Polyprenol formation in the yeast *Saccharomyces cerevisiae:* effect of farnesyl diphosphate synthase overexpression

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Abstract Biosynthesis of polyprenols was followed in the *erg* mutants of *Saccharomyces cerevisiae* impaired in various steps of the mevalonate pathway. The end products of the enzymatic reaction carried out in vitro, in the wild type yeast and all mutants tested, were identified as dehydrodolichols (α-unsaturated polyprenols) whereas in vivo, yeast synthesize dolichols (α-saturated polyprenols) (Biochimie, 1996.78:111–112.) The strain defective in the farnesyl diphosphate (FPP) synthase, (coded by the *erg20-2* gene) required the presence of exogenous FPP for synthesis of dehydrodolichols to occur in vitro. Overexpression of the *ERG20* gene restored synthesis of polyprenols in vitro indicating that FPP is the allylic "starter" for *cis*-prenyltransferase in yeast. Overexpression of the *ERG20* gene in the *erg 9* mutant, defective in squalene synthase activity, not only restored synthesis of dehydrodolichols in vitro, but also increased the synthesis of dolichols in vivo, almost 10-fold in comparison with wild type yeast. On the other hand overexpression of the mutated FPP synthase, coded by the gene *erg20-2* in the same genetic background, resulted in a 100-fold increase of the amount of dehydrodolichols. Interestingly, in addition to the family of typical for yeast C_{60}–C_{80} compounds, dehydrodolichols of chain length up to C_{135} were synthesized both in vitro and in vivo.—**Szkopińska A., K. Grabińska, D. Delourme, F. Karst, J. Rytka, and G. Palamarczyk.** Polyprenol formation in the yeast *Saccharomyces cerevisiae:* effect of farnesyl diphosphate synthase overexpression. *J. Lipid Res.* 1997. **38:** 962–968.

Supplementary key words *S. cerevisiae* • farnesyl diphosphate synthase • dehydrodolichols • dolichols

Dolichol phosphate is the substrate for a variety of enzymes forming mono- and oligosaccharide derivatives of this lipid, and there are several indications that the level of glycosylation in cells and tissues depends on its amount. Hence, the regulation of dolichol biosynthesis is of a considerable importance. Its synthesis branches from the biosynthetic pathway of other isoprenoid lipids (sterols and ubiquinones, **Fig. 1**). A *cis*-prenyltransferase is considered to be the first enzyme committed to dolichol biosynthesis. It catalyzes successive *cis* additions of isopentenyl diphosphate to the allylic substrate which, as we have shown in the present paper, is farnesyl diphosphate (FPP). Multiple condensations yield dehydrodolichol/dolichol diphosphates and/or their respective alcohols (1) ranging in size from 6 to 24 isoprene units, although longer chain species have been also found (2). In yeast and animal tissues, dolichols and their phosphates were identified as the end products of the biosynthetic pathway.

FPP, the product of the reaction catalyzed by FPP synthase, is a precursor for the synthesis of sterols and dolichols. Although the enzymatic steps leading to the isoprenoid lipids synthesis are known (Fig. 1), the mechanism of the regulation of the pathway is far from being understood.

To have a better insight into dolichol formation in yeast, we have measured synthesis of dolichols and dehydrodolichols in yeast strains defective in FPP synthase.

The *ERG20* gene encoding FPP synthase is an essential gene in yeast, therefore strains with deletion in this gene cannot be constructed. However, a yeast mutant partially defective in FPP synthase has been isolated and characterized. The mutant bears the defective *erg20-2* allele with a Lys^{197} to Glu substitution.

The viability of strains carrying the *erg20-2* allele is absolutely dependent on the presence of the second mutation affecting the isoprenoid pathway in order to diminish the formation of geraniol, the major end

Abbreviations: FPP, farnesyl diphosphate; HPLC, high performance liquid chromatography.

