EUKARYOTIC CELL, Dec. 2002, p. 1041–1044 Vol. 1, No. 6
1535-9778/02/$04.00+0 DOI: 10.1128/EC.1.6.1041–1044.2002

NOTE

ROX1 and *ERG* Regulation in *Saccharomyces cerevisiae*: Implications for Antifungal Susceptibility

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Received 5 August 2002/Accepted 19 September 2002

Yeasts respond to treatment with azoles and other sterol biosynthesis inhibitors by upregulating the expression of the *ERG* genes responsible for ergosterol production. Previous studies on *Saccharomyces cerevisiae* implicated the *ROX1* repressor in *ERG* regulation. We report that *ROX1* deletion resulted in 2.5- to 16-fold-lower susceptibilities to azoles and terbinafine. In untreated cultures, *ERG11* was maximally expressed in mid-log phase and expression decreased in late log phase, while the inverse was observed for *ROX1*. In azole-treated cultures, *ERG11* upregulation was preceded by a decrease in *ROX1* RNA. These inverse correlations suggest that transcriptional regulation of *ROX1* is an important determinant of *ERG* expression and hence of azole and terbinafine susceptibilities.

In fungi, the sterol biosynthesis pathway leads to the formation of ergosterol, with many steps in the pathway being essential (3, 16). Indeed, sterol biosynthesis inhibitors (SBIs) are widely used as antifungal agents in medicine and agriculture. The most important group of SBIs is the azoles, which target the *ERG11*-encoded enzyme lanosterol 14α-demethylase. As clinical use of these agents increased, so did the isolation of azole-resistant mutants, and one of the major resistance mechanisms involves constitutive *ERG11* upregulation (18, 20, 24, 29). Furthermore, many strains of *Candida albicans* and related yeasts display "trailing" growth in azole susceptibility assays (21–23). One potential mechanism for trailing is *ERG11* upregulation, and consistent with this idea, it has been shown that exposure of *Candida* species to SBIs upregulates the expression of *ERG11* and other genes in the ergosterol biosynthesis pathway (4, 10). SBI-dependent *ERG* upregulation has also been demonstrated in the *Saccharomyces cerevisiae* genetic model (2, 6, 7, 13, 25, 26), and mutations that alter sterol biosynthesis have a similar effect (1, 2, 6, 8, 13, 19, 25, 26).

Previous studies examined regulatory elements within selected *ERG* promoters (1, 6, 25, 28) and the role of specific transcription factors in *ERG* expression (13, 28). *ERG11* is positively regulated by the heme-activated transcription factor Hap1p and negatively regulated by the oxygen-responsive repressor Rox1p, while *ERG9* is similarly regulated by these two factors along with Yap1p and Ino2p-Ino4p. *ROX1* is autoregulated and Rox1p has a short half-life (<10 min), which are important characteristics as *ROX1* overexpression may be lethal (5, 12, 30). Recently, DNA arrays have identified Rox1p-regulated genes under aerobic and anaerobic conditions (15, 27), confirming that selected *ERG* genes are regulated by this repressor. We show here that *ROX1* is an important determinant of SBI susceptibility and that *ROX1* and *ERG* expression are inversely correlated in response to growth phase and SBI treatment.

Initial studies employed two distinct azoles at concentrations three- to fivefold higher than the drugs' MICs. Log-phase cultures of *S. cerevisiae* W303-1A (*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) in YPD medium (1% yeast extract, 2% peptone, 2% dextrose) at 30°C were exposed to

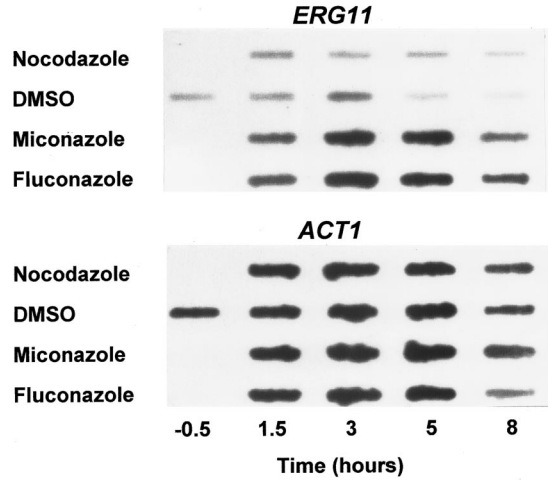

FIG. 1. Azole-dependent upregulation of *ERG11*. RNA slot blot hybridization was used to examine *ERG11* expression in strain W303-1A following treatment with nocodazole (3 μg/ml), fluconazole (20 μg/ml), miconazole (0.3 μg/ml), or dimethyl sulfide (DMSO; drug vehicle) for the times indicated in the figure. *ACT1* expression was employed as a loading control. Cells were cultured in YPD medium. Probes were prepared from PCR products generated with the primers indicated in Table 1.

