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CYTOCHROME *P*450 LANOSTEROL 14α-DEMETHYLASE (CYP51): INSIGHTS FROM MOLECULAR GENETIC ANALYSIS OF THE *ERG11* GENE IN *SACCHAROMYCES CEREVISIAE*

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Summary—Eukaryotes characteristically express a cytochrome *P*450-catalyzed sterol 14α-methyl demethylase as an essential step in the production of membrane sterols. Lanosterol 14α-demethylase of *Saccharomyces cerevisiae* is the best characterized representative of these enzymes among fungi and provides a model system for the molecular genetic analysis of the reaction. The gene for this *P*450 and the gene for the *S. cerevisiae* NADPH–cytochrome *P*450 reductase have been examined by mutational inactivation and for their regulation of expression. Our results have contributed to a better understanding of sterol biosynthesis in relation to mechanisms of resistance to fungicidal demethylase inhibitors, and promote the rationale for using *S. cerevisiae* in the further characterization of structure function relationships among sterol 14α-demethylases.

OUTLINE

Demethylation of 14α-methyl sterols is a cytochrome *P*450-catalyzed reaction essential for the biosynthesis of sterols by eukaryotes. In recent years, the yeast *Saccharomyces cerevisiae* has gained prominance as a model system for the molecular genetic analysis of this reaction. Moreover, this analysis has taken on added importance because of the rapidly increasing need for more effective antifungal agents. This demethylase reaction is inhibited by a major portion of the antifungal agents in current use, which makes this enzyme system an attractive target for structure function studies leading to the development of still more useful fungicides.

This paper reviews some of the basis for these statements, summarizes pertinent findings from this laboratory, and provides speculations upon directions of research using *S. cerevisiae*, for the further characterization of structure function relationships among sterol 14-methyl demethylases.

1. THE *S. CEREVISIAE* GENE *ERG11* ENCODES LANOSTEROL 14α-DEMETHYLASE, CYP51

Lanosterol 14α-demethylase of *S. cerevisiae* is the most extensively characterized of all fungal *P*450s, and simultaneously is the best understood among the sterol 14α-demethylases. The initial studies on this enzyme have been extensively reviewed [1, 2] and only specific advances are cited here. The presence of a *P*450 spectrum in growing cultures of *S. cerevisiae* was detected as early as 1964 [3]. By 1978, Aoyama and Yoshida and coworkers had shown that the

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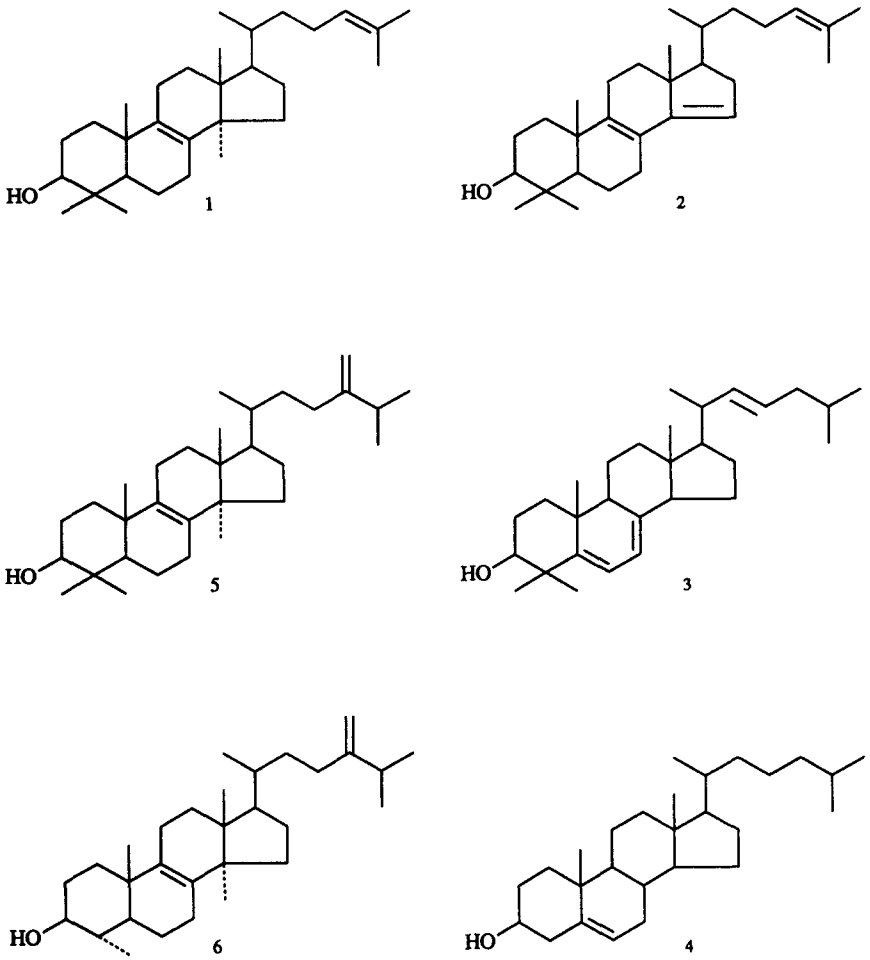