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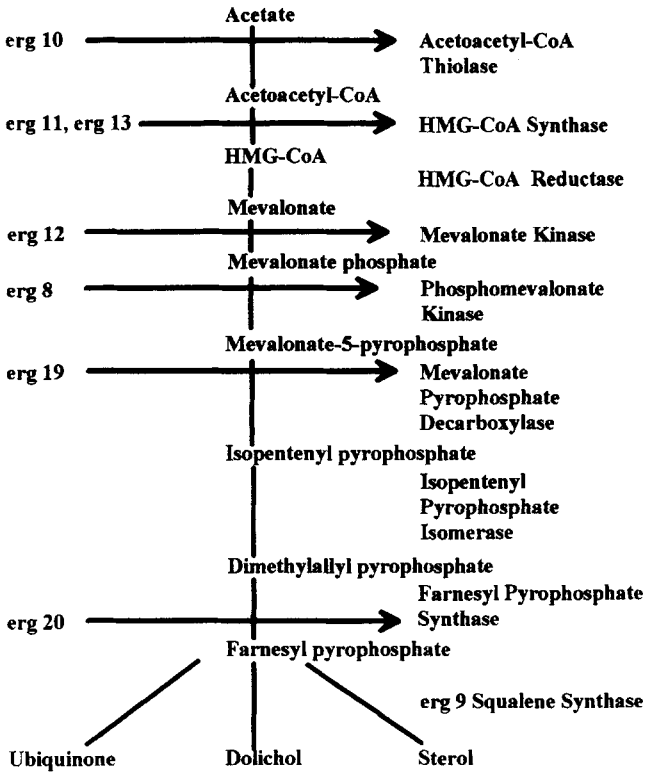

Fig. 1. Metabolic pathway leading to the synthesis of isoprenoic lipids. The *erg* mutants of *Saccharomyces cerevisiae*, deficient in the synthesis of ergosterol, and corresponding defective enzymes are listed.

product of the defective enzyme, that is toxic for yeast (3). In the two defective mutants tested, the *erg20-2* allele was accompanied by mutations affecting mevalonate kinase (*erg12*) or squalene synthase (*erg9*) genes.

We have also used the strains in which biosynthesis of the wild type and mutated form of FPP-synthases has been highly elevated by introducing the respective genes (*ERG20* and *erg20-2*) on multiple copy plasmids under inducible promoters. The earlier results from our laboratory indicated that in the three *erg* mutants: *erg8*, *erg12*, and *erg9* blocked in phosphomevalonate, mevalonate kinases, and squalene synthase, respectively (Fig. 1), mannosylation of endogenous dolichol phosphate was 2- to 4-fold lower as compared to the parental strain. These differences could be overcome when exogenous lipid carrier (dolichol phosphate) was added to measure glycosylation in vitro, indicating that the activity of the respective glycosyl transferase in these mutants was not affected (4). The results obtained suggested that the synthesis of dolichyl phosphate in *erg* mutants was impaired. Therefore, we have undertaken the studies on polyprenol formation in the wild type yeast and some *erg* mutants.

MATERIALS AND METHODS

Chemicals

All reagents were of analytical grade. Dehydrodolichol and dolichol standards were obtained from the Collection of Polyprenols of the Institute of Biochemistry and Biophysics (Warsaw). [^{14}C]isopentenyl diphosphate was from Amersham.

Yeast *S. cerevisiae* strains

The strains used in this study are listed in **Table 1** Plasmids: pDD plasmids are the derivatives of a high copy number plasmid pDP51 Not I (5). Plasmid pDD5 carried the wild type gene *ERG20* and pDD9 mutated allele *erg20-2*, under *GAL10/CYC1* promoter with *PGK* terminator. The genes were ligated in EcoR1–BamH1 sites.

Media and growth conditions

Yeast cells were grown in YP medium (1% yeast extract, 1% bactopeptone) with 1% glucose (YPG), 2% galactose (YPGal), or 2% ethanol (YPE) as a carbon source. Cells were grown at 28°C with vigorous agitation. Media for the *erg* mutants were supplemented with ergosterol (2 mg/l) in 1% Tween 80. The yeast membrane fraction served as an enzyme source and it was prepared as described previously (6).

Chromatography

Thin-layer chromatography was performed on silica gel plates Kieselgel 60 in benzene–ethyl acetate 95:5 (v/v) and HPTLC RP-18 precoated plates with concentrating zone in acetone containing 50 mM H_3PO_4.

