Sterols, such as cholesterol in mammalian cells and ergosterol in yeast, are important constituents of all eukaryotic cells (33, 34, 36, 48). They are synthesized in the endoplasmic reticulum (ER) but enriched ~5- to 10-fold in the plasma membrane (PM) (30, 46); in the yeast Saccharomyces cerevisiae, ~70% of total cellular ergosterol is located in the PM (3, 49). The mechanism by which sterols are transported between the ER and PM is poorly understood. Since the spontaneous diffusion of hydrophobic sterol molecules through an aqueous environment is thermodynamically unfavorable, cells must have a mechanism to transport sterols between membranes (33, 38). Studies with yeast and mammalian cells indicate that sterol transport between the ER and the PM does not depend on a functional vesicular transport pathway but requires ATP and is blocked at low temperatures (3, 18, 24, 29, 32, 45, 50).

Proteins required for nonvesicular sterol transport between the ER and PM have not been identified convincingly (33, 38). There are numerous known sterol-binding proteins that could function in nonvesicular sterol transport, either directly as carriers or in an accessory role. Proteins with lipid-binding START domains (2) are attractive candidates for cytoplasmic sterol carriers. One member of the START family, the ceramide transporter CERT, was shown to catalyze the ATP-dependent accumulation of ceramide from the ER to the Golgi apparatus (14, 15). Recent structural analyses indicate that CERT specifically sequesters ceramide in a hydrophobic binding pocket to shield the lipid from the aqueous environment during transit through the cytoplasm (28). Targeting motifs within CERT enable it to engage the ER and Golgi apparatus specifically, providing a mechanism to restrict the ceramide transport pathway to these two organelles. Yeast cells lack proteins with START domains but contain other proteins with lipid-binding motifs. The seven oxysterol binding protein homologs, or Osh proteins (4), are prominent examples of soluble yeast proteins with putative lipid-binding domains. One of the Osh proteins, Osh4/Kes1, has been analyzed by X-ray crystallography in complex with different sterols (25) and shown to transport sterols between donor and acceptor vesicles in vitro (39). Elimination of the Osh proteins in particular combinations influences sterol transport in vivo (39, 49). However, it remains to be seen whether the true function of the Osh proteins is to transport sterols or to act in other ways, for example, as sterol sensors in regulating sterol distribution within the cell (10, 55).

A recent study attempted to identify proteins involved in sterol transport by using a visual screen for the transport-dependent accumulation of a fluorescent analog of cholesterol (40). The screen was limited to genes required for anaerobic growth. While the study discovered an unexpected role for mitochondria in PM-ER sterol transport, it did not identify a sterol transporter. Here we report a tritium suicide selection strategy designed to identify yeast mutants with defects in sterol transport, thus providing an unbiased way to identify components of the nonvesicular sterol transport system. The selection operates on the premise that cells that are unaffected in a function/pathway of interest will accumulate large amounts of a tritiated substrate, whereas those with defects in that function or pathway will not. When a mutagenized culture of cells is incubated with an appropriate tritium-labeled substrate and stored at ~80°C, cells that efficiently accumulate the radiolabeled probe undergo radiation-induced DNA damage and die, leaving behind an enriched pool of mutants.

We exploited the transport-dependent accumulation of
exogenous [3H]cholesterol in Upc2-1 cells mutagenized with a transposon library, in conjunction with tritium suicide selection, to uncover mutants defective in the incorporation of exogenous sterols. Analysis of the survivors uncovered numerous genes involved in the uptake and transport of exogenous sterols. We identified the transcription factor Mot3 as a repressor of AUS1 and PDR11, genes that encode PM-localized ATP-binding cassette (ABC) transporters involved in sterol uptake. We also identified the previously uncharacterized open reading frame (ORF) Ydr051c, which we now refer to as DETI (decreased ergosterol transport). Transport of sterols between the ER and PM was significantly reduced in det1Δ cells, indicating that the cytoplasmic protein encoded by DETI is involved in intracellular sterol transport.

**MATERIALS AND METHODS**

**Materials.** All radiolabeled compounds were purchased from American Radiolabeled Chemicals (St. Louis, MO). Methyl-β-cyclodextrin (MβCD), ergosterol, cholesterol, Tween 80, FM4-64, calcofluor white, Nile red, and sodium diolabeled Chemicals (St. Louis, MO). All radiolabeled compounds were purchased from American Radiochemicals (Shelton, CT). Antibodies against Sec61, Vph1, and Gas1 were gifts from T. Rapoport (Harvard Medical School). The digested product was ligated into pRS316 and confirmed by sequencing. Aus1-GFP and Pdr11-GFP were expressed as C-terminal fusion proteins under the control of their endogenous promoters from the plasmids pWP1220 and pWP1251, respectively (32).

