Farnesol-induced growth inhibition in *Saccharomyces cerevisiae* by a cell cycle mechanism

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The growth of budding yeast, *Saccharomyces cerevisiae*, was inhibited in medium containing 25 µM farnesol (FOH). The FOH-treated cells were still viable, and were characterized by a transition from budded to unbudded phase as well as a significant loss of intracellular diacylglycerol (DAG). FOH-induced growth inhibition could be effectively prevented by the coaddition of a membrane-permeable DAG analogue which can activate yeast protein kinase C (PKC). However, yeast cell growth was not initiated upon addition of the PKC activator when the cells had been pretreated with FOH for 20 min. The failure in cell growth recovery was believed to be due to a signalling-mediated cell cycle arrest in FOH-pretreated cells. Differential display analysis demonstrated that the expression of cell cycle genes encoding DNA ligase (CDC9) and histone acetyltransferase (HAT2) was strongly repressed in FOH-treated cells. Repression of the expression of these genes was effectively cancelled when cells were grown in medium supplemented with DAG. The authors propose an interference with a phosphatidylinositol-type signalling which is involved in cell cycle progression as a cause of FOH-induced growth inhibition in yeast cells.

**Keywords:** *Saccharomyces cerevisiae*, farnesol, diacylglycerol, signal transduction, cell cycle

INTRODUCTION

Isoprenoids are involved in the biosynthetic pathways of sterols, dolichols, haems and ubiquinones in a variety of organisms (Goldstein & Brown, 1990), as well as those for isoprenoid membrane lipids in Archaea (Zhang & Poulter, 1993). Isoprenoids play another essential role in post-translational modification of oncogenic RAS proteins and other G proteins in which farnesylation and geranylgeranylation moieties function in determining their inner-membrane location and activation for signal transduction (Glomset et al., 1990). Farnesyl pyrophosphate and geranylgeranyl pyrophosphate are commonly found as the intermediates in the sequential reactions for isoprenoid biosynthesis and are also used as the substrates in other reactions for protein prenylation. Farnesol (FOH) and geranylgeraniol (GGOH) can thus be endogenously generated, possibly by enzymic dephosphorylation of the corresponding pyrophosphate esters in mammalian cells (Bansal & Vaidya, 1994). Unexpectedly, exogenous FOH and GGOH were found to inhibit the proliferation of human acute leukaemia CEM-C1 cells and human promyelocytic HL-60 cells, respectively, thereby inducing apoptotic cell death as reflected in the predicted DNA fragmentation pattern (Melnykovych et al., 1992; Haug et al., 1994; Voziyan et al., 1995; Ohizumi et al., 1995).

Phosphatidylinositol-type signalling is widely conserved among eukaryotic cells, including yeasts, to regulate cell proliferation or growth in response to the cellular diacylglycerol (DAG) level. Protein kinase C (PKC)-dependent signal transduction has been studied with the aid of a membrane-permeable DAG analogue, 1-oleoyl-2-acetyl-sn-glycerol (OAG) which can activate the enzyme under physiological conditions. FOH-treated mammalian cells were protected against apoptotic cell
death by the coaddition of DAG analogue, with a parallel loss of DNA fragmentation (Haug et al., 1994; Voziyan et al., 1995). This suggests that FOH induces apoptosis of mammalian cells possibly by reducing the intracellular level of DAG so as to cause inactivation of PKC.

In a previous study we found an inhibitory effect of a long-chain alkyl derivative of UMP on the pheromone-induced sexual agglutination of Saccharomyces cerevisiae, an initial step of the mating process between cells of a and z mating type (Machida et al., 1997). The presence of the UMP derivative allowed both a and z haploid cells to grow normally in a vegetative manner, indicating a non-cytotoxic effect as a cause of the above biological event. Although the exogenous addition of FOH inhibited agglutination more effectively than the growth inhibition in S. cerevisiae in terms of its relation to a phosphatidylinositol-type signalling function in cell cycle regulation.

METHODS

Strains and media. Unless stated otherwise, S. cerevisiae X2180-1A (MATa) (Machida et al., 1997) was used throughout. Some other yeast strains, filamentous fungi and bacteria (see Results and Discussion) were also used for FOH antimicrobial activity assays by the serial broth dilution method as described previously (Akeda et al., 1995). Growth properties of S. cerevisiae X2180-1A cells were examined at 30 °C in YPD medium, which contained 1% yeast extract, 2% polypeptone and 2% glucose.

