *erg6*Δ::*LEU2* cells are cold sensitive only if they are also defective for tryptophan biosynthesis. The most likely explanation for this phenotype, that tryptophan uptake by *erg6*Δ::*LEU2* cells is defective, was confirmed in subsequent experiments (see below).

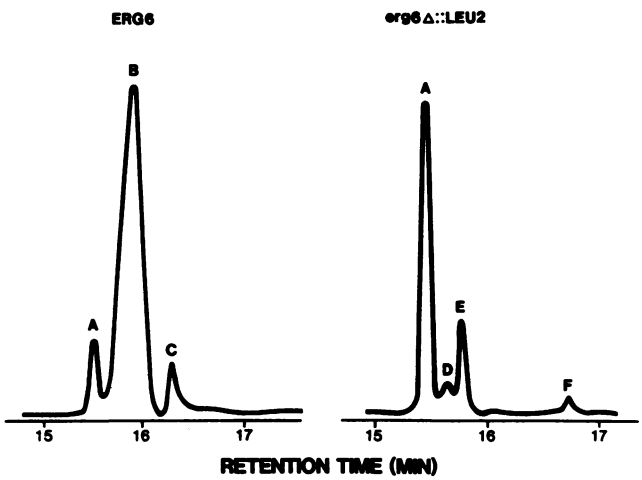

FIG. 3. Profiles of sterols extracted from CG378 (*ERG6*) and BY48-5C (*erg6Δ::LEU2*) strains. Peak A, Zymosterol present in both strains; peaks B and C, the methylated sterols, ergosterol and ergostadiene, respectively; peaks D, E, and F, nonmethylated cholestane-type sterols. GC-MS was carried out on a model HP5988A with an HP-1 column.

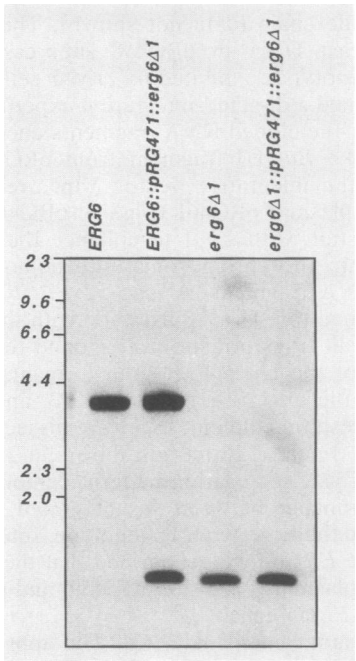

FIG. 4. Southern blot analysis of *ERG6*, *ERG6*::pRG471, and *erg6Δ1* genomic DNA. DNA from *ERG6*, *ERG6*::pRG471, and *erg6Δ1* strains was digested with *Bam*HI and *Eco*RI prior to electrophoresis and blotting to the filter. The 4.1-kb *Bam*HI-*Eco*RI fragment from plasmid pRG461 was used as the probe. Low-stringency washing conditions used to remove nonspecifically bound [^{32}P]dATP-labeled probe are described in Materials and Methods. Lane 1, Wild-type *ERG6* strain R757; lane 2, *ERG6*::*URA3* transformant of strain R757 (R757/pRG471); lane 3, Ura$^-$ Erg$^-$ (Nys^r) mitotic segregant of R757/pRG471; lane 4, Ura$^+$ Erg$^-$ (Nys^r) mitotic segregant of R757/pRG471. The Ura$^+$ Erg$^-$ (Nys^r) strain analyzed in lane 4 is a probable *erg6Δ1::URA3::erg6Δ1* gene convertant of strain R757/pRG471.