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TABLE 1. Primers used in this study[a]

Gene	GenBank accession no.	Position (bp)	Primer pair	Sequence (5′ to 3′)
ACT1	L00026	1297–1811	ACT2000F	ACCGAAGCTCCAATGAATCCAAAATCC
			ACT2516R	GTTTGGTCAATACCAGCAGCTTCCAAA
ROX1	X60458	545–1295	ROX1F	CAATCAACAATGAATCCTAAATC
			ROX1R	TTACCGGTGTTTGACTGCTG
ROX1, His5$^+$	X60458	503–1701	ROX1ΔF	AGAAAATACTAATACTTCACACAAAAGAAACGCAGTAGACAATCAAC GGATCCCCGGGTTAATTAA
			ROX1ΔR	ATAATATATATAACGGAAAGAAGAAATGGAAAAAAAAAAATCATTTCG GATGAATTCGAGCTCGTTTACAC
ERG11	M18109	1762–2266	ERG11F	ATTGGTATTCTTATGGGTGGTCAACATAC
			ERG11R	CCCAATACATCTATGTCTACCACCACC
ERG1	M64994	1085–1747	ERG1F	TTGACAATTAGTTGTGATGGTAT
			ERG1R	CTTTGGAAATATTTGAAACAACC
ERG3	M62623	1275–1793	ERG3F	CCWMTTTGAAAAACCAAATG
			ERG3R	GAATTGACCGTAGTTGTAGTTGAA
ERG7	U04841	2424–2988	ERG7F	TATCCATACGTGGAATGTAC
			ERG7R	TGTATAWACCTAATGCCTTAAT
ERG9	X59959	778–1231	ERG9F	AAAATGGGTAATGGTATGGC
			ERG9R	CTTGYGGAATYGCACAAAAT

[a] For each gene, the GenBank accession number and the corresponding termini (base pair position) of the PCR product are indicated. Nucleotides that are complementary to the regions flanking the *His5*$^+$ gene of plasmid pFA6a-His5MX6 are underlined. ROX1ΔF corresponds to the *ROX1* sequence immediately upstream of the start codon; ROX1ΔR overlaps the stop codon.

fluconazole (20 μg/ml) or miconazole (0.3 μg/ml), and RNA levels were examined by slot blot hybridization as previously described (9). *ERG11* expression was upregulated about twofold at 1.5 h and threefold at 3 h by these azoles, while a third drug with a different mechanism of action (the microtubule inhibitor nocodazole) had no effect (Fig. 1). By 5 h the control culture was in late log phase and *ERG11* expression had noticeably declined; in contrast, *ERG11* expression remained elevated in the presence of miconazole and fluconazole.

To examine potential mechanisms for *ERG* regulation, two *S. cerevisiae* strains with *ROX1* deletions were studied. Strain YJN433 (derived from W303-1A and with the same genotype) was transformed with a PCR product generated with the primers ROX1ΔF and ROX1ΔR (Table 1) and the template pFA6a-His5MX6 (17). Transformants were selected on His− DOB medium (Bio 101, Carlsbad, Calif.), and PCR was used to confirm *ROX1* deletion. In mid-log-phase cultures, the expression of *ERG1, ERG11,* and *ERG3* as measured by RNA hybridization and densitometric analysis increased 2.3- to 2.5-fold in the *rox1*Δ strain compared to that in the YJN433 parent strain (Fig. 2). The expression of *ERG9* and *ERG25* also modestly increased, while *ERG7* expression was essentially unchanged. In late-log-phase cultures, the expression of all *ERG* genes examined decreased relative to that in mid-log-phase cultures (Fig. 2). *ROX1* deletion again resulted in the increased expression of all but one of these *ERG* genes; indeed, the increase was proportionately greater than that observed in mid-log-phase cultures. Specifically, *ERG1*, *ERG11, ERG3*, and *ERG25* expression increased 3.4- to 7.9-fold, while *ERG7* expression was essentially unchanged. *S. cerevisiae* strain