Measurements of cell growth. Unless otherwise stated, yeast cells were grown overnight in YPD medium at 30 °C with vigorous shaking and were inoculated into freshly prepared medium to give an initial cell density of approximately 10^6 cells ml^-1. Portions were withdrawn at intervals to measure the cell density (OD610) and the number of c.f.u. by plating appropriately diluted samples on YPD agar and incubating at 30 °C. A yeast cell suspension of 10^7 cells ml^-1 gave an OD610 value of approximately 0.1. The relative cell growth was calculated using OD610 values as previously described (Machida et al., 1997). The proportions of budded cells to total cells were measured by counting 600 cells per sample under an Olympus BX 50 microscope, as described by Smith et al. (1995).

Lipid extraction. Yeast cells were inoculated into YPD medium at an initial cell density of 10^7 cells ml^-1 and grown at 30 °C with or without addition of FOH. At various times, 30 ml portions were withdrawn and cells were collected by centrifugation. Cell pellets were then suspended in 1:1 ml of 0.2 M KCl containing 5 mM EDTA, and the lipids extracted by a modification of the method of Bligh & Dyer (1959), as follows. Cells were disrupted by repeated vortexing with 500 mg glass beads (500 μm diameter) for five 30 s periods. The supernatant obtained after centrifugation was then mixed with 3:75 ml chloroform/methanol (1:2, v/v). After extensively stirring the monophase, 1.25 ml chloroform and 1.25 ml 0.2 M KCl containing 5 mM EDTA were added to break the phase. The resulting two phases were separated by centrifugation at 3000 g for 2 min. The lower chloroform phase was evaporated under N_2 and the pellet was stored in a solution of benzene/hexane (1:1, v/v) at -20 °C until use.

Quantitative assay of DAG. DAG contents in the lipid fractions were measured by a radioenzymatic assay employing DAG kinase, which quantitatively converts DAG to the corresponding [32P]phosphatidic acid in the presence of [γ-32P]ATP (Preiss et al., 1986). A DAG assay kit (Amersham) was used. [32P]Phosphatidic acid formed in the reaction was measured by a BAS 1000 bio-imaging analyser (Fuji Film Co.) after separation by thin-layer chromatography using a silica gel plate (Merck 60) and a solvent of chloroform/methanol/acetate (65:15:5, by vol.).

RNA isolation and differential display analysis. The yeast cells were grown at 30 °C for 30 min with or without FOH and collected as described in the section on lipid extraction. Cells were then disrupted by ten 30 s periods of vortexing at 4 °C, and total RNA was isolated by a modified guanidine thiocyanate method using a Quick Prep total RNA extraction kit (Pharmacia Biotech). Total RNA fractions thus obtained were employed for the following differential display analysis using a differential display assay kit (Display Systems) after treatment with RNase-free DNase. This assay kit provides 24 upstream primers and 9 downstream primers and we employed a total of 24 x 9 PCR reactions using RNA fractions isolated from yeast cells with or without FOH treatment. The first-strand cDNA was synthesized in 30 μl RT-PCR buffer (Takara, Japan) containing total RNA (6–9 μg), 20 μM of each dNTP, 2 mM MgCl2, 30 units RNase inhibitor (Takara), 2.5 μM of the arbitrary downstream primer and 7.5 units AMV reverse transcriptase XL (Takara) at 55 °C for 30 min followed by the annealing at 30 °C for 10 min. The mixture was heated at 95 °C for 5 min to inactivate of reverse transcriptase, diluted with 80 μl TE buffer (10 mM Tris/HCl pH 8.0, 1 mM EDTA) and stored at -80 °C until use. The reaction mixture (20 μl) for PCR contained 1 μl cDNA solution prepared as above, 05 μM of the arbitrary upstream primer, 2.5 μM of the arbitrary downstream primer, 2 μM of each dNTP, 3 mM MgCl2, [x-32P]dATP (1.85 MBq ml^-1) and 10 units Taq DNA polymerase in the PCR buffer (Takara). The mixture was subjected to 30 cycles (95 °C for 30 s for denaturation, 37 °C for 1 min for low-stringency annealing of arbitrary primer, and 72 °C for 1 min for second-strand extension), followed by three cycles (95 °C for 30 s, 46 °C for 1 min and 72 °C for 1 min), three cycles (95 °C for 30 s, 43 °C for 1 min and 72 °C for 1 min), another three cycles (95 °C for 30 s, 40 °C for 1 min and 72 °C for 1 min) and an additional final extension at 72 °C for 5 min. The amplified cDNAs (10 μl) were separated on a 6% polyacrylamide gel in 0.375 M Tris/HCl (pH 8.9) using 0.25 M Tris/0.192 M glycine running buffer as described by Ito et al. (1993). Band patterns were visualized by autoradiography with a BAS 2000 bio-imaging analyser (Fuji Film Co.). The overall step was run in duplicate and reproduced bands were used in the next step.