To assess the effect of the *erg6Δ::LEU2* mutation on other aspects of the yeast life cycle, a homozygous *erg6Δ::LEU2/erg6Δ::LEU2* diploid (strain BKY51) was generated by mating strains BKY48-3A and BKY48-3B. Sporulation and subsequent growth of the haploid progeny from this diploid at 22 and 37°C were indistinguishable from those of wild-type cells. Thus, the *erg6Δ::LEU2* mutation has no effect on normal mitotic growth, meiosis, or sporulation. These results suggested that *ERG6* is not essential and supported the interpretation of Parks and colleagues (17) that *ERG6*-mediated SMT activity, catalyzing the C-24 methylation of yeast sterols, is not required for viability. The C-28 methyl group is evidently not an essential structural component of the putative sparking sterol. This conclusion relies on two assumptions. The first is that *erg6Δ::LEU2* cells do not contain SMT activity, and the second is that the *erg6Δ::LEU2* mutation is a null allele of *ERG6*.

To test the first assumption, the sterols present in *ERG6* and *erg6Δ::LEU2* cells were analyzed. The data presented in Fig. 3 represent the sterol profiles obtained from wild-type and *erg6Δ::LEU2* strains. Peaks A, B, and C found in the profile of the wild-type strain (CG378) represent zymosterol (M_r, 384), ergosterol (M_r, 396), and an ergostadiene (M_r, 398), respectively. Within the limits of detection, the *erg6*-disrupted strain BKY48-5C was found to contain only nonmethylated sterols. Peaks D and E, found in the *erg6Δ::LEU2* strain profile, are two cholestatrienes each having a molecular weight of 382. Peak E is the major cholestatrienol and is therefore presumably cholesta-5,7,24-trien-3β-ol. This interpretation is consistent with a previous description of this sterol (4). While we have not observed other cholestatrienols before, the minor component peak D may also correspond to cholesta-5,7,22-trien-3β-ol. Peak F has an M_r of 380 and is cholestatetraen-3β-ol. Whereas zymosterol, the normal substrate for C-24 methylation, is only a minor peak in the wild-type strain, it is the major sterol in the *erg6Δ::LEU2* strain. Finally, UV profiles of the sterols that accumulate in the *erg6Δ::LEU2* strain are identical to those previously reported for *erg6-5* strains (4; data not shown).

The second assumption outlined above, that the *erg6Δ::LEU2* mutation is a null allele of *ERG6*, was considered plausible because the deletion-substitution mutation removed nearly 300 base pairs of DNA required for *ERG6* function and inserted the 1.9-kb *LEU2* gene in its place. However, because it was formally possible that the *erg6Δ::LEU2* mutation retained residual activity, we constructed a second *erg6Δ* mutation that removed a much larger region of *ERG6*. The 2.6-kb fragment defined by the *Kpn*I site proximal to the *Bam*HI site and the *Kpn*I site proximal to the *Pvu*II site was deleted from plasmid pRG461; the resulting mutation was designated *erg6Δ1*. This deletion removes approximately 90% of the minimal 2.9-kb DNA fragment shown to contain the functional *ERG6* gene (Fig. 1) and thus in all likelihood represents a null allele.

Haploid strains containing the *erg6Δ1* mutation were constructed as described in Materials and Methods. Low-stringency Southern blot analysis of genomic DNA prepared from *ERG6*, *ERG6*::pRG471, and *erg6Δ1* haploid cells indicates that *ERG6* is a unique gene in *S. cerevisiae* (Fig. 4). *erg6Δ1* haploids are viable and exhibit the Nys^r Cyh^s phenotype. The viability of *erg6Δ1* haploid cells demonstrates that *ERG6* is nonessential.