HPLC was performed on a column 6 cm × 4.5 mm Hewlett-Packard, ODS hypersil 3 μm at a flow rate 1.5 ml/min in gradient A: methanol–isopropanol–water 12:8:1 (by vol); B: hexane–isopropanol 7:3 (by vol)

In vitro synthesis of dehydrodolichols

The incubation mixture contained, in a final volume of 250 μl, 50 mM sodium phosphate buffer, 0.5 mM $MgCl_2$, 20 mM β-mercaptoethanol, 10 mM KF, 3 × 10^5 cpm [^{14}C]IPP and 500 μg of membrane protein. After a 90-min incubation at 30°C, the reaction was terminated by the addition of 4 ml of chloroform–methanol 3:2. The protein pellet was removed by centrifugation and the supernatant was washed three times with 1/5 volume of 10 mM EDTA in 0.9% NaCl. The organic

TABLE 1. Strains used in this work

Strain	Genotype	Source
FKerg8	*MAT* α-*erg8-1 ura3-1*	ref. 11
CC 25	*MAT* α-*erg20-2 erg12-2 ura3-1 trp1-1*	ref. 17
FK 5188	*MAT* α-*erg9-1 ura2-33 ura3-1*	ref. 11
FL 100	*MAT* a	ATCC 28383
DD 104	*MAT* α-*erg9-1 erg20-2 his3 leu2 ura3 ade2*	F. Karst
DD 95	*MAT* α-*erg9-1 erg20-2 his3-1 leu2-3 ura3-1 ade2-1* [pDD5]	F. Karst
DD 94	*MAT* α-*erg9-1 erg20-2 his3-1 leu2-3 ura3-1 ade2-1* [pDD9]	F. Karst

phase was concentrated under stream of nitrogen and subjected to thin-layer chromatography.

Characterization of polyisoprenoids synthesized in vivo

The membrane fraction was extracted with chloroform–methanol 3:2. Denatured protein was discarded by centrifugation. The organic supernatant was washed three times with 10 mM EDTA in 0.9% NaCl and evaporated to dryness. Extracted lipids were suspended in hexane and applied to a silica gel column equilibrated with hexane. A step gradient of 3, 8, 12, 15, and 18% of diethyl ether in hexane allowed recovery of an almost pure fraction of polyisoprenoids eluting at 15% diethyl ether. The amount of polyprenol alcohols was estimated by comparison with an internal standard of 0.1 mM Dol_{23} during HPLC. The chain length was determined by HPLC.

RESULTS

Thorough analysis of the products of *cis*-prenyltransferase activity in vitro in wild type yeast revealed synthesis of a family of unphosphorylated dehydrodolichols, ranging in chain length from C_{60} to C_{80} with the predominant C_{75} and C_{80} species (7).

To have a better insight in dolichol biosynthesis in yeast, we measured the formation in yeast strains defective in FPP synthase activity. The results presented in **Fig. 2** indicate that strain CC 25 (*erg20-2,erg12*) is defective in this activity. In contrast, sterol auxotrophs defective elsewere in the pathway, such as strains *FKerg8* and *FK 5188* (*erg9*) defective in phosphomevalonate kinase and squalene synthase activity, respectively, synthesize free polyprenol alcohols typical for the wild type yeast. The addition of farnesyl diphosphate to an incubation mixture containing the membrane fraction of CC 25 (*erg20-2,erg12*) restores the synthesis of polyprenol alcohols in vitro; thus polyprenol formation is not affected in this strain.

HPTLC analysis of the lipids isolated from the membranes of mutants with *erg20-2* mutation indicates that the strains synthesize constitutive amounts of polyprenol alcohols in vivo (not shown).

To find out whether overexpression of the *ERG20* (farnesyl diphosphate synthase encoding) gene would influence biosynthesis of dolichols in yeast, strain DD 104 (*erg20-2,erg9*) was transformed with multicopy pDD5 plasmid bearing the *ERG20* gene under the inducible *Gal 10/CYC 1* promoter (strain DD 95). The advantage of DD 104 is that it carries an *erg9* defective (squalene synthase) allele in addition to *erg20-2*, thus the overexpression of *ERG20* would theoretically not only restore the biosynthesis of polyprenols in vitro but would also increase the biosynthesis of dolichols in vivo, due to the defect in ergosterol biosynthesis.