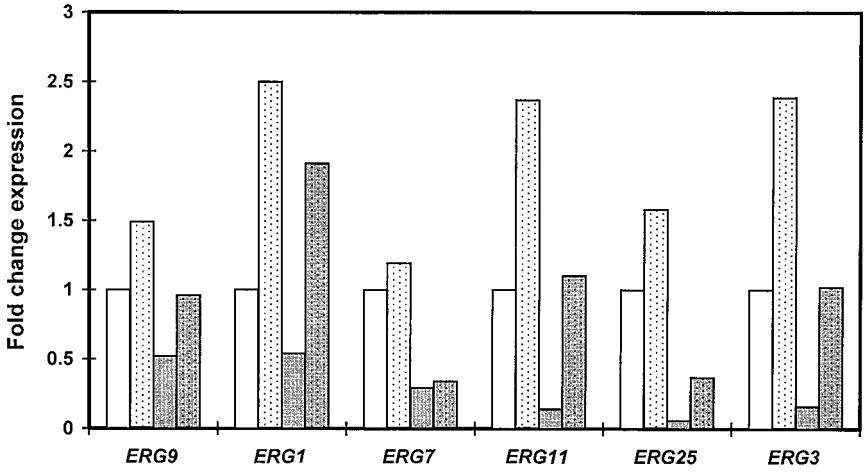

FIG. 2. *ROX1* deletion results in increased expression of multiple *ERG* genes. RNA slot blot hybridization was used to measure the expression of the indicated *ERG* genes in strains YJN433 (solid bars) and YJN433 *rox1*Δ (stippled bars). RNA was extracted from mid-log-phase cultures (2×10^7 cells/ml) (unshaded bars) or late-log-phase cultures (1×10^8 cells/ml) (shaded bars). Autoradiographs were densitometrically scanned; values shown represent the fold change in RNA relative to the levels in mid-log YJN433 cultures, with normalization to *ACT1* RNA levels.

TABLE 2. SBI susceptibilities of *S. cerevisiae* strains and *rox1*Δ derivatives[a]

Inhibitor	IC_{50} (μg/ml)			
	RZ53-6		YJN433	
	ROX1	*rox1*Δ	*ROX1*	*rox1*Δ
Fluconazole	6.2	23	1.1	8.3
Itraconazole	0.30	5.1	0.34	5.2
Miconazole	0.17	0.40	0.09	0.23
Terbinafine	3.0	11	0.06	0.56

[a] Susceptibilities were determined by serial dilution in 96-well plates as previously described (10), except that YPD medium at 30°C was employed. IC_{50}s were estimated by extrapolation from the results for the two wells spanning the 50% growth point (control absorbance × 0.5).

RZ53-6 (*MAT***a** *trp1-289 ura3-52 leu2-3,112 ade1-100*) and its *rox1::LEU2* derivative (obtained from R. Zitomer [5]) similarly demonstrated increased expression of the *ERG* genes noted above (1.7- to 2.2-fold in mid-log-phase cultures, 2.4- to 4.9-fold in late-log-phase cultures), again with the exception of *ERG7* (data not shown).

Since *ROX1* deletion resulted in the increased expression of multiple *ERG* genes, it was of interest to test the effects of this deletion on susceptibility to SBI antifungals that target ergosterol biosynthesis. Indeed, in both strain backgrounds described above, *ROX1* deletion resulted in decreased susceptibilities to azoles and the Erg1p-targeted allylamine terbinafine (Table 2). Specifically, for the *rox1*Δ derivatives, 50% inhibitory concentrations (IC_{50}s) of fluconazole, itraconazole, and miconazole increased an average of 5.6-, 16-, and 2.5-fold, respectively, and the IC_{50} of terbinafine increased an average of 6.5-fold. For comparison, the RZ53-6 strains were tested for sensitivity to the microtubule inhibitor nocodazole and the protein synthesis inhibitor cycloheximide; there were no significant differences associated with *ROX1* deletion (data not shown).

The data above indicate that *ERG* transcription and SBI susceptibility are regulated by *ROX1*. It is likely that *ROX1* itself is transcriptionally regulated, since Rox1p has a short half-life (<10 min) and the *ROX1* promoter includes known regulatory elements (30, 31). Reverse transcriptase PCR (RT-PCR) analysis (10) of an untreated *S. cerevisiae* W303-1A culture making a transition from mid-log to late log phase demonstrated that *ROX1* expression increased as *ERG11* expression decreased (Fig. 3, lanes 1 to 11). Specifically, the ratio of *ERG11* RNA to *ROX1* RNA (determined by densitometric analysis of the RT-PCR products) decreased 2-fold after 1 h but ≥10-fold after 2 or 5 h of incubation. This inverse correlation suggests that transcriptional regulation of *ROX1* mediates the transcriptional regulation of *ERG* genes.