Fig. 1. Chemical structure of sterols. 1: lanosterol; 2: 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol, the immediate product of the lanosterol 14α-demethylase reaction; 3: ergosterol; 4: cholesterol; 5: 24-methylene-24,25-dihydrolanosterol; and 6: obtusifoliol.

majority of this *P*450 spectrum was accounted for by a single enzyme, which catalyzed the 14α-demethylation of lanosterol [4, 5]. The purified enzyme, named *P*45014DM [6], required only NADPH–cytochrome *P*450 reductase (CPR1) as an electron carrier protein, in a membrane bound system catalyzing the reaction indicated below [7]. Structures of the sterols cited in this paper appear in Fig. 1.

$$\text{lanosterol} + 3\text{NADPH} + 3O_2 + 3H^+ \rightarrow 4,4\text{-dimethyl-5}\alpha\text{-cholesta-8,14,24-trien-3}\beta\text{-ol} + \text{HCOOH} + 3\text{NADP}^+ + 4H_2O$$

This reaction is the first step in the biosynthetic conversion of lanosterol to the yeast sterol ergosterol; in mammals an orthologous enzyme catalyzes the identical reaction during cholesterol synthesis [8, 9].

The action of ketoconazole (Kc) as a yeast inhibitor was shown in 1980 to occur through binding to *P*45014DM [10; see also 11]. We used this inhibitor to clone the *S. cerevisiae* *P*45014DM gene from a library contained on a high copy plasmid, in a procedure based upon increased ketoconazole resistance in yeast transformants that over-expressed the gene [12]. Sequence characterization of the gene revealed a *P*450 amino acid coding sequence with the same N-terminal sequence determined for *P*45014DM by Yoshida and co-workers [13, 14]. This *P*450 is the first member of the CYP51 *P*450 family, i.e. CYP51A1 [15].

Based upon physiological properties of mutant alleles described in the next section, a *S. cerevisiae* gene, previously identified in the literature as *ERG11*, has been identified as the structural gene for this enzyme. Thus, for this paper, ERG11 is used when referring to the *P*45014DM protein of *S. cerevisiae*, while for other organisms, with no history of pertinent genetic nomenclature, *P*45014DM enzymes that are known to be coded by genes of

the same subfamily are identified as CYP51A1. We have recently shown *ERG11* to be linked by known DNA sequence to the gene for manganese superoxide dismutase on chromosome VIII [16].

2. MOLECULAR GENETIC ANALYSIS OF THE *IN VIVO ERG11 P450* SYSTEM

2.1. Nystatin resistance and erg mutants produced by gene targeting

S. cerevisiae has been the microorganism of choice in the study of sterol biosynthesis, particularly in the overall path from lanosterol to ergosterol, which requires 10 or more enzymes in addition to CYP51 [8, 17]. That research has benefitted from the use of sterol biosynthetic mutants isolated on the basis of nystatin resistance. Nystatin is a polyene antimycotic agent known to bind to membrane sterols leading to loss of membrane integrity; resistance is dependent upon the synthesis of novel membrane sterols that are thought to have reduced affinity for the drug [18]. These novel sterols arise as the result of mutational changes in the *ERG* genes encoding the lanosterol to ergosterol pathway.