The identification of isoprenoids produced by strain DD 95 (*erg20-2,erg9*/pDD5*ERG20*) was established in two ways: one, by cochromatography with polyprenol and dolichol standards of equivalent chain length.

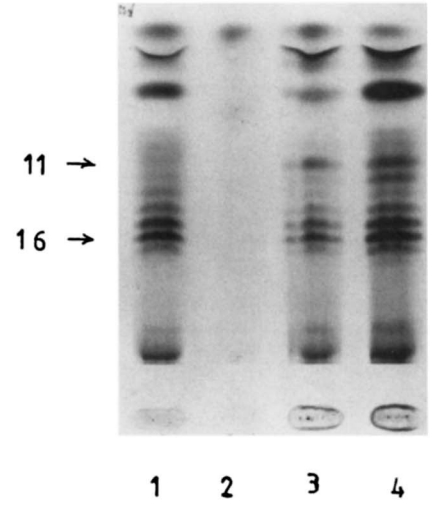

Fig. 2. Autoradiogram of HPTLC (RP-18) plates of polyprenol alcohols synthesized in vitro by wild type and ergosterol yeast mutants. FL 100 (lane 1); CC 25 (*erg20-2,erg12*) (lane 2); *FKerg8* (lane 3); FK 5188 (*erg9*) (lane 4). The lipophylic incubation products were isolated and subjected to HPTLC as in Materials and Methods. Plates were developed in acetone containing 50 mM H_3PO_4. ^{14}C-labeled compounds were visualized by autoradiography. Arrows show polyprenols of indicated chain length.

TABLE 2. Synthesis of isoprenoid alcohols in wild type and mutants of *Saccharomyces cerevisiae*

Strain	Relevant Genotype	Isoprenoid Alcohols	
		Dolichol	Dehydrodolichol
		μg/mg protein	
FL 100	*ERG20,ERG9*	0.1	
DD 104	*erg20-2,erg9*	0.11	
DD 95	*erg202,erg9/*pDD5*ERG20*	1.18	
DD 94	*erg20-2,erg9/*pDD9*erg20-2*	0.80	11.2

Isoprenoid alcohols were extracted and prepared for HPLC as in Materials and Methods. Their amount was estimated against internal standard 0.1 mM Dol_{23}.

Chromatography on Silica Gel plates distinguishes the two types of isoprenoids (see ref. 1). Additionally, we compared the retention times of isoprenoids synthesized by strain DD 95 with retention times of standards of prenols and dolichols of corresponding chain length using HPLC. Both analyses indicated that isoprenoids synthesized by strain DD 95 were dolichols. Quantitative analysis of the lipids (see Materials and Methods) of DD 95 strain indeed revealed a 10-fold increase of dolichol content as compared to the wild type strain (**Table 2**).

We have also performed the lipid analysis (as described above) in strain DD 104 (*erg20-2,erg9*) transformed with the mutated allele of FPP synthase gene (*erg20-2*) (strain DD 94). Data presented in Table 2 indicate a further increase in the amount of isoprenoids synthesized (about 100-fold higher than the control). Moreover, as shown in **Fig. 3B,** lane 1, the pattern of polyprenol alcohols synthesized by DD 94 in vitro in addition to typical C_{60} to C_{80} (compare Fig. 2) shows polyprenols up to C_{110} and in vivo very long chain polyprenol alcohols composed of even up to 25 isoprene units (C_{130}) (Fig 3A). For the synthesis in vitro to occur, addition of exogenous farnesyl diphosphate is necessary (Fig. 3B lane 2). The synthesis of polyprenols of unusual chain length was only noticeable when the DD 94 strain was grown in the induced condition. The level of induction was significantly higher in cells grown on galactose than on ethanol. Thus it can be concluded that biosynthesis of polyprenols of unusual chain length correlates with the level of induction of the *erg20* gene (**Fig. 4**).