Rearrangement, cloning, sequencing of DNA fragments, and Northern analysis. Gel pieces containing the bands of interest were excised from the gel using a blade, and added directly into 50 μl PCR mixture containing 0.2 μM upstream primer, 0.2 μM downstream primer, 2 mM MgCl2, 50 μM of each dNTP, and 2.5 units Taq DNA polymerase in the PCR buffer (Takara). The corresponding DNA fragments were reamplified with the same condition as for the differential display PCR.
FOH-induced growth inhibition of S. cerevisiae

Table 1. Inhibitory effects of FOH and related isoprenoids on the growth of S. cerevisiae

Yeast cells were grown at 30 °C for 4 h in YPD medium in which the initial cell density was adjusted to 10^5 ml^{-1} (OD_{600} approx. 1.00).

<table>
<thead>
<tr>
<th>Addition</th>
<th>Conc (µM)</th>
<th>Relative cell growth (%)^*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>GOH</td>
<td>400</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>98</td>
</tr>
<tr>
<td>Nerol</td>
<td>400</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>101</td>
</tr>
<tr>
<td>Linalool</td>
<td>400</td>
<td>101</td>
</tr>
<tr>
<td>FOH</td>
<td>6.25</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>GGOH</td>
<td>400</td>
<td>98</td>
</tr>
<tr>
<td>Farnesyl acetate</td>
<td>400</td>
<td>83</td>
</tr>
<tr>
<td>Squalene</td>
<td>400</td>
<td>101</td>
</tr>
</tbody>
</table>

^* The OD_{600} value corresponding to 100% was 4.36 ± 0.8. All measurements were done in triplicate and the mean values are given.

X2180-1A was almost completely inhibited in shaking culture with 25 µM FOH. GOH, nerol, linalool, squalene and GGOH did not show apparent growth-inhibitory effects even at 400 µM. Farnesyl acetate inhibited yeast cell growth slightly at 400 µM. Isoprenoid alcohols may exhibit different biological activities depending on the tested organism, although the corresponding pyrophosphate esters are commonly involved in the initial step of their isoprenoid metabolism (Goldstein & Brown, 1990; Zhang & Poulter, 1993). GOH was evaluated as a lipophilic agent which can increase cytoplasmic membrane fluidity, thereby inhibiting the growth of Candida albicans and S. cerevisiae at high concentrations around 3 mM (Bard et al., 1988). GOH could enhance potassium leakage from whole cells of S. cerevisiae under the above conditions. FOH was likely to exhibit a different type of yeastcidal activity since the yeast cells significantly lost the ability to form colonies on YPD agar at 48 h cultivation when they had been exposed to only 25 µM FOH for 15 min or less (Fig. 1a). However, the FOH-treated cells still kept the ability to divide so that they could form visible colonies after 96 h cultivation on the agar plate. As shown in Fig. 1(b), FOH-treated cells were all viable even after 6 h incubation in the medium with 25 µM FOH as judged from the fact that the numbers of c.f.u. were comparable to those in the original inoculum. This means that FOH-treated cells were prevented from normal cell division for an extremely extended period. Microscopic observation revealed a marked increase in the proportion of unbudded cells in the population at

acts. Upstream primers and downstream primers which could reamplify each band were as follows: band (a), upstream primer 7, downstream primer 3; band (b), upstream primer 14, downstream primer 3; band (c), upstream primer 19, downstream primer 4; band (d), upstream primer 15, downstream primer 6; band (e), upstream primer 7, downstream primer 4. Reamplified DNA fragments were then cloned into the pCR-Script AmpSK(+) cloning vector (Strategene). Among the transformants obtained, clones bearing the bands of interest were identified as described by Adati et al. (1996).