**Materials.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>WPY361</td>
<td>MAta upc2-1 ura3-1 his3-11.15 32</td>
<td></td>
</tr>
<tr>
<td>DSY104</td>
<td>WPY361/pMO73-URA3</td>
<td>This study</td>
</tr>
<tr>
<td>C209</td>
<td>WPY361 det1::Mn3</td>
<td>This study</td>
</tr>
<tr>
<td>BY4741</td>
<td>MATA his3-1 leu2-0 ura3-2 54</td>
<td>EUROSCARF</td>
</tr>
<tr>
<td>det1Δ</td>
<td>BY4741 det1::KanMX4</td>
<td>54; EUROSCARF</td>
</tr>
<tr>
<td></td>
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</tbody>
</table>

**Uptake of cholesterol by yeast on plates.** To assess cholesterol uptake of multiple colonies simultaneously, the protocol of Lewis et al. (31) was used. Briefly, colonies were spotted onto YPD plates, grown for 2 days at room temperature, and then replica plated onto a YPD plate overlaid with a nitrocellulose membrane. After allowing the cells to grow for 2 days at room temperature, the disc was overlaid onto a YPD plate containing 1% (vol/vol) Tween 80–ethanol (1:1 [vol/vol]) and 0.5 μCi/ml [3H]cholesterol. After 4 h, the disc was removed and treated to remove nonspecifically bound [3H]cholesterol. This was accomplished by first placing the disc with the colony side up on Whatman paper soaked with 0.5% (wt/vol) NP-40 and then on dry paper towels. After repeating the treatment thrice, the colonies on the disc were transferred to Whatman paper by the two together pieces of paper towels. The disc was sandwiched between two sheets of Whatman paper and dried. The Whatman paper was wrapped in plastic wrap and exposed to a phosphorimagere plate overnight to visualize [3H]cholesterol uptake by the individual colonies.

**Uptake of cholesterol by cells in suspension.** Measurement of cholesterol uptake by suspension-grown yeast cells was done by a procedure adapted from the work of Li and Prinz (32). Briefly, overnight cultures were diluted to 0.5 OD600 unit/ml in 2 ml of YPD. After 2 h (roughly one doubling period), the cultures were shifted to 37°C and preincubated for 15 min before adding a mixture of cholesterol and [3H]cholesterol in 1:1 (vol/vol) Tween 80–ethanol. The final concentration (each) of Tween 80 and ethanol was 0.5% (vol/vol), and the final concentration of cholesterol was 1 μM, with 10 μCi of [3H]cholesterol. After incubation at 37°C for 1 h, 1.5 ml of each culture was removed and centrifuged. Cell pellets were washed three times with 1 ml of 0.5% (vol/vol) Tween 80 in water and then resuspended in 200 μl ice-cold water. Acid-washed glass beads (200 μl) were added, and the cells were disrupted by being vortexed (six times for 30 s each, interspersed with 30-s cooling periods on ice). The resulting cell homogenate was collected, and the beads were washed with 800 μl of water. The homogenate and wash were combined and then extracted with 5 ml of hexanes-isopropanol (3:2 [vol/vol]). After thorough mixing of the sample, the organic phase was allowed to separate and then recovered and replaced with 2 ml of hexanes. The combined organic phases were dried and dissolved in 100 μl of chloroform-methanol (1:1 [vol/vol]), from which 10 μl was removed for scintillation counting to determine the total amount of radioactivity taken up by the cells and 35 μl was analyzed by thin-layer chromatography (TLC) to quantify the amount of label incorporated into cholesterol esters and free cholesterol. For TLC, the samples were spotted onto silica gel 60 plates and resolved using hexanes-diethyl ether-acetic acid (70:30:1 [vol/vol/vol]) as the solvent system. Free cholesterol and cholesterol esters had Rf values of 0.15 and 0.75, respectively. The relative amounts of radioactivity in the peaks were quantitated using a Berthold LB2842 TLC scanner.