The correlation of individual *ERG* genes with specific synthetic steps has been based primarily upon differences in the GC or GC/MS-generated profiles of total sterols extracted from the mutants. In practice these assays may fail to detect differences between "null" mutants, i.e. strains with a totally non-functional *erg* allele, and "leaky" mutants expressing only low level activity of the gene product. In fact, interpretations have required the action of leaky mutants in some models of yeast sterol structure and function. Recently, however, the successive cloning of *ERG* genes has allowed the gene-engineered construction of *bone fide erg* null mutants, produced by gene targeting the wild type *ERG* chromosomal allele for replacement by an inactive gene sequence [19]. Three of the *ERG* genes that have now been replaced by inactive variants have been shown to be non-essential for viability (*ERG2, ERG3* and *ERG6* [20]); this was not the case with *ERG11.*

2.2. Erg11 null mutants

Isolation of the *ERG11* DNA allowed us to target the chromosomal locus to produce *erg11* null mutant haploid strains. These mutant strains are obligate anaerobes, i.e. *ERG11* is essential for aerobic growth [13]. Since both ergosterol and unsaturated fatty acids are required by *S. cerevisiae* and since synthesis of these substances is oxygen dependent, they are included as constituents in anaerobic growth media; under these conditions, both wild type *ERG11* and *erg11* mutant strains grow equally well. However, in the presence of air *S. cerevisiae* cannot import ergosterol added to the medium and *erg11* mutants do not grow.

The *erg11* null mutants are recessive: restoration of aerobic growth occurs in strains expressing wild type *ERG11* on an autonomous plasmid, and in heterozygous diploids, which segregate 2:2 [21]. However, restoration to aerobic growth in the absence of added ergosterol was observed to occur spontaneously. An anaerobically grown *erg11* null mutant was harvested and plated on synthetic agar media [22] but lacking ergosterol. After several days incubation in air, the faint lawn of quiescent cells spontaneously gave rise to a few colonies per plate. Each of the clones tested retained its disrupted *erg11* allele and we demonstrated that for these strains the original failure to grow aerobically is suppressed by another recessive mutation in an unlinked locus. We termed the gene for this trait suppressor of lanosterol demethylase deficiency, *sld1* [21]. These *erg11 sld1* strains retained resistance to Kc, as expected since the *P*450 target for that agent is absent, and were also resistant to nystatin.

The properties of *erg11* and *erg11 sld1* strains are of interest in relation to earlier models. A specific case is the proposal that a low level of ergosterol or some other sterol product is essential for growth by *S. cerevisiae* [23, 24], possibly as an effector required for the G1 to S transition during mitosis [25]. Our observations have not resolved this intriguing model, which clearly warrants further research. However, the fact that *sld1*-suppressed *erg11* null mutants grow aerobically in the absence of added ergosterol indicates that ergosterol itself is not required for any essential hormonal function.

On the other hand, our results directly support the current model that nystatin resistant *erg11* deficient strains are only isolated with the presence of a second unlinked defect in sterol synthesis [26–29]. This second block occurs after the ERG11 reaction and lowers or prevents the Δ5,6-desaturaton of sterols. The structural gene of this enzyme has been designated *ERG3.* Properties of *sld1* appear to be identical to those of some known *erg3* alleles [21, 30]. Now that the cloning and sequence characterization of the

Table 1. Effect of DMI on *cpr1* null mutant strains containing high copy vector constructs

DMI	Strains[a]			
	WT	*cpr1* (YEp24)[b]	*cpr1* (*ERG11*)	*cpr1* (*CPR1*)
	MIC (μg/ml)			
Kc	5	0.02	0.02	10
Flu	40	<0.04	<0.04	80

[a] WT, wild type *CPR1*; (YEp24), (*ERG11*), and (*CPR1*) indicate the presence in strain *cpr1* of the cloning vector YEp24 or YEp24 containing a functional *ERG11* or *CPR1* gene, respectively.
[b] Similar MIC values were obtained for strain *cpr1* not containing YEp24.