HPLC analysis of polyprenoids obtained from the membranes of the DD 94 strain (*erg20-2,erg9/*pDD9*erg20-2*) confirmed occurrence of the very large amount of unexpected chain length polyprenol alcohols, all α-unsaturated, while the amount of dolichols typical for yeast C_{50}–C_{60} (retention time 10.38–10.50

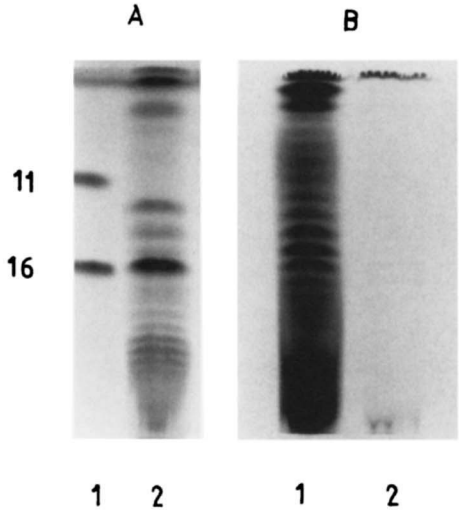

Fig. 3. HPTLC (RP-18) of polyprenol alcohols synthesized by strain DD94(*erg20-2,erg9/*pDD9*erg20-2*. A: Polyprenol standards of indicated chain length (lane 1). Lipids synthesized by DD94 in vivo (lane 2). Products were visualized with iodine vapor. B: ^{14}C-labeled lipids obtained after incubation of [^{14}C]IPP with the DD94 membranes (lane 1 in the presence, lane 2 in the absence of exogenous FPP). [^{14}C]-labeled lipids were detected by autoradiography. Incubation, lipids purification, and HPTLC as in Materials and Methods.

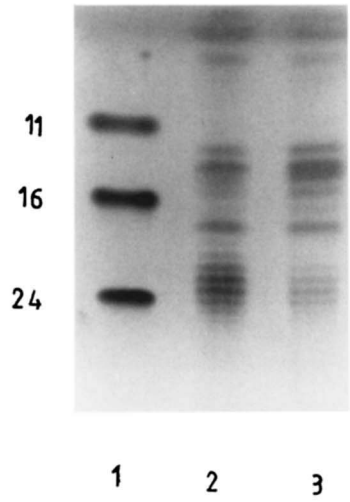

Fig. 4. Induction of biosynthesis of polyprenol alcohols in vivo in DD 94. DD 94 strain (*erg9, erg20-2,* double mutant with plasmid bearing *erg20-2* gene under *GAL10/CYC1* promoter) was cultivated in the medium containing galactose (lane 2) or ethanol (lane 3) as a sole carbon source; lane 1, polyprenol standards of indicated chain length. Lipids were isolated and analyzed by HPTLC (RP-18) as in Fig. 2 and visualized with iodine vapor.