Consistent with results of previous studies, treatment of *S. cerevisiae* with fluconazole upregulated *ERG11* expression (Fig. 3, lanes 1 and 13 to 21). This increase was maximal after 1 to 2 h of treatment. Conversely, fluconazole treatment resulted in decreased *ROX1* expression that appeared to precede (minimum at 30 min) this increase in *ERG11* RNA. Consequently, the ratio of *ERG11* RNA to *ROX1* RNA increased ninefold by 1 h after treatment. (By 5 h, however, the fluconazole-treated culture had resumed growth and entered late log phase, resulting once again in increased *ROX1* expression and decreased *ERG11* expression.) Similar results were obtained in RNA hybridization studies of strain YJN433 (data not shown).

The SBI-dependent *ERG* upregulation demonstrated here and previously is predicted to reduce SBI susceptibility, just as constitutive *ERG* upregulation (due to currently uncharacterized mutations) contributes to SBI resistance in many clinical isolates. Understanding the mechanism behind this SBI response could lead to much needed improvements in antifungal therapy and a greater understanding of resistance mechanisms. Since disruption of ergosterol biosynthesis by SBI treatment or genetic lesion at any of several different steps in the pathway results in the upregulation of multiple *ERG* genes, there is likely to be a common mechanism for their transcriptional control. The data presented here, combined with those from previous studies, indicate that the repressor Rox1p is a promising candidate. Potential Rox1p binding sites (31) can be identified upstream of most *ERG* promoters; specifically, 17 of 22 *ERG* promoters but only 5 of 22 randomly selected non-*ERG* promoters include at least one copy (allowing for two mismatches) of the YYYATTGTTCTC consensus binding site (unpublished data).

A *C. albicans* gene, *RFG1*, with limited homology to *ROX1* was recently reported; however, its deletion did not alter the expression of oxygen-regulated genes but rather blocked hypha-to-yeast morphogenesis (11, 14). *C. albicans* may therefore

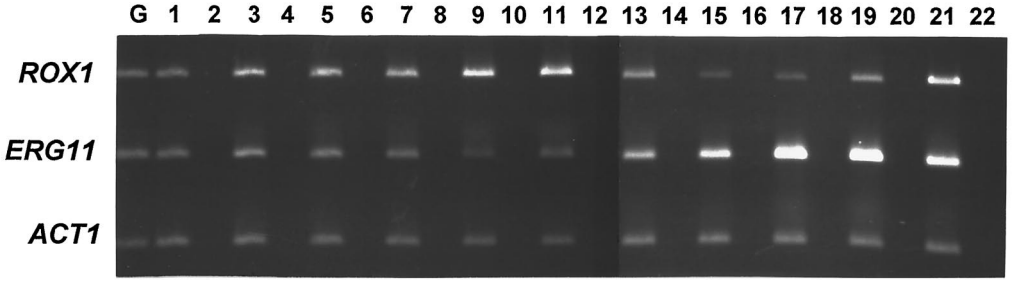

FIG. 3. *ERG11* expression is inversely correlated with *ROX1* expression. RT-PCR (odd-numbered lanes) was used to examine *ROX1*, *ERG11*, and *ACT1* expression in untreated (lanes 3 to 11) or fluconazole-treated (9 μg/ml) (lanes 13 to 21) cultures of strain W303-1A. Log-phase cultures (3×10^7 cells per ml) were sampled after incubation for 0 h (lane 1), 0.25 h (lanes 3 and 13), 0.5 h (lanes 5 and 15), 1 h (lanes 7 and 17), 2 h (lanes 9 and 19), or 5 h (lanes 11 and 21). Control reactions which lacked RT (even-numbered lanes) confirmed that the observed bands were not due to genomic DNA contamination. Lane G is a positive control from a reaction mixture containing genomic DNA. Amplification was for 23 (*ROX1* and *ERG11*) or 25 (*ACT1*) cycles, which was within the logarithmic range. Primers are indicated in Table 1.

regulate its *ERG* genes by mechanisms that are at least partially distinct from those employed by *S. cerevisiae*. Other clinically important species, such as *Candida glabrata*, are more closely related to *S. cerevisiae* and even more problematic in terms of azole resistance. Examining the role of *ROX1* homologs in these species would therefore be of interest.

This study was funded by National Institutes of Health grants AI46768 and AI47718 to T.D.E. and HL67401 to J.T.N.

We thank R. Zitomer for providing strains.

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