Effect of *erg6Δ::LEU2* on tryptophan transport (uptake). The cold sensitivity of *erg6 trp1* cells, described above, suggested that tryptophan transport might be adversely affected by the loss of C-28 sterols in the membrane. The effect of the *erg6Δ::LEU2* mutation on tryptophan uptake was examined directly by measuring the rate of [^{14}C]tryptophan uptake by cultures of isogenic *ERG6* and

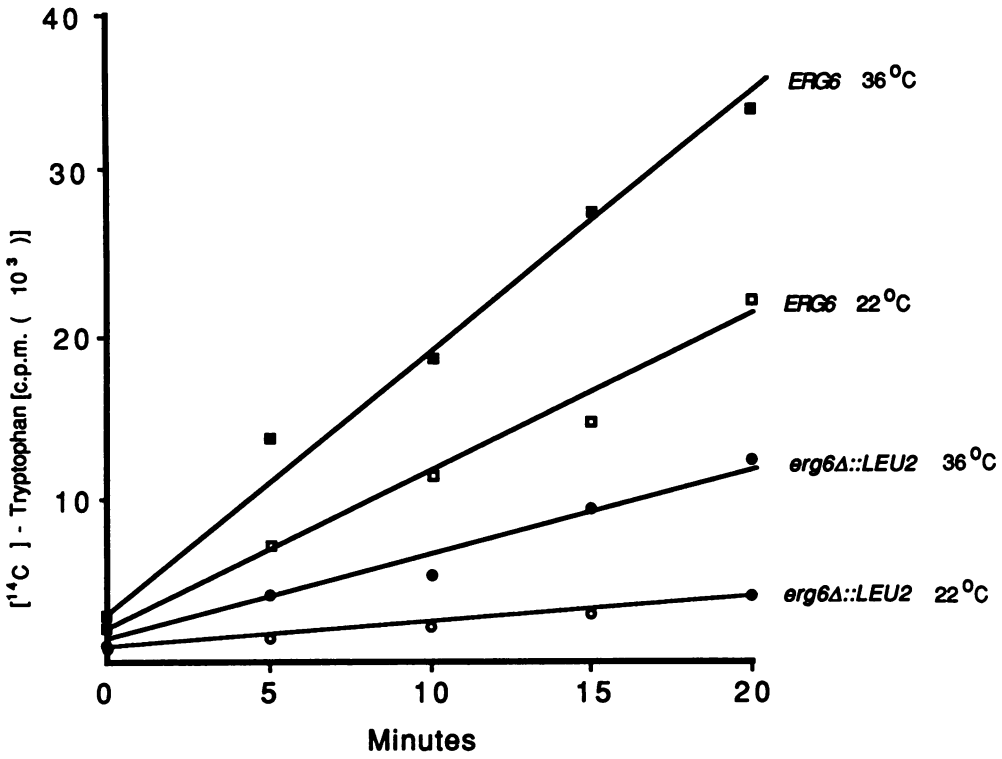

FIG. 5. Tryptophan uptake by isogenic *ERG6* (BKY36-2A) and *erg6Δ::LEU2* (BKY36-2A-T4) cells. [^{14}C]tryptophan uptake was measured in YNB medium plus 0.2 mM tryptophan. The data in the figure represent an average of values from five experiments at 22°C and two experiments at 37°C. At a given temperature, the results did not vary significantly from one experiment to another.

erg6Δ::LEU2 strains BKY36-2A and BKY36-2A-4T, respectively. The results of several independent experiments demonstrated that [^{14}C]tryptophan uptake from YNB + 0.2 mM tryptophan at 22°C is reduced approximately sixfold in *erg6Δ::LEU2* cells as compared with *ERG6* cells (Fig. 5). At 37°C, the relative rate of tryptophan uptake by *erg6Δ::LEU2* cells is reduced only 2.5-fold. The increased rate of uptake by *erg6Δ::LEU2* cells at the elevated temperature is sufficient to support growth of *erg6Δ::LEU2 trp1* cells. The reduced [^{14}C]tryptophan uptake is not the effect of an inherently slower growth rate of the *erg6Δ::LEU2* cells. Isogenic *ERG6* and *erg6Δ::LEU2* strains prototrophic for tryptophan (BKY36-3-6B and BKY36-3-6B-T1, respectively; Table 1) grew at rates indistinguishable from one another at 22 or 37°C (data not shown).