Following isolation by the alkaline-SDS method and subsequent polyethylene glycol precipitation (Sambrook et al., 1989), cloned DNA fragments were sequenced by the dideoxy chain-termination method (Sanger et al., 1977) using an ABI Prism 373 Dye Terminator Cycle Sequencing Core kit (Applied Biosystems). Sequence data were subjected to homology searching at the nucleotide level using the BLAST program on the S. cerevisiae Genome Database at Stanford. For Northern analysis, total RNA fractions were again isolated from yeast cells grown at 30 °C for 30 min with or without 100 µM FOH as described above. Total RNAs (10 µg) were resolved on a 1% agarose/22 M formaldehyde gel, blotted onto a Hybond-N membrane filter (Amersham) and then hybridized with α-32P-labelled probes by the standard procedure (Sambrook et al., 1989). The ACTI (yeast actin gene) transcript was used as an internal control for loading. In the Northern analysis, the total RNA fractions were also prepared from cells grown in YPD medium containing both 100 µM FOH and 30 µM OAG. DNA probes used were a 110 bp PstI fragment for CDC9, a 230 bp PstI fragment for HAT2, and a 520 bp Styl fragment for ACTI, which were isolated with the QiaQuick Gel Extraction kit (Qiagen) after purification by gel electrophoresis. Band patterns were visualized by autoradiography with a BAS 2000 bio-imaging analyser (Fuji Film Co.).

Chemicals. GOH (trans-geraniol), nerol (cis-GOH), linalool, squalene, FOH, farnesyl acetate and GGOH were purchased from either Aldrich or Sigma. These chemicals were kept as solutions in ethanol. Phospholipids and OAG were from either Sigma and then hybridized with α-32P-labelled probes by the standard procedure (Sambrook et al., 1989). The ACTI (yeast actin gene) transcript was used as an internal control for loading. In the Northern analysis, the total RNA fractions were also prepared from cells grown in YPD medium containing both 100 µM FOH and 30 µM OAG. DNA probes used were a 110 bp PstI fragment for CDC9, a 230 bp PstI fragment for HAT2, and a 520 bp Styl fragment for ACTI, which were isolated with the QiaQuick Gel Extraction kit (Qiagen) after purification by gel electrophoresis. Band patterns were visualized by autoradiography with a BAS 2000 bio-imaging analyser (Fuji Film Co.).

**RESULTS AND DISCUSSION**

**Effects of FOH and other isoprenoids on yeast cell growth and viability**

A potent antimicrobial activity of FOH was first observed with the follicular bacterium Propionibacterium acnes (Kubo et al., 1994) and more recently with a halophilic archaeon, Halofexerax volcanii (Tachibana et al., 1996). A step of mevalonate biosynthesis was proposed to be one of the FOH-sensitive sites in H. volcanii. However, FOH did not show any antibacterial activity against Escherichia coli IFO 3545, Staphylococcus aureus NCTC 8530 or Bacillus subtilis IFO 3007 in our experiment using the serial broth dilution method. FOH was also inactive against the filamentous fungi Aspergillus niger ATCC 6275, Penicillium chrysogenum IFO 4626 and Mucor mucedo IFO 7684. In contrast, FOH inhibited the growth of various yeast strains such as S. cerevisiae X2180-1A (MATa), S. cerevisiae X2180-1B (MATα) and Schizosaccharomyces pombe IFO 0342, with minimum inhibitory concentrations less than 25 µM. As shown in Table 1, the growth of S. cerevisiae

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Fig. 1. Growth-inhibitory effects of FOH on cells of S. cerevisiae. Cells were grown in YPD medium containing FOH at 0 (○), 12.5 (●), or 25 μM (■). Cell viability was expressed as the number of c.f.u., counted after 48 h (a) and 96 h (b) cultivation on YPD agar.