ERG3 gene has been reported [20], the relationship of these genes can be tested by producing strains disrupted for both *erg11* and *erg3*. This work is now in progress.

2.3. *Cpr1 null mutants*

We made use of the published sequence of the *S. cerevisiae* gene for NADPH–cytochrome *P*450 reductase, *CPR1* [31] and produced *cpr1* null mutant strains by gene targeting [32]. It was generally accepted that NADPH–*P*450 reductase is required for the function of all membrane bound *P*450s [33], including ERG11 [7]. Thus, these *cpr1* null mutants would be expected to lack a lanosterol 14α-demethylase reaction, i.e. as is the case with *erg11* null mutants, these *cpr1* mutant strains would be obligate anaerobes.

However, our experiments demonstrated that this *CPR1* gene is not essential for aerobic growth in the absence of ergosterol. No evidence for a second *CPR* gene was found using tests of DNA homology or immunological cross reaction, suggesting that some alternative electron carrier in *cpr1* null mutant strains provides electrons to the ERG11 *P*450 [32]. The protein basis of this alternate reductase activity has not been established.

Nevertheless, these *cpr1* mutants do show that CPR1 is an important enzyme in *S. cerevisiae* [32]. Cells deleted for the gene contain decreased levels of ergosterol and grow more slowly than wild type. They also show increased sensitivity to agents known to inhibit ERG11 [32]. Table 1 presents data obtained recently using Kc and fluconazole (Flu), two demethylase-inhibitory agents (DMI) in clinical use. Minimal inhibitory concentrations (MIC) were determined on a wild type and its isogenic *cpr1* null mutant variant. Loss of the *CPR1* allele resulted in a >100-fold increase in sensitivity to either drug. It was also observed that the presence of multiple copies of the *ERG11* gene in the *cpr1* strain had no effect upon these drug sensitivities, while the presence of multiple copies of the *CPR1* gene completely restored resistance (Table 1).

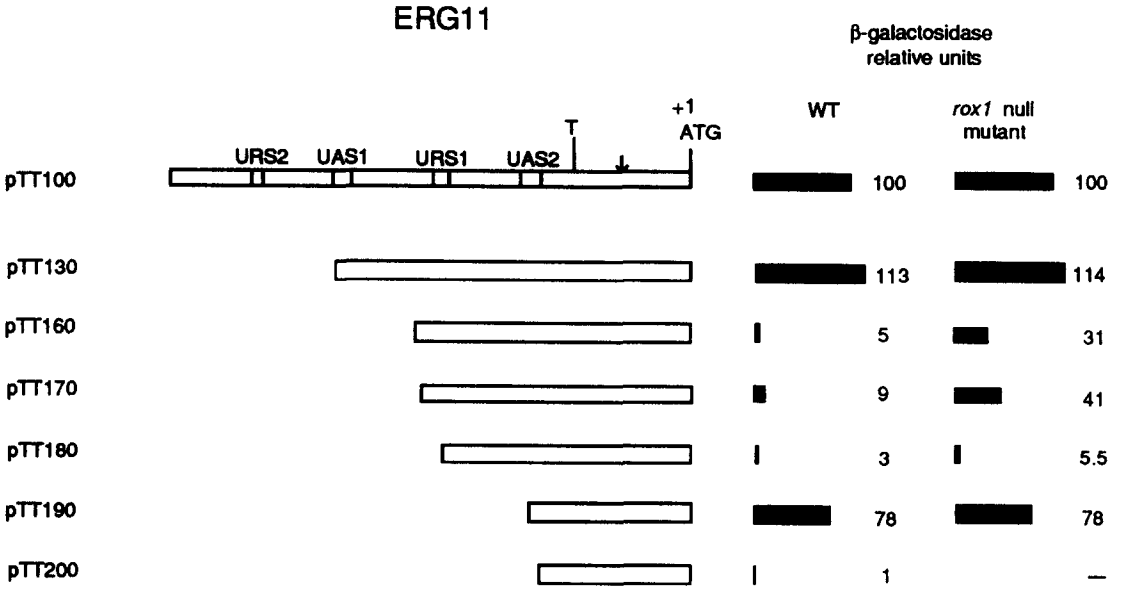

Fig. 2. The *ERG11* promoter URS1 includes a ROX1-dependent element. Deletion constructs in the *ERG11* promoter-β-galactosidase reporter plasmid were tested in a wild type and a *rox1* strain. Coordinates of the deletion end points are identified by their wild type base numbers 5′ to the *ERG11* ATG, with that A designated as +1. The location of the proposed UAS and URS segments, the proposed TATA Box (T), and the transcription initiation sites (↓) are indicated on the diagram of plasmid pTT100. The activity of each plasmid, assayed in either strain, appears numerically and as a histogram. See [34] for description of assays and additional data.