The addition of high concentrations of tryptophan to YPD medium suppressed the cold sensitivity of *erg6Δ::LEU2* cells (data not shown). To determine whether the *erg6-5* mutation conferred a similar defect tryptophan transport, strain JR5 was crossed with an *ERG6 trp1Δ1* strain (R1838). The *erg6-5 trp1Δ1* meiotic spores obtained from this cross exhibited cold sensitivity for growth on YPD medium. Although auxotrophy for histidine, lysine, or uracil did not confer cold sensitivity for growth on YNB+AA or YPD medium, it is not known whether their transport is decreased in *erg6* cells.

Effect of the *erg6Δ::LEU2* mutation on conjugation. During the course of generating homozygous *erg6Δ::LEU2/erg6Δ::LEU2* diploids, we noticed that mating mixtures of *erg6Δ::LEU2 MAT***a** and *erg6Δ::LEU2 MATα* cells yielded aberrant-looking zygotes. Matings between *erg6Δ::LEU2* and *ERG6* strains also showed marked deficiencies in the rate and frequency of zygote formation. This mating defect cosegregated with the disrupted *erg6* allele in tetrads from such crosses. The conjugation defect resulting from the *erg6Δ::LEU2* disruption was analyzed further through quantitative mass-mating assays by the method of Trueheart et al. (30). The results of the mass-mating experiments are presented in Table 2. After 2 h of mating at 30°C, there were approximately threefold-fewer diploids (conjugative pairs) formed in *erg6/erg6* matings than in *ERG6/ERG6* wild-type matings. The results show that *erg6Δ::LEU2 MATα* cells are more defective in conjugation than *erg6Δ::LEU2 MAT***a** cells but that the defect is greatly enhanced in homozygous *erg6Δ::LEU2* × *erg6Δ::LEU2* matings. The mating defect in *erg6Δ::LEU2* × *ERG6* matings is most obvious after relatively short periods of mating (2 h), and such matings are almost indistinguishable from *ERG6* × *ERG6* matings after 4 h (Table 2). The molecular basis of this mating defect is not yet known.

Genetic mapping of *ERG6*. Hybridization of the 1.16-kb *Kpn*I fragment purified from plasmid pRG458 to yeast chromosomes immobilized on filters (6) resulted in the detection of a single band corresponding to chromosome XIII (data not shown). The single band was identified following stringent washing of the hybridized blot, suggesting that the *ERG6* gene is not duplicated on any of the other chromosomes. Southern analysis confirmed that *ERG6* is present in a single copy in the yeast genome (described above).

Precise mapping of the *ERG6* locus was determined by crossing an *erg6Δ::LEU2* sec59 strain (BKY135-1C) with a *pet8* strain (A121-3D; Table 1). Tetrad analysis of the meiotic progeny obtained from this cross confirmed that *ERG6* is linked to both the centromere and *sec59* on chromosome XIII (Fig. 6).

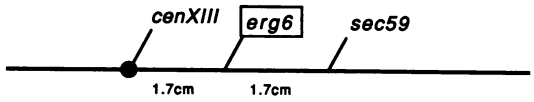

FIG. 6. Genetic map of the right arm of chromosome XIII. The genetic distance between *erg6* and the linked markers on the right arm of chromosome XIII is indicated in centimorgans (cm). The genetic map positions are derived from tetrad analysis of meiotic progeny from a cross between BKY135-1C and A121-3D (Table 1). Among 117 asci analyzed, 113 showed first-division segregation for the *erg6-pet8* marker pair while 4 showed second-division segregation. The sec59-pet8 marker pair exhibited 109 first-division segregation:8 second-division segregation, and the *erg6-sec59* marker pair exhibited 113 parental ditype: 4 tetratype: 0 nonparental ditype. The four asci that showed second-division segregation for *erg6* (by comparison with *pet8*) were parental ditype for the *erg6-sec59* marker pair, and the four *erg6-sec59* tetratype asci showed first-division segregation for *erg6* but second-division segregation for *sec59*, consistent with the order *cenXIII-erg6-sec59*. It is unlikely that *pet8* underwent any recombination with its centromere in this sample size, since we have observed none among over 400 *pet8-trp1* asci previously analyzed in this strain background (R. Gaber, unpublished observation).

