FEBS Letters 470 (2000) 102–106 FEBS 23440

Sterol metabolism and *ERG2* gene regulation in the yeast *Saccharomyces cerevisiae*

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Received 28 January 2000; received in revised form 23 February 2000

Edited by Horst Feldmann

Abstract Certain exogenously-supplied sterols, like ergost-8-enol, are efficiently converted into ergosterol in yeast. We have taken advantage of this property to study the regulation of the Δ8-Δ7-sterol isomerase-encoding *ERG2* gene in an ergosterol auxotrophic mutant devoid of squalene-synthase activity. Ergosterol starvation leads to an 8–16-fold increase in *ERG2* gene expression. Such an increase was also observed in wild-type cells either grown anaerobically or treated with SR31747A a sterol isomerase inhibitor. Exogenously-supplied zymosterol is entirely transformed into ergosterol, which represses *ERG2* transcription. By contrast, exogenously-supplied ergosterol has little or no effect on *ERG2* transcription.

Key words: Sterol uptake; Sterol isomerase; Transcription; Ergosterol; *Saccharomyces cerevisiae*

1. Introduction

The yeast *ERG2* gene encodes Δ8-Δ7-sterol isomerase, an enzyme of the sterol biosynthetic pathway. This enzyme is the target for several active agents exhibiting antifungal [1], immunosuppressive [2], and/or neuroprotective properties [3]. Sterol isomerase together with 14-sterol reductase constitute the two main targets for the morpholine and piperidine derivative family of sterol biosynthesis inhibitors. This group includes several structurally-related molecules, such as fenpropimorph and fenpropidine, that are widely used in agriculture against cereal powdery mildew [4].

SR31747A is a highly selective Δ8-Δ7-sterol isomerase inhibitor that is effective against the yeast enzyme [5] as well as against the structurally and phylogenetically unrelated mammalian Δ8-Δ7-sterol isomerase [2,6,7]. SR31747A displays immunosuppressive properties [8] that appear to be mediated by the *ERG2* ortholog in mammals, namely the subtype 1 of the so-called 'sigma receptors' (sigma1) also known as SR31747A-binding protein [3,9]. This drug also exhibits anti-proliferative properties in mammalian cells, that are obtained through the inhibition of the high-eukaryote-specific type of Δ8-Δ7-sterol isomerase [7]. Finally, inhibition of neuronal cholesterol biosynthesis is thought to induce the long-term neuroprotective properties exhibited by sterol isomerase ligands [2]. Thus, sterol isomerase appears to be a target of paramount importance for both agricultural and pharmaceutical issues.

In this work, we have checked whether *ERG2* gene expression is regulated at the transcriptional level in yeast. Unlike in previous studies [10–12], we have used a sterol auxotrophic strain to examine if gene expression could be up-regulated by ergosterol deprivation, or down-regulated by sterol intermediates accumulating at high concentrations. In addition, as the sterol uptake and intracellular trafficking mechanisms are not well understood, we have examined the uptake and the biotransformation of various ergosterol precursors.

With the exception of lanosterol, all the sterol species tested were efficiently converted into ergosterol. We could demonstrate that ergosterol, when taken up directly from the growth medium, had little or no effect on *ERG2* transcription, whereas ergosterol synthesised from an exogenously-supplied precursor, like zymosterol, dramatically repressed *ERG2* transcription.

2. Materials and methods

2.1. Construction of the LacZ-expressing cassette placed under the control of the ERG2 promoter

Strain EMA4 (*MAT*a, *ura3*-251, *ura3*-373, *ura3*-328, *trp1*-4) is a congenic derivative of FL100 (ATCC 28383). Plasmid pEMR1465 is an integrative plasmid that contains a wild-type (wt) version of the *URA3* gene and the *lacZ* gene placed under the control of the *ERG2* promoter. The *ERG2* promoter-encompassing region that flanks *lacZ* starts at coordinate 1930 of chromosome XIII (Accession number Z48755). The 3′-end of this chromosome XIII-derived fragment includes the sixth codon of the *ERG2* coding sequence fused in frame with the *LacZ* sequence derived from Yep 358 [13]. *Stu*I-linearised pEMR1465 DNA was used to transform EMA4 to Ura$^+$. One transformant, namely EMA90-3, was chosen, that harboured four copies of pEMR1465 integrated as direct repeats inside the *URA3* locus.