2.4. Regulation of expression of *ERG11* and *CPR1*

Understanding the control of *ERG11* expression is of special interest. This yeast *P*450 was first detected in cells grown semi-anaerobically [3]. Since then, whole cell studies using spectral analysis, and *in vitro* enzymatic and immunological assays, have shown that this *P*450 is present in low levels in oxidatively respiring cells, and appears in elevated levels during strict fermentative, semi-anaerobic growth. The *P*450 levels decrease rapidly upon switching the culture to growth on a non-fermentable carbon source, or upon entry of the cells into stationary growth phase. This physiological data suggested that *ERG11* expression might be useful as a model of oxygen-related regulation. Additionally, *S. cerevisiae* strains vary up to 10-fold in the level of intracellular ERG11 *P*450 they can attain. Such strain specific differences can be of use in the analysis of gene regulation [12, 34].

We have recently examined the regulation of *ERG11*, using mRNA-Northern blot hybridization and the expression of β-galactosidase under control of different segments of the *ERG11* promoter [34]. Some of the data and two of our conclusions from that work are presented here. One conclusion is that *ERG11* is a member of a hypoxic gene family. *ERG11* message levels increase during growth on glucose, in the presence of heme, during oxygen-limiting growth conditions, and during anaerobic growth. During anaerobiosis, of course, the enzyme is non-functional since molecular oxygen and heme are required for activity. Another finding is that *CPR1*, encoding the other membrane-bound protein of this *P*450 system, is coordinately regulated with *ERG11* [34]. Data supporting these conclusions are shown here.

Deletion analyses were performed on the *ERG11* promoter-*lacZ* reporter construct in cells grown fermentatively under conditions known to yield high ERG11 levels. Figure 2 shows relative β-galactosidase expression data for a subset of the *ERG11* 5′ promoter deletion constructs. Maximum expression was obtained using the entire *ERG11* promoter, i.e. in plasmid pTT100, with a value of approx. 100-fold the base line level obtained with the extensive deletion plasmid pTT200. The studies detected several *cis*-acting elements positioned between these limits, two upstream activating sequences UAS1 and UAS2 and an upstream repressor sequence URS1, plus a possible URS2. The approximate locations of these upstream regulatory elements are indicated on plasmid pTT100 in Fig. 2. This figure also presents evidence for a role of the ROX1 protein as a *trans*-acting regulator of this promoter. ROX1 protein is known to be involved in the repression of anaerobically induced genes during aerobic growth and in the repression of aerobic genes during anaerobiosis [35]. In this case, the wild type *ERG11* construct on plasmid pTT100 gave about the same activity when it was expressed in either the *ROX1* wild type or in a *rox1* null mutant. Also, the *rox1* mutant had no effect on transcriptional activation requiring either UAS1, i.e. from plasmid pTT130; or UAS2, from plasmid pTT190. However, increases in β-galactosidase activity were seen from plasmids pTT160 and pTT170 when they were expressed in the *rox1* null mutant. These data indicate a role for the wild type ROX1 protein, in a *trans*-acting repressor mechanism involving URS1.

The effects of *rox1* on *ERG11* expression were also examined by Northern hybridization; results for a pair of isogenic strains *ROX1* and *rox1-a1*, appear in Fig. 3. Total RNA was prepared from aerobic (high), semi-anaerobic (low), and anaerobically ($-O_2$) grown cells. The RNA blot was probed with an *ERG11* fragment and then sequentially with the constitutively expressed yeast actin gene (*ACT1*) and the *CPR1* gene. Intensities of the mRNA bands were quantitated with a scanning densitometer. The values for *ERG11* and *CPR1* presented are normalized to the level of expression of the wild type *ROX1* strains grown in higher aeration. For this *rox1* mutant, compared to the (repressed) wild type, expression of both *ERG11* and *CPR1* during aerobic growth was elevated 7-fold.