Table 2. Transition of budded to unbudded phase of FOH-treated S. cerevisiae cells

<table>
<thead>
<tr>
<th>FOH (μM)</th>
<th>Incubation time (h)</th>
<th>Proportion of budded cells (%)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2</td>
<td>69±7</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>76±6</td>
</tr>
<tr>
<td>12.5</td>
<td>2</td>
<td>68±8</td>
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<td></td>
<td>4</td>
<td>73±5</td>
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<tr>
<td>25</td>
<td>2</td>
<td>26±4</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>28±7</td>
</tr>
</tbody>
</table>

a Values are means ± SD in triplicate assays.

both 2 and 4 h incubation with 25 μM FOH (Table 2). About 70% of the cells were budded in the medium without FOH. FOH-induced growth inhibition of yeast cells was characterized as a static but a potent growth-arresting effect, as is the case with Li+-induced cell cycle arrest in G1 of S. cerevisiae cells, which was assessed by a similar transition from budded to unbudded phase (Smith et al., 1995). Fig. 1 also shows a remarkable difference between the response of cells in the medium with 12.5 and 25 μM FOH. A similar result has been reported for the growth-inhibitory effect of FOH on human acute leukaemia CEM-1 cells (Haug et al., 1994). The mammalian cells were mostly viable (84.0%) in the medium with 25 μM FOH but drastically lost viability to 0.6% of the original level when exposed to 30 μM FOH. It remains to be solved how such a modest change in FOH concentration could evoke such a strong response.

DAG, which also functions as a modulator of phosphatidylinositol-type signalling in yeast (Ogita et al., 1990), in the presence or absence of added FOH. As shown in Fig. 2, the intracellular DAG level was drastically decreased by 5 min incubation with 25 μM FOH, correlating with the failure in cell division or budding of the cells thus treated. DAG was maximally decreased to 40–50% of the original level at 10 min of incubation in the medium containing FOH at 25 μM or higher. In α-pheromone-treated cells of S. cerevisiae, the cellular content of DAG was similarly reduced to the lowest level, around 150 pmol per 107 cells, along with a drastic decrease in the budded cell population (Marini et al., 1996). DAG was again increased up to 300 pmol per 107 cells in the following synchronous recovery from the mating-pheromone-induced cell cycle arrest. It is unlikely that FOH-treated cells could not reinitiate the cell cycle upon the following removal of exogenous FOH (see Fig. 1). This would require a long-lasting FOH-induced event (as discussed in the following section) since FOH itself can be metabolized into the corresponding isoprenoid compounds via the formation of farnesyl pyrophosphate (Crick et al., 1997).

Protective effects of OAG on FOH-induced growth inhibition

In mammalian cells, the FOH-induced inhibition of cell proliferation could be restored with either exogenously added phosphatidycholine (PC) or OAG, a physiological activator of PKC (Melnikovych et al., 1992). OAG is also known to activate PKC from S. cerevisiae cells as effectively as the enzyme from mammalian cells (Ogita et al., 1990). As shown in Fig. 3, the exogenous addition of PC was effective in preventing the growth-inhibitory effect of FOH; a weak protective effect was observed with phosphatidylserine but no protective effect with phosphatidylinositol, phosphatidylethanolamine or...
pretreated with 25 μM FOH at 30 °C for the indicated periods, without 25 μM FOH at 30 °C for 4 h. Cells were also grown in YPD medium containing 25 μM FOH and 100 μM PC (○), 25 μM FOH and 100 μM phosphatidylserine (△), 25 μM FOH and 10 μM OAG (●), 25 μM FOH and 30 μM OAG (▲), 25 μM FOH and 100 μM phosphatidylethanolamine (▲), 25 μM FOH and 100 μM phosphatidylethanolamine (▲), and 25 μM FOH and 100 μM phospha-

dlysinositol (▲). The yeast cell growth was more effectively restored with OAG than PC, which can give DAG as a result of phospholipase action (Melnikovych et al., 1992). These findings suggested that FOH arrested the yeast cell growth by interfering with a phosphatidylinositol-type signalling.

Fig. 3. Protective effects of phospholipids and OAG on FOH-induced growth inhibition of S. cerevisiae. Cells were grown in YPD medium with (■) or without (□) 25 μM FOH. Cells were also grown in YPD medium containing 25 μM FOH and 100 μM PC (○), 25 μM FOH and 100 μM phosphatidylserine (△), 25 μM FOH and 10 μM OAG (●), 25 μM FOH and 30 μM OAG (▲), 25 μM FOH and 100 μM phosphatidylethanolamine (▲), and 25 μM FOH and 100 μM phospha-

dlysinositol (▲).