2.2. Construction of EK9-2 sterol auxotroph

Strain EMA90-3 was crossed with the *ERG9* gene disruptant MK 5306 (*MAT*α, *erg9::HIS3*, *aux32*, *ura3*-1, *leu2*-1, *ade2*-1, *his3*-1). The latter strain is devoid of squalene synthase and therefore requires sterol for growth. The diploid was induced to sporulate, asci were dissected, and single-spore colonies were obtained under anaerobic conditions on complete medium supplemented with 10 ml/l of a solution of Tween 80/ethanol (v/v), containing 4 mg/ml ergosterol and 10 mg/ml oleate. Sterol auxotrophy was tested under aerobic conditions and β-galactosidase production was ascertained by the X-Gal assay. Clone EK9-2 (*erg9::HIS3*, *aux32*, *URA3::pERG2-LacZ*, *leu2*-1) was retained for further studies.

2.3. Sterol uptake and metabolism

Strain EK9-2 was grown in YPD medium supplemented with either ergosterol, lanosterol, 24-demethyl sterols or Δ8-sterols. Demethyl-24 sterols were prepared from an *ERG6* gene disruptant devoid of 24-methyl transferase. A mixture of Δ8-sterols was prepared from a double *ERG2*, *FEN1* disruptant. *FEN1* gene disruption allows *ERG2* gene disrupted cells to proliferate in the absence of ergosterol [2]. Zymosterol was prepared from a double *ERG2, ERG6* disruptant.

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PII: S0014-5793(00)01300-4

For uptake studies, sterols (10 μg/ml, final concentration) were supplied by dilution of a stock solution (4 mg/ml) in a mixture of Tergitol NP40 (Sigma)-ethanol (v/v). Cells were cultured at 30°C for 30 h, then harvested and freeze dried. After saponification, the ergosterol/ergosta-5,7-dienol and the overall sterol amounts were determined by UV spectrophotometry and gas chromatography (GC), respectively [14].

2.4. β-Galactosidase assay

β-Galactosidase activity was assayed directly on entire cells permeabilised by *N*-lauroylsarcosine treatment, as already described [15]. Activity was expressed in arbitrary units (OD/min/cell density unit).

2.5. Northern blot

Analyses were performed as described [16]. The *ERG2* and *ACT1* probes were obtained by PCR amplification of an *ERG2* gene segment (nt positions 266–977 of sequence M74037 in GenBank/EMBL database) and of an *ACT1* gene segment (nt positions 426–1150 of sequence V011288 in GenBank/EMBL database), respectively. PCR products were labelled with ^{32}P-γ-ATP by a conventional nick-translation procedure [16].

3. Results

3.1. Sterol transport and metabolism in yeast

To facilitate the study of the effects of various sterol species on yeast *ERG2* gene transcription, we used an *ERG9* gene disrupted strain, MK 5306. The *ERG9* gene encodes squalene synthase, the enzyme catalysing the first specific step of the sterol branch of the isoprenoid pathway. Thus, cells deleted for *ERG9* do not synthesise sterols de novo and are strictly dependent on uptake to proliferate. Yeast cells are generally unable to take up exogenous sterols in the presence of oxygen. A way to restore aerobic viability in an Erg$^-$ mutant is to block heme biosynthesis [17] or to over-express certain genes, like *SUT1* [18], both strategies resulting in sterol uptake competency. In our strain MK 5306, sterol uptake capability was conferred by the presence of the leaky *aux32*-1 mutation that affected the pyridoxal phosphate oxidase gene and consequently decreased heme biosynthesis [19].