3. RELATIONSHIP OF CYP51 TO OTHER STEROL 14α-DEMETHYLASES

Sterol 14α-demethylase: an ancient and extensive *P*450 family? Since production of membrane sterol is characteristic of essentially all eukaryotes, it follows that the genetic basis of the sterol 14α-demethylation step existed before or at the time of the emergence of eukaryotes, at least 1–1.5 billion years ago [36], or possibly much earlier [37]. Some indication of the evolutionary diversity of the current genes for sterol 14α-demethylase can be gained by comparative assays of cellular sterol composition and enzyme substrate specificity. Based upon the patterns summarized in Table 2, it is apparent that

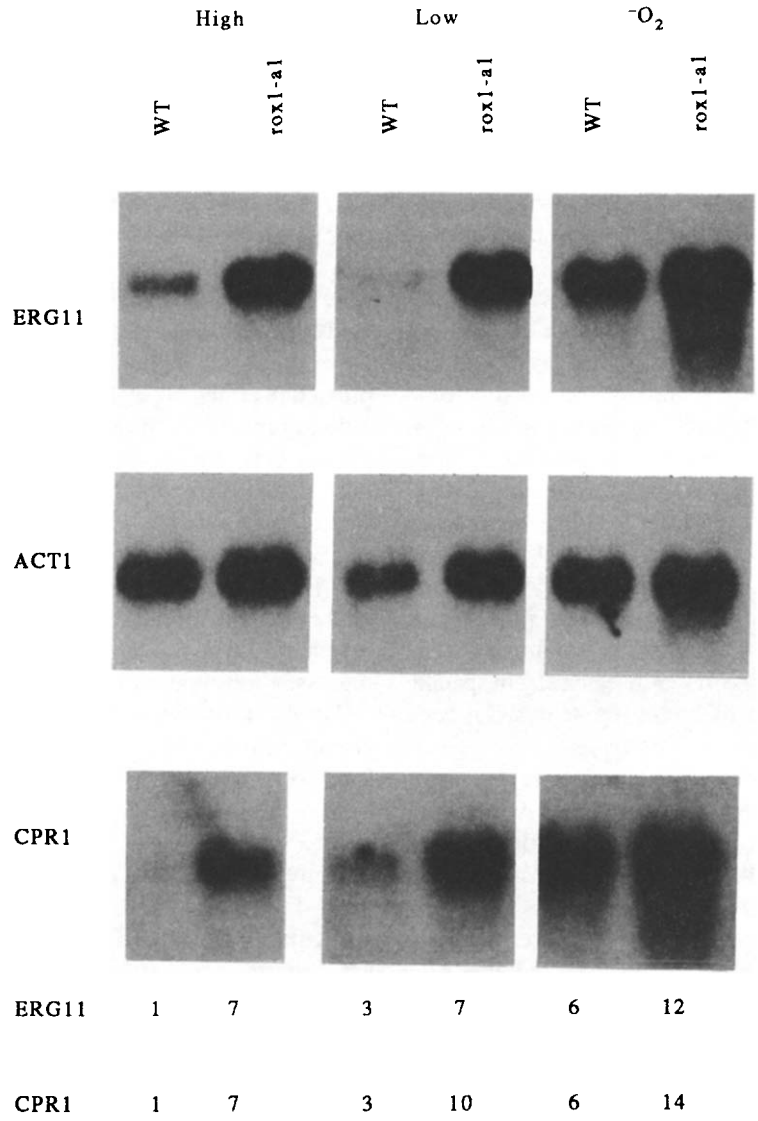

Fig. 3. *ERG11* and *CPR1* are derepressed during aerobic growth in the absence of ROX1. A wild type and an isogenic *rox1* strain were grown under high aeration, low aeration or strict anaerobiosis ($-O_2$). Northern blots of total RNA were probed with DNA fragments specific for *ERG11*, *ACT1*, and *CPR1*. This figure was modified from Ref. [34].