Table 3. Time-dependent loss of growth recovery in FOH-induced-growth-inhibition of S. cerevisiae cells

In control assays, cells were grown in YPD medium with or without 25 μM FOH at 30 °C for 4 h. Cells were also pretreated with 25 μM FOH at 30 °C for the indicated periods, and then grown for a further 4 h following the addition of 30 μM OAG.

![Graph showing growth over time](image)

<table>
<thead>
<tr>
<th>Addition</th>
<th>Period of pretreatment (min)</th>
<th>Growth (OD₆₅₀)</th>
<th>Relative cell growth (%)^a</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>—</td>
<td>4.60 ± 0.23</td>
<td>100</td>
</tr>
<tr>
<td>FOH</td>
<td>—</td>
<td>1.03 ± 0.15</td>
<td>1</td>
</tr>
<tr>
<td>FOH/OAG</td>
<td>0</td>
<td>4.67 ± 0.16</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4.26 ± 0.18</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1.82 ± 0.21</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1.09 ± 0.21</td>
<td>3</td>
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</tbody>
</table>

^a Initial OD₆₅₀ values were adjusted to about 1-00. Values are means ± s.d in triplicate assays.

phosphatidic acid. The yeast cell growth was more effectively restored with OAG than PC, which can give DAG as a result of phospholipase action (Melnikovych et al., 1992). These findings suggested that FOH arrested the yeast cell growth by interfering with a phosphatidylinositol-type signalling.

FOH-treated cells seemed to reinitiate growth as soon as OAG was added to the medium. From this we might assume that the yeast cell growth was only statically repressed due to a nutritional requirement for this neutral lipid. Cells were preincubated in YPD medium with FOH and washed with PBS. FOH-treated cells were then suspended at a density of 10⁷ cells ml⁻¹ in freshly prepared YPD medium containing 30 μM OAG and grown for another 4 h. As shown in Table 3, OAG could protect the FOH-induced growth inhibition only when it was added after 5 min of FOH pretreatment. The turbidimetric assay found no sign of cell growth initiation following addition of OAG after cells had been pretreated with FOH for at least 20 min. We believe that the failure in cell growth recovery was not caused by an induction of FOH on a nutritional requirement for DAG but suggested a signalling-mediated cell cycle arrest in FOH-pretreated cells.

Differential display analysis of gene expression under FOH-induced growth inhibition

We employed the differential display technique to identify the genes differentially expressed in response to the FOH treatment. As shown in Fig. 4, each mixture of arbitrary primers gave a unique fingerprint composed of various distinct bands. One of these bands (band e) exhibited an increasing expression pattern with FOH whereas the other four bands (a, b, c, d) showed decreasing expression patterns in both the duplicate analyses. These five bands were subjected to the following analysis to identify a genetic event with closer relation to the FOH treatment (although clearly these bands represented a limited population of the genes differentially expressed with or without FOH). After purification of these five bands, reamplification and cloning into the plasmid vectors, each cDNA insert was sequenced by the dideoxy chain-termination method. Comparison of their partial nucleotide sequences with the genome sequence of S. cerevisiae revealed the cDNA sequences to be identical to the coding regions of CDC9 (a), HAT2 (b), YMR116C (c), YMR226C (d) or PDR5 (e).

YMR116C is a hypothetical ORF with similarity to the G-protein β subunit family containing seven Trp-Asp domains. YMR226C is also a hypothetical ORF which has a high homology to the mammalian β-oesestradiol dehydrogenase gene. The biological significance of downregulation of these hypothetical ORFs is unknown. PDR5, the only gene identified with upregulation, is one of many multidrug-resistance genes in yeast. Expression of this gene can be stimulated by various environmental factors such as heat shock, cycloheximide (Miyahara et al., 1996a) and metal ions (Miyahara et al., 1996b). PDR5 could be upregulated simply to export FOH out of the cell as a toxic drug. However, PDR5 expression was decreased to the normal level when OAG was added.
to the medium (data not shown), suggesting the involvement of signal transduction in regulation of expression. CDC9 encodes a protein with DNA ligase activity. Downregulation of this gene can cause a disorder in DNA replication and thus results in cell cycle arrest at the RAD9 checkpoint in response to DNA damage (Tomkinson et al., 1992). HAT2 encodes a protein required for high-affinity binding of acetyltransferase to histone H4 (Parthun et al., 1996; Wade et al., 1997). In eukaryotic cells, histone must be newly synthesized and acetylated in the cytoplasm during DNA replication so that acetylated products enter the nucleus for deposition onto the newly replicated DNA. Downregulation of HAT2 should also cause a disorder in DNA replication because of a decrease in the cytoplasmic level of Hat2p.