DISCUSSION

The recent discovery that ergosterol plays an essential cell-cycle-specific role in *S. cerevisiae* (7) has reemphasized the significance of the so-called sparking function mediated by this sterol. The inability of cholesterol or cholestanol to completely replace ergosterol as the sparking sterol has focused investigations into those structural components that are specific to ergosterol. To assess the role of specific structural features of the sparking sterol, we have taken a genetic approach, altering the sterol biosynthetic pathway in well-defined ways. We have targeted the yeast SMT, because it catalyzes formation of the ergosterol-specific C-28 methyl group.

The available evidence suggests that *ERG6* is the structural gene encoding SMT in *S. cerevisiae*. A number of independent mutations have been isolated that are severely deficient in SMT and all are allelic to *ERG6* (17; Bard, unpublished). However, it is possible that *ERG6* is a regulatory gene controlling SMT expression or activity. If so, *ERG6* would represent a positive-acting factor that is responsible for a thousandfold increase in SMT activity (17).

We have cloned the *ERG6* gene, have shown that it is centromere proximal to *sec59* on chromosome XIII, and have generated haploid yeast cells that harbor *erg6* null alleles. Although the *erg6Δ* mutations confer pleiotropic effects on the cell, *ERG6* is a unique, nonessential gene.

TABLE 2. Effect of the *erg6* disruption on the frequency of diploid formation during mating[a]

Mated strains	% of diploids formed after[b]:		
	2 h	3 h	4 h
*MAT***a** *ERG6* × *MATα* *ERG6*	50.6 ± 5.9	60.7 ± 13.4	60.9 ± 22.0
MATα erg6Δ × *MAT***a** *ERG6*	35.1 ± 8.3	53.2 ± 16.4	55.3 ± 18.9
*MAT***a** *erg6Δ* × *MATα* *ERG6*	47.6 ± 7.4	62.1 ± 16.2	63.8 ± 17.0
*MAT***a** *erg6Δ* × *MATα* *erg6Δ*	18.7 ± 5.0	24.2 ± 11.5	27.0 ± 2.8

[a] See Materials and Methods. Strains tested for mating ability were Erg$^+$ (*ERG6*) and Erg$^-$ (*erg6Δ::LEU2*) isogenic derivatives of BKY36-3-6B (*MAT***a**) and BKY36-2-8A (*MATα*). Complete genotypes are presented in Table 1.

[b] Mean ± standard deviation. Percentages reflect averages of three independent experiments.

Our results suggest that the structural requirements of the regulatory sterol(s) involved in the cell cycle sparking function do not include the C-28 methyl group. McCammon et al. (17) were unable to detect C-24 methylated sterols in the nonsaponifiable fraction of *erg6-5* cells. On the basis of these results, they suggested that the regulatory sterol does not require the C-28 group to satisfy the sparking function. However, a cautious interpretation of these results was imperative because of the possibility that *erg6-5* is a leaky mutation that results in a very low level of SMT activity. Indeed, they estimated that *erg6-5* mutants might still contain 4×10^4 ergosterol molecules per cell. This could satisfy the low levels of the putative sparking sterol thought to be required to enter the cell cycle at G1.