vascular plants, yeasts, filamentous fungi and mammals express differences in their sterol 14α-demethylases. Taton and Rahier [38] have studied substrate requirements of a microsomal enzyme system of maize which shows a high degree of specificity for removal of the 14α-methyl group of obtusifoliol. This enzyme preparation is inactive for either lanosterol or 24-methylene-24,25-dihydrolanosterol(24-methylene DL). Using purified enzyme from *S. cerevisiae*, Aoyama and Yoshida [39] showed the 14α-demethylation of both lanosterol and 24-methylene DL even though the 24-methylene DL does not occur in *S. cerevisiae*: instead, it is a constituent of filamentous fungi, where it is thought to be the natural substrate for this reaction [40]. Although lanosterol is the natural substrate in mammals as well as in *S. cerevisiae*, Aoyama and Yoshida [39] have shown significant substrate activity differences for the 14α-demethylase enzyme purified from this yeast and from rat liver.

Protein amino acid sequence data are available only for the lanosterol 14α-demethylases from the yeasts *S. cerevisiae*, *Candida tropicalis* and *C. albicans*. The gene from both *Candida sp.* was isolated using *S. cerevisiae ERG11* DNA as probe [41, 42]. These three proteins share high similarity [43, 44], and are classified as orthologous CYP51A1 enzymes [15].

Moreover, the use of DNA probes based upon the *S. cerevisiae* and/or *Candida* genes has

Table 2. Sterol 14α-demethylases

Primary substrate	Organism	Membrane sterol
Lanosterol (1)[a]	Mammals	Cholesterol (4)
Lanosterol	Yeast	Ergosterol (3)
24-Methylene-24,25-dihydrolanosterol (5)	Filamentous fungi	Ergosterol
Obtusifoliol (6)	Vascular plants	24-Methylcholesterol
		Sitosterol
		Stigmasterol

[a]Chemical structures of the numbered sterols appear in Fig. 1.

recently been reported for the cloning of a homologous sterol 14α-demethylase gene of the filamentous fungus *Penicillium italicum*. Translation of a partial sequence of the *P. italicum* gene generated a deduced polypeptide with 75% sequence similarity to an amino acid sequence encoded by the *C. tropicalis CYP51A1* gene [44]. Other researchers have reported work in progress using probes based upon these same yeast *CYP51A1* genes for the isolation of a homologous gene in the filamentous fungus *Erysiphe graminis* (powdery mildew [46]), and in maize (P. Benveniste, personal communication).

Isolation of a mammalian gene for lanosterol 14α-demethylase has not been reported. A gene probe based upon *S. cerevisiae ERG11* showed little hybridization to rat genomic DNA (this laboratory) or to rat mRNA (Trzaskos, personal communication).

4. ANTIFUNGAL RESISTANCE AND STRUCTURE FUNCTION ANALYSES OF STEROL 14-DEMETHYLASES IN *S. CEREVISIAE*

Sterol 14α-demethylase has been recognized as a prime target for antifungal agents and a wide array of demethylase inhibitors (DMI) has been developed [47]. Included among these DMI are several antifungal agents in primary use, both in clinical and animal medicine, and in agriculture for crop protection from phytopathogenic fungi.

Nevertheless, practical inadequacies in present day DMI applications in both of these areas have led to increased recognition of the need for more effective agents. In agriculture, the problem is the emergence of resistance in fungal pathogens that were formerly sensitive. A 1988 review listed five phytopathogenic fungi against which DMI fungicides were reported to be failing to sustain disease control [48]. Two more fungi were added to that list in 1990 (D. Hollomon, personal communication).

Medically, the status of mycotic infections over the past several years is characterized by unremittant increases in number and severity [49]. This experience has its roots in the emphasis upon antibiotics for bacterial infections, with relatively scant attention to the development of agents for use against medically important fungi. Imbalances in normal flora induced by the use of broad spectrum bacterial antibiotics contribute to the problem, creating niches that favor overgrowth by fungi. Systemic mycoses have been a result of the increasing use of drugs with immunosuppressive effects, and of the increased success in tertiary treatment of patients with other life-threatening disease. These factors also contribute to fungal infections in patients with acquired immunodeficiency syndrome. The available antifungal agents often are not curative in such cases, and patients require prolonged maintenance on these drugs for their fungistatic effects [50]. Thus, there are broad and increasing needs for more effective fungicidal drugs for use against infections in humans as well as in crop protection. Development of a next generation of DMI is an attractive possibility to meet these needs, and for this the evolutionary and structure function relationships among the sterol 14α-demethylases may be of great practical importance.