We checked the efficacy of sterol uptake and metabolisation in strain MK5306. The first observation is that the ergosterol level remains three-fold lower than in the congenic wt strain FL100, even when the cells are supplied with ergosterol at saturating concentrations (Table 1). The conversion of cholesta-5,7,24-trienol into ergosterol is a three-step process that involves a 24-transmethylase localised in lipid droplets [20] and a Δ24-sterol reductase and a Δ22 desaturase localised in the endoplasmic reticulum (ER). Therefore, sterol trafficking between plasma membrane, ER and lipid droplets is required for metabolisation into ergosterol. The conversion of ergost-8-enol into ergosterol requires three enzymatic steps: Δ8-Δ7-sterol isomerase, Δ5-sterol desaturase and Δ22-sterol desaturase, these three enzymes being localised in the ER membranes. Cholesta-5,7,24-trienol on the one hand and ergost-8-enol on the other hand, are converted into ergosterol with an efficiency of about 90% (Table 1). This result indicates that these sterol species are transported from the plasma membrane to the various subcellular compartments containing sterol biosynthetic enzymes. However, the total sterol level is about three-fold lower than that in wt, whatever the type of sterol supplied, which reveals that uptake is the rate-limiting step in the in vivo conversion of precursors into ergosterol. Lanosterol was found not to be metabolised at all and did not support growth in MK5306. As an attempt to estimate the speed of sterol conversion, the wt strain FL100 was cultured overnight in the presence of fenpropimorph at a concentration sufficient to significantly inhibit ergosterol biosynthesis without arresting cell proliferation (0.7 μM), then the cells were washed and suspended in fresh medium without inhibitor. The fate of the two major sterol species that had been accumulated during treatment with the inhibitor, ergost-8-enol and ergosta-8,14-dienol (ignosterol) was then followed by GC analysis. Surprisingly, ergost-8-enol and ignosterol amounts remained constant even in cells grown in drug-free medium for 23 h (Table 2,

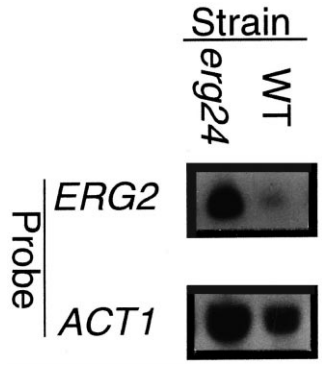

Fig. 1. Northern blot analysis of *ERG2* and ACTIN mRNAs in wt and in a *ERG24* gene disruptant. Steady state levels of actin mRNA were supposed to be constant in both strains, so variations in actin mRNA were supposed to represent differences in loaded material amounts. Signal intensity of each band was quantified by a phosphoimager apparatus, and variations in *ERG2* signal intensity from one strain to another were corrected by the corresponding actin signal variations.

Table 1
Sterol uptake and metabolism during growth in MK 5306 (*erg9::HIS3*) strain

Sterol supplied	Growth Yield	5,7-Sterol level	Sterol Uptake	GC Analysis
Ergosterol	208	0.30	62%	Ergosterol 100%
Cholesta-5,7,24-trienol[a]	216	0.32	69%	Ergosterol 100%
Ergost-8-enol[a]	204	0.23	65%	Ergosterol 72% Zymosterol 25% Ergost-8-enol 3%
Lanosterol	8	NT	NT	NT
–	1	NT	NT	NT

Growth yield is expressed in mg dry weight, after a 30 h growth period at 28°C. 5,7-Sterol level (sum of ergosterol and ergosta-5,7-dienol) was determined from the UV spectrum of non-saponifiable lipid fraction and expressed in percent of cell dry weight (in wt strain the 5,7-sterol level is 0.76%). Sterol uptake is expressed in percent relative to total sterol amount provided in growth medium. The GC analysis gives the percent of each individual sterol. NT = not tested

[a]Main sterols in mixtures of 24-demethyl-sterols (cholesta-5,7,24-trienol 79% and cholesta-5,7,22,24-tetraenol 21%) and Δ8-sterols (ergost-8-enol 75%, ignosterol 10% and zymosterol 15%). Sterols were supplied at 10 μg/ml final concentration in 100 ml YPD medium.