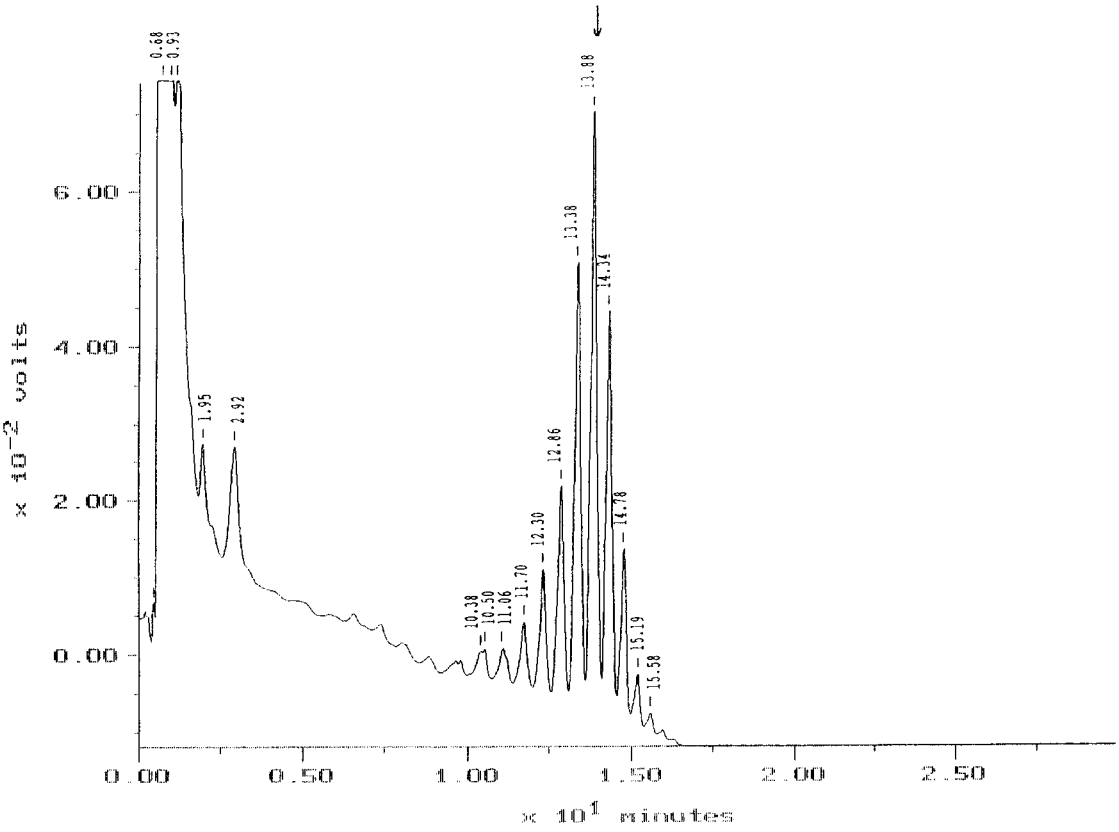

Fig. 5. HPLC analysis of polyprenol alcohols isolated from the membranes of DD 94 strain (*erg20-2,erg9/*pDD9*erg20-2*). Lipids were extracted from the membranes of DD 94 strain with chloroform–methanol 3:2 and washed with 10 mM EDTA in 0.9% NaCl. The organic extract was evaporated to dryness and resuspended in hexane followed by chromatography on a Silica Gel column equilibrated with hexane. A nearly pure fraction of polyisoprenoids was eluted with 15% diethyl ether in hexane and subjected to HPLC analysis on ODS hypersil column. The polyprenol alcohol, composed of 22-isoprene units (C_{110}), served as the internal standard (position of the standard is indicated by the arrow). Numbers over the peaks indicate retention times.

min) was relatively small as shown in **Fig. 5.** Thus, the α-saturation step in the DD 94 strain was also affected.

DISCUSSION

In this study we have used *S. cerevisiae* strains defective in the ergosterol biosynthetic pathway to follow synthesis of polyprenol alcohols as a means of focusing on the role of FPP synthase. As sterols and dolichols share a common biosynthetic pathway, we assumed that the defect in one branch of the pathway might influence the other. It appeared that among the mutants tested, deficient in the synthesis of farnesyl diphosphate, strains CC 25 (*erg20-2,erg12*), DD 104 (*erg20-2,erg9*) and DD 94 (*erg20-2,erg9/*pDD9*erg20-2*) required addition of allylic "starter" for the synthesis of polyprenols in vitro. FPP synthase is mainly present in cytoplasm. However, recent investigations demonstrate that FPP synthase activity is associated with other subcellular compartments. Runquist et al (8) and Ericsson et al. (9) found that extensively washed rat liver microsomes contained FPP activity and that FPP produced could be used by both squalene synthase and *cis*-prenyltransferase present in the membranes.

The synthesis of polyprenols in vitro increases to a different level after addition of FPP depending on the strain. In strains with *erg20-2* mutation, the synthesis is possible only in the presence of exogenous FPP (CC25, DD 94). In strain DD 95 (with *ERG 20* on the plasmid under inducible galactose promoter) it has no influence, while in the wild type strain FL 100 it stimulates formation of polyprenols approximately 4-fold. As CC 25 strain (*erg20-2, erg12*) has been reported to synthesize geranyl diphosphate (GPP) in amounts comparable to the level of FPP in the wild type strain (3), the above results suggest that in yeast, FPP is the allylic primer for polyprenol chain elongation, and that it cannot be replaced by GPP, thus confirming the results of the in vitro studies that FPP is the best substrate for polyprenol formation (10).