At this juncture, there is a considerable body of knowledge and understanding of ERG11 as a model sterol 14α-demethylase. Using purified ERG11 protein, Yoshida and Aoyama and coworkers have examined structural requirements for substrates in assays of *in vitro* activity [51–53] and have proposed a possible architecture of the active center [53, 54]. Additionally, they have begun to test the fit of azole DMI agents with their structural model [54]. Related studies have modeled the conformation of an enzymatically inactive variant produced by *S. cerevisiae* expressing the mutated allele *erg11-1* [29]. Morris and Richards [55] have incorporated these results in their molecular modeling for a proposed three dimensional structure for the same enzyme based upon the structure of *P*450cam [55]. Their model for the active site is being tested further for structural compatibility with a diverse array of DMI

agents ([55] and G. Morris personal communication). These authors identify specific amino acid residues which they propose to be critical for catalysis. These residues are attractive targets for site-directed mutagenesis ([55] and G. Morris personal communication).

S. cerevisiae itself provides a model organism for physiological *in vivo* expression of the *ERG11* *P*450 system, both for gene engineered mutant alleles of *ERG11* and for naturally occurring "evolutionary variants" of sterol 14α-demethylases from among other organisms. As discussed above, our studies of this model have shown multiple mechanisms of increased DMI resistance, e.g. overexpression of *ERG11* based upon gene dosage [12] and bypassing of the requirement for ERG*11* function in *erg11 sld1/erg3* strains [22, 30]. Additionally, the data for multiple *cis*- and *trans*-acting elements as regulators of *ERG11* transcription [34] suggest that DMI resistance may arise during semi-anaerobic fermentation through the induction of elevated *ERG11* and *CPR1* expression, or during more respirative growth through mutation to permit constitutive transcription. At the same time, the greatly enhanced azole sensitivity of *cpr1* mutants shown in Table 1 suggests that the yeast *P*450 reductase could represent an additional target for selective antifungal agents. Drugs that decrease the available CPR1 activity would be expected to act synergistically with DMI agents.

Presently, the use of wild type and mutant *S. cerevisiae* for the *in vivo* analysis of mutant or foreign *CYP51* genes is still in its earliest stages. The *CYP51A1* genes of *C. tropicalis* and *C. albicans* are the only additional sterol 14α-demethylase genes characterized: both of these genes have been functionally expressed in wild type *S. cerevisiae* and the *C. tropicalis* gene is known to complement *erg11* null mutants to restore aerobic growth [41]. It is important that the orthologous genes be cloned from additional human pathogenic yeast and fungi such as *Histoplasma capsulatum*, *Cryptococcus neoformans*, and *Aspergillus fumigatus*, and from the human host. Based upon our observations on CPR1 as part of the CYP51 electron transport system, the *CPR* genes from these pathogens should also be isolated. These could be used as gene replacements of the *S. cerevisiae* *CPR1* in conjunction with *in vivo* assays of the *CYP51* gene from the respective source. Fortunately, there now is increased interest in the pursuit of such studies, not only for clinically important fungi, but as has been noted earlier, for phytopathogenic fungi and for their host organisms as well.

Expression of these coding sequences in *S. cerevisiae* is likely to be a powerful tool for studies of their structure and function. In some cases, the sterol 14α-demethylases are likely to restore lanosterol demethylation to *S. cerevisiae* *erg11* mutants, as has been observed with the *C. tropicalis* orthologue. Other enzymes, of which the maize obtusifoliol demethylase appears to be an example, may not replace ERG11 function. However, in all cases, *S. cerevisiae* is expected to serve as a versatile host for the production of protein components of sterol 14α-demethylases, and for molecular genetic analyses of structure and function of this crucial eukaryotic system.

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