Table 2
Metabolism of ergost-8-enol and ignosterol after fenpropimorph treatment

Time (h)	Cell yield	5,7-Dienols	GC analysis; total sterols (%)				
			Lanosterol	Zymosterol	Ergost-8-enol	Ignosterol	Ergosterol
Part A: wt strain							
0	32	0.08	17	ND	41	41	<1
1.5	32	0.09	17	ND	43	40	<1
3	32	0.10	18	ND	44	37	<1
6	32	0.29	16	ND	45	39	<1
23	162	0.49	1	12	46	24	12
Part B: Strain ISA1 (*fen1::URA3*)							
0	186	0.09	2	9	73	16	<1
1	199	0.15	4	10	65	21	<1
2	234	0.29	3	12	51	17	16
3	371	0.49	1	14	44	7	34

Cells were inoculated at OD (700 nm) 0.1 unit/ml in 500 ml YPD containing 0.7 μM fenpropimorph and cultured under shaking at 28°C during 15 h. Cells were collected by centrifugation and washed two-fold with YPD medium, then inoculated in 500 ml YPD medium and cultured at 28°C. At the time indicated, aliquots of 100 ml were collected. Freeze dried cells were saponified and the sterol content analysed. Cell yield was expressed in mg dry weight. 5,7-Dienol level was determined from the UV spectrum and expressed in percent of cell dry weight.

part A). The experiment was repeated with a *FEN1* gene disruptant, namely ISA1 [21]. Loss of Fen1p relieves cells from the fenpropimorph-induced antiproliferation arrest, although fenpropimorph induces the accumulation of the same sterol species whether Fen1p is present or not [2,21]. In *fen1* as in wt, ergost-8-enol and ignosterol that were accumulated upon fenpropimorph treatment were not converted into ergosterol, after removal of the anti-fungal agent, although Δ8-Δ7-sterol isomerase and Δ14-sterol reductase were functional as indicated by the restoration of ergosterol biosynthesis (Table 2, part B). Therefore, the conversion speed could not be assessed by this approach.

3.2. *ERG2 mRNA steady state levels increase in a mutant impaired in ergosterol biosynthesis*

To test whether *ERG2* gene expression could be modulated transcriptionally, *ERG2* mRNA levels were compared in a Erg^+ strain and in a congenic *ERG24* gene disruptant. In the latter strain, which lacks 14-sterol reductase, the sterol pathway ends with ignosterol, and no ergosterol is produced. Loss of Erg24p correlated with a 8–9-fold increase in *ERG2* mRNA level (Fig. 1). As a control, loss of Fen1p or Sur4p, two similar ER membrane proteins that are required for sensitivity to the antiproliferative effects of various sterol biosynthesis inhibitors, had no consequence on *ERG2* mRNA steady state levels (not shown).

Table 3
β-Galactosidase activity in EK9-1 cells fed with various sterols

Sterol supplied	β-Galactosidase specific activity	
	Exp. 1	Exp. 2
Cholesterol	19.5	17.8
Ergosterol	17.2	20.3
Zymosterol	7.7	8.7

Cells were grown in YPD supplemented with Tergitol NP40 (Sigma) and 25 mg/l cholesterol for 30 h, then an aliquot was used to inoculate growth medium differing only by the sterol source as indicated. Starting OD value was 0.05 units/ml. Cells were grown for 24 h and then tested for β-galactosidase activity. Exp.1 and Exp.2 are two independent experiments. In each case, cell ergosterol amounts were quantified. Ergosterol was below level of detection in cholesterol-fed cells, whereas it represented about 0.4% of cell dry weight in ergosterol or zymosterol-fed cells.