Our results and those of McCammon et al. (17) conflict with reports that suggest that *ERG6*-dependent C-24 methylation of sterols is essential for cell viability. Nes and colleagues tested the ability of different exogenous sterols to promote the growth of cells unable to synthesize their own sterols and found only sterols that contain C-28 methyl groups to be effective (20, 21). However, in these experiments the synthesis of both heme and sterols was blocked, as the cells were grown anaerobically in the presence of a metabolic inhibitor of squalene cyclase in order to induce uptake of exogenous sterols. In fact, in all cases in which the sparking function has been studied, both ergosterol and heme biosynthesis have been inhibited, either in this fashion or by using heme-ergosterol double mutants (*hem erg*). Since heme is a cofactor in the cytochrome P-450 reaction required for lanosterol demethylation (1), sterol biosynthesis is blocked at a relatively early step. With these approaches, the degree to which the structural requirements of the sparking sterol can be determined, merely by adding one or more exogenous sterols such as ergosterol, is limited. The feeding of various sterol intermediates to such cells can produce ambiguous results due to the interconversion of these molecules as well as to the nonlinearity of sterol biosynthesis downstream from lanosterol demethylation.

An additional complication, inherent in feeding experiments that depend on the uptake of exogenous sterols, is that of differential rates of uptake. Ergosterol uptake is 5- to 10-fold more efficient than that of cholesterol (27, 29). As a consequence, contaminating amounts of ergosterol or ergosterollike molecules taken up in feeding experiments can be significantly underestimated. Thus, feeding experiments alone could miss a C-28 requirement.

Generating null alleles of genes encoding the relevant enzymes allows specific structural requirements of the sparking sterol to be tested directly. In this regard, our results strongly suggest that methylation at C-24 is not essential for functional activity of the sparking sterol. The inability to detect C-24-methylated sterols in *erg6Δ::LEU2* haploid cells supports the interpretation that a null allele has been generated in a unique gene. However, it is possible that trace levels of C-24-methylated sterols exist in *erg6Δ::LEU2* cells. Perhaps these molecules could arise from low levels of SMT activity due to a partially functional *erg6Δ::LEU2* allele. To remove this ambiguity, we generated haploid strains that contained deletions of approximately 90% of the minimal DNA fragment known to contain the functional *ERG6* gene. The resulting mutation, *erg6Δ1*, is in all likelihood a null allele of *ERG6*. The viability of haploid *erg6Δ1* strains confirms that *ERG6* is nonessential. If another gene

encoding SMT exists in *S. cerevisiae*, it is nonhomologous, as we were unable to detect *ERG6*-related DNA sequences by hybridization of *ERG6* to genomic DNA. Nevertheless, if putative cryptic essential SMT genes exist, *erg6Δ* strains may be used to detect conditional mutations in these genes.

The effects of lethal or sublethal mutations in the sterol biosynthetic pathway can sometimes be masked by the presence of a second mutation. For example, it was shown that an *erg7* mutation greatly enhances the ability of *hem1* mutants to grow on exogenously supplied sterols (29). In another case, a *hem3* mutation was found to be essential for viability of *erg12* strains (10). In both examples, the second mutation was thought to enhance the uptake of exogenous sterols. Results from our genetic crosses demonstrate that *erg6Δ::LEU2* cells are not dependent on other unlinked mutations for viability. Since haploid strains containing the *erg6Δ::LEU2* or the *erg6Δ1* mutations grow as well as isogenic wild-type strains and do not require exogenously added sterols, we conclude that the *ERG6* gene is not essential for growth and, therefore, cannot be required for the sparking function.

The *erg6Δ* mutations result in a number of defects that appear to be related to alteration of membrane fluidity and permeability. These include hypersensitivity to cycloheximide, resistance to nystatin, decreased mating frequency, decreased transformation frequency, and decreased tryptophan uptake. We have not determined whether the transport of compounds other than tryptophan is similarly affected. Although the transport of leucine, lysine, uracil, and histidine is sufficient to confer normal growth of the appropriate auxotrophic strains on typical yeast media, one might predict that the function of a number of integral membrane proteins involved in transport is affected by the altered membrane environment created by *erg6Δ* mutations.

The reduced tryptophan uptake in *erg6* mutants at typical incubation temperatures might have been responsible for the low frequency of *trp1 erg6-5* recombinants observed by McCammon et al. (17) that led them to conclude that *erg6* was linked to *trp1* on chromosome IV. We have demonstrated that *erg6* is linked to *sec59* on chromosome XIII.

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