In vivo, however, the level of polyprenols in the *erg 20* defective cells was only 20% lower than in the wild type strain. As sterol auxotrophs usually do not require sterol supplementation at a permissive temperature, although in vitro the activity of the defective enzymes is often impaired (11,12), the latter result is understandable. We have also used the strains in which activity of the wild type and mutated form of farnesyl diphosphate synthase is overexpressed in the genetic background of *erg20-2* and *erg9* mutations. In these transformants, *ERG20* and *erg20-2* genes were introduced on a multicopy plasmid under the inducible promoter *GAL10/CYC1*. The substitution of lys197 by glu in the *erg20-2* mutant leads to excretion of short chain prenyl alcohols such as farnesol and geraniol (3). Introduction of the *erg9* mutation into the strain insures impairment of squalene synthase and, in consequence, a markedly reduced level of ergosterol biosynthesis.

As is demonstrated in the Figs. 4 and 5, the pattern of polyprenol alcohols synthesized both in vitro and in vivo by the DD 94 (*erg20-2,erg9/*pDD9*erg20-2*) strain is distinctly different from that synthesized by the wild type strain. In addition to the compounds typical for yeast, long chain polyprenol alcohols are synthesized, all α-unsaturated, whereas in wild type yeast only dolichols were found. Hence, it can be concluded that overexpression of the *erg20-2* gene together with the impairment of ergosterol biosynthesis leads to a defect in the final step of conversion of polyprenols to dolichols catalyzed by α-saturase and to the perturbance in the termination of polyprenol chain synthesis. Nevertheless, it is unclear how and whether these two effects are related.

Analysis of the products of the reaction in vitro catalyzed by *cis*-prenyltransferase from yeast indicates that polyprenol diphosphates synthesized undergo immediate dephosphorylation (7). A similar conclusion was reached for the rat liver system (13) and the yeast system (14). As we have demonstrated that endogenous dolichols are phosphorylated by CTP-dependent dolichol kinase (15) it is reasonable to assume that the conversion of dehydrodolichols to dolichols proceeds rephosphorylation step.

It should be noted that overexpression of the FPP-synthase gene (without shutting off sterol synthesis) has no effect on the dolichol level (A. Szkopińska, unpublished results) indicating the higher affinity for FPP of the enzymes involved in biosynthesis of ergosterol, as compared to *cis*-prenyltransferase. The occurrence of dehydrodolichols in the DD 94 strain (*erg20-2,erg9/*pDD9*erg20-2*) instead of dolichols typical for yeast coincides with the massive increase of their synthesis. The latter might suggest that the α-saturation step is a rate-limiting factor in conversion of dehydrodolichols. A similar observation was already made when studying dolichol synthesis in mutants of Chinese hamster ovary cells (16).

In the FPP-synthase defective strains where an increase in the FPP level is achieved by overexpression of the *erg20-2* gene, the FPP and ergosterol levels are not restored to the amount characteristic for the wild type (3). Therefore, accumulation of polyprenol alcohols due to the overexpression of FPP-synthase and to an impaired sterol biosynthetic pathway cannot simply result from the increase in FPP formation. The latter results allow us to assume that the FPP-synthase itself could be a part of the complex involved in polyprenol formation.

The deficiency in the conversion of dehydrodolichols to dolichols is not observed upon induction of "wild type" FPP-synthase in the DD 95 strain (*erg20-2,erg9/*pDD5*ERG20*) although a 10-fold increase in the synthesis of dolichols is measured. These results also suggest that *cis*-prenyltransferase activity requires interaction with FPP-synthase. Introduction of the mutated form of the enzyme, coded by the *erg20-2* gene, leads to the impairment of this interaction and in turn to the perturbed synthesis of dolichols typical for yeast.

Our results demonstrate that using yeast, a convenient model for genetic and molecular biology manipulations, offers a good chance to answer questions concerning the regulation of dolichol biosynthesis that cannot be answered in other systems.

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