3.3. *β-Galactosidase assays confirm that ERG2 transcription is regulated*

Northern blot results indicated that the steady state level of sterol isomerase-encoding mRNA was enhanced in cells in which ergosterol biosynthesis was impaired. This result suggested that *ERG2* expression could be controlled at the transcriptional level. To verify this point, we tested the effect of SR31747A, at various concentrations, on β-galactosidase production in EMA90, an Erg^+ strain that harbours four direct repeats of the bacterial *LacZ* gene placed under the control of the *ERG2* gene promoter at the *URA3* locus. The addition of 10 nM SR31747A into the culture medium was sufficient to enhance β-galactosidase production by three-fold. This factor still increased in a SR31747A-dependent manner, to reach a plateau at drug concentrations exceeding 0.5 μM (Fig. 2). At these concentrations, β-galactosidase production was 15 to 16-

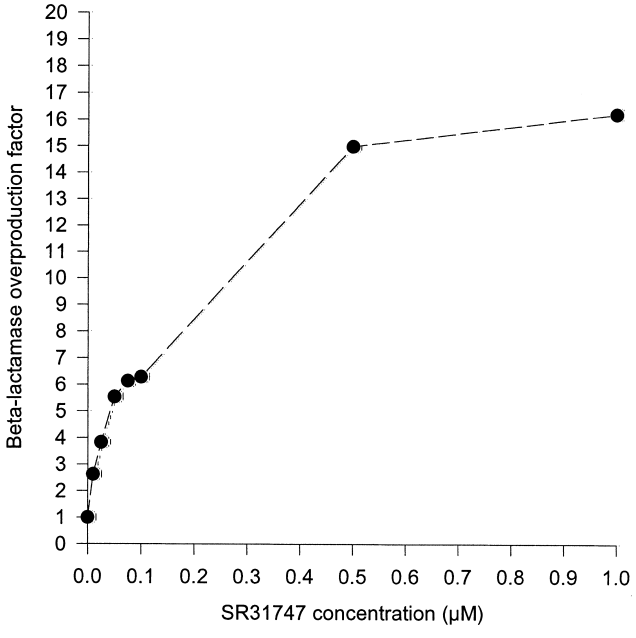

Fig. 2. SR31747 effect on *ERG2* promoter-controlled *lacZ* gene expression in yeast.

Table 4
β-Galactosidase activity in EMA90 cells grown in the presence or in the absence of oxygen

Growth conditions	β-Galactosidase specific activity (ratio activity in the absence versus in the presence of oxygen)				
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5
Aerobiosis	1.2	0.9	1.1	0.9	0.7
Anaerobiosis	4.7 (4)	4.6 (5)	5.3 (5)	5.2 (6)	8.5 (12)

Cells were grown either aerobically or anaerobically in synthetic SD medium (Exp. 1–4) or in YPD (Exp. 5). Media were supplemented with 1 ml/l of a mixture of Tween 80 containing ergosterol (4 mg/l) and oleic acid (10 mg/l).