We focussed on CDC9 and HAT2 because the expression of these genes is inevitable in the cell cycle progression and thus may be closely related to FOH-induced growth inhibition. The expression patterns of these two cell cycle genes were examined by Northern blot analysis. As shown in Fig. 5(a), mRNA levels of CDC9 and HAT2 were significantly decreased in cells treated with 25 μM FOH but not in those treated with 12.5 μM FOH. Although we used 100 μM FOH for differential display analysis, 25 μM FOH was sufficient to decrease the mRNA levels of CDC9 and HAT2, this being in agreement with the concentration of FOH critical for growth arrest and loss of DAG. Downregulation of CDC9 and HAT2 expression was effectively cancelled when cells were grown in medium supplemented with OAG (Fig. 5b). These results strongly support the idea that FOH repressed expression of CDC9 and HAT2 by reducing the intracellular level of DAG. In S. cerevisiae, PKC1 functions to activate the mitogen-activated protein (MAP) kinase cascade, a regulator of gene expression at the G1 to S phase transition of the cell cycle (Madden et al., 1997). It was reported that yeast cyclin-dependent kinase encoded by CDC28 stimulated a cell-cycle-dependent hydrolysis of PC to DAG at the START control point committing the cells to enter S phase and complete the next cell cycle (Marini et al., 1996). FOH-induced growth inhibition may reflect an inhibition of cell cycle transition via PKC-dependent activation of the MAP kinase cascade. This was supported by the fact that yeast cell cycle was arrested in G1 with Li⁺ as an inhibitor of phosphoinositide metabolism (Smith et al., 1995). Downregulation of CDC9 and HAT2 expression should reflect a cell cycle mechanism such as G1 arrest in FOH-induced growth inhibition of S. cerevisiae cells. We propose an interference with a phosphatidylinositol-type signalling which is involved in cell cycle progression as a cause of FOH-induced growth inhibition in yeast cells.

**Fig. 4.** Differential display of cDNAs from yeast cells grown with (F) or without (C) 100 μM FOH. Each cDNA was subjected to differential display analysis using different random primers as described in Methods. Because yeast transcripts are fewer than those of mammalian cells, the PCR reaction was carried out using two different upstream primers and one downstream primer in order to increase the number of differentially expressed transcripts in one reaction. In the PCR mixture loaded in the left panel, upstream primers 7 (5' GATCATGGTC 3') and 14 (5' GGAACCAATC 3'), and downstream primer 3 (5' T,AG 3') were used. In the PCR mixture loaded in the middle panel, upstream primers 7 and 19 (5' TACAACGAGG 3') and downstream primer 4 (5' T,CA 3') were used. In the PCR mixture loaded in the right panel, upstream primers 15 (5' GATCAATCGC 3') and 19, and downstream primer 6 (5' T,CG 3') were used. Following duplicate electrophoresis on non-denaturing polyacrylamide gel, the fingerprints were visualized by autoradiography with a BAS 2000 bio-imaging analyser. The letters (a) to (e) denote cDNA fragments representing differentially expressed transcripts.

**Fig. 5.** Effects of FOH on CDC9 and HAT2 mRNA levels in the absence (a) and presence (b) of OAG. Cells were treated in YPD medium containing FOH at the indicated concentrations; the asterisks (*) in (b) indicate that cells were treated with 25 μM FOH and 30 μM OAG. Each RNA sample was analysed by Northern blotting as described in Methods. The blots were probed with radiolabelled CDC9 cDNA and HAT2 cDNA in addition to ACT1 cDNA to demonstrate equivalent loading of samples.
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Received 9 July 1998; revised 12 October 1998; accepted 15 October 1998.