fold higher than in the absence of the drug. Taken together, these results indicated that a block in the sterol biosynthetic pathway, achieved either by deleting *ERG24* gene or by inhibiting Δ8-Δ7-sterol isomerase, provoked an 8–16-fold increase in *ERG2* gene transcription. As this block leads to the accumulation of distinct sterol species, Δ8-14 and Δ8-sterols, respectively, it seemed likely that it was ergosterol, and not the intermediate compounds accumulated, that modulated *ERG2* gene transcription. To address this issue, we constructed a squalene synthase-defective strain that contained four copies of the *ERG2*-controlled lacZ construct. This strain, namely EK9-2, was unable to synthesise sterols and proliferated only in the presence of exogenously-supplied sterols. This strain was starved for ergosterol by subsequent cultures in medium containing cholesterol as the sole source of sterol. No sterol species other than cholesterol was detected in these cells (not shown), which was expected as cholesterol is not metabolised by *Saccharomyces cerevisiae* [18]. The β-galactosidase level in these ergosterol-starved cells was found to be maximal, and similar to that observed in wt cells treated with high concentrations of SR31747A (Table 3). This result indicated that intermediates of the ergosterol biosynthesis pathway were not required for maximal induction of sterol biosynthesis gene expression. However, when cholesterol was replaced by ergosterol, β-galactosidase production did not decrease at all, although exogenously-supplied ergosterol was incorporated at a level reaching approximately 40% that normally found in wt cells. This result suggested that exogenously-supplied ergosterol was not competent for sterol biosynthesis gene regulation. When fed with zymosterol, these cells biosynthesised ergosterol which reached a level similar to that found in ergosterol-fed cells. However, β-galactosidase production was reduced by two to three-fold in zymosterol-fed cells, suggesting that ergosterol had to be biosynthesised, rather than provided exogenously, to exert a regulatory role on sterol biosynthesis. Similar results were obtained by comparing the β-galactosidase levels in an Erg^+ strain grown under aerobic and anaerobic conditions. Under the latter condition ergosterol biosynthesis is blocked at the squalene epoxidase step, no sterol is produced and ergosterol has to be provided as an exogenous source. β-Galactosidase production was enhanced 5–11-fold in cells grown in the absence of oxygen (Table 4), suggesting here again, that exogenous ergosterol exerts no inhibitory effect on *ERG2* gene transcription.

4. Discussion

Using a squalene synthase-defective strain, we could show that exogenously-supplied sterol molecules, except lanosterol, were efficiently converted into ergosterol. These results unambiguously demonstrate that sterols taken up from the medium by yeast cells can reach the subcellular compartments where sterol biosynthesis enzymes are localised. The lack of lanosterol bioconversion, as already described by Bard et al. [22], might point to a specific defect in lanosterol uptake or intracellular trafficking rather than to a defect in metabolism, since *aux32* cells are indeed able to synthesise ergosterol [23]. We have shown that the intermediate sterols accumulated upon fenpropimorph treatment, are not metabolised after drug removal, even when ergosterol biosynthesis is restored. It has already been shown that these sterol intermediates are accumulated chiefly under an esterified form [24]. As steryl esters are stored in lipid droplets [20,25], it can be deduced that these sterol intermediates are accumulated chiefly in lipid droplets, instead of being used as constituents of the plasma membrane. Our experiments suggest the absence of steryl ester transfer from lipid droplet to ER membrane. This observation contrasts with the postulated trafficking of free sterol from lipid particles to ER membrane during sterol biosynthesis. This apparent contradiction could point to the existence of (a) transporter(s) that specifically transfer free sterol, and not steryl esters, from lipid droplets to ER membrane. It is worth noting that the normal routing for steryl esters is from lipid particles to plasma membrane, which enables their utilisation as shown by Taylor and Parks [26].

ERG2 transcription is induced in the absence of ergosterol, or repressed in its presence, to an extent similar to that reported for *ERG3*. Our data are in good agreement with a recent work showing that *ERG2* gene is the most strongly induced gene amongst those in isoprenoid pathway, in yeast cells treated with sterol biosynthesis inhibitors [12]. We show however that ergosterol supplied exogenously has little or no effect on this regulation. This observation is consistent with previous results from Servouse and Karst [27] who demonstrated that exogenously-supplied ergosterol did not affect the specific activity of various sterol biosynthetic enzymes, including acetoacetyl-CoA thiolase, HMG-CoA synthase and HMG-CoA reductase. A possible explanation is that ergosterol, or an immediate metabolite, has to be present in a particular cell compartment, possibly the ER membrane or the nuclear envelope, to correctly regulate expression of its own biosynthetic genes. Exogenously-supplied ergosterol would be partly incorporated in plasma membrane, and the excess stored as steryl ester in lipid droplets, which would impede traffic back to the ER membrane and its perinuclear prolongation.

Acknowledgements: we thank Dr A. Akers (BASF, Limburgerhof, Germany) for the gift of fenpropimorph; during the course of this work I. Soustre was supported by a grant from de French 'Ministère de l'Education Nationale, de la Recherche et de la Technologie'.

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