**Transport of ergosterol from the ER to the PM.** Transport assays were done as described by Baumann et al. (3) at 37°C with a 15-min preincubation in prewarmed medium. Briefly, cells were pulse labeled with [3H-methyl]methionine and then chased. Aliquots were then resuspended in 200 μl of YPD and incubated with MβCD on ice to extract PM ergosterol. Ergosterol in the MβCD extract, as well as that in the cell sample, was quantitated by organic solvent extraction and high-performance liquid chromatography (HPLC) analysis. Fractions were collected and taken for scintillation counting to determine the amount...
of [3H]ergosterol, whereas bulk ergosterol was quantitated by an in-line UV detector at 280 nm. The specific radioactivity (SR) of [3H]ergosterol was determined in each case, and the SR of the MipCD sample was normalized to the SR of the cell sample to determine relative specific radioactivity (RSR) (RSR = SR of sample/SG of cell). The RSR directly compares the specific radioactivity of PM-localized ergosterol extracted by MipCD with that of total cellular ergosterol; plots of RSR versus time provide a measure of the rate at which ergosterol is transported from the ER to the PM.

Subcellular distribution of ergosterol. Total membranes from ~20 OD600 units of cells (grown to an OD600 of ~1 at room temperature and then incubated for 15 min at 37°C prior to homogenization) were fractionated on step gradients of ReneoCal-76 exactly as described previously (3, 7, 32). Sterol content in the pooled intracellular and PM fractions (identified by sodium doxyl sulfate-polyacrylamide gel electrophoresis/immoblotting using organelle-specific antibodies) was determined using reverse-phase HPLC as described above. Sterol-specific radioactivity was determined using lipid extracts obtained by methanol extraction (16) and desalted by n-butanol–water partitioning. Sterol was quantitated using HPLC as described below, and lipid phosphorus was quantitated exactly as described previously (43).

QPCR. Overnight cultures were diluted to 0.5 OD600 unit/ml and incubated for 2 h at 25°C. Cells were harvested, transferred to prewarmed YPD, and incubated at 37°C for 45 min. RNA was isolated from 2 OD600 units of cells by use of an RNeasy Mini kit (Qiagen), following the protocol for mechanical disruption of cells using glass beads. cDNA was prepared using Brilliant SYBR green QRT-PCR AffinityScript two-step master mix (Stratagene) following the provided protocol. Quantitative PCR (QPCR) amplification of cDNA was performed using the same kit according to the manufacturer’s protocol. The primers used are listed in Table 2. QPCR was carried out using Stratagene’s MX3005P and MX4000 QPCR systems. The thermal profile was as follows: 10 min at 95°C and 40 cycles of 30 s at 95°C, 1 min at 50°C, and 30 s at 72°C. Fluorescence readings were taken at the end of each 50°C annealing cycle. A melting curve from 50°C to 95°C was performed in each experiment after the last cycle to determine the specificity of the primers for a single sequence. Data were analyzed using Stratagene’s MsPro software and normalized to ACT1 mRNA levels.

Microscopy. For examination of the cellular distribution of Aus1-GFP and Pdr11-GFP, cells from exponentially growing cultures carrying the indicated green fluorescent protein (GFP) constructs were grown in selective media lacking the appropriate nutrient and visualized on coverslip dishes at room temperature in the same media. For Nile red staining, exponentially growing cultures were harvested, washed once, and resuspended in 1 ml of water. Two microliters of 1-mg/ml Nile red in acetone was added and incubated for 5 min at room temperature. Cells were then collected, washed twice with water, and resuspended to 1.5 OD units/ml in water. One hundred microliters of cells was added to coverslip dishes before visualization.

All images were acquired at a magnification of ×63, using an oil immersion lens on a Zeiss Axiophot 200 wide-field microscope equipped with a MicroLux interline charge-coupled device camera driven by MetaMorph Imaging System software (Universal Imaging Corp.). Images were taken with the same exposure times and processed to maintain intensity differences using Adobe Photoshop CS2 (Adobe Systems, Mountain View, CA).

### RESULTS

#### Suicide selection of sterol transport mutants.
Under aerobic conditions, wild-type *S. cerevisiae* cells do not take up exogenous sterol into their membranes (a phenomenon termed aerobic sterol exclusion) and instead synthesize their own (9). However, the WPY361 strain (Table 1), which has a gain-of-function point mutation in the transcription factor Upc2, is able to incorporate sterols from the growth medium. WPY361 cells (henceforth referred to as *Upc2-1* cells) express a number of proteins that are not normally expressed under aerobic conditions. These proteins include the cell wall mannoprotein Dan1 and two ABC transporters, Aus1 and Pdr11, that have been shown to be involved in the uptake of exogenous sterols (1, 53).

When *Upc2-1* cells are supplied with [3H]cholesterol, they convert it efficiently to [3H]cholesteryl esters that accumulate in lipid droplets (32). It is likely that the exogenously supplied sterol is first incorporated into the PM via a mechanism involving Dan1, Aus1, and Pdr11 and then transported to the ER, where it is esterified by the ER-localized yeast acyl-coenzyme A cholesterol acyl transferases Are1 and Are2 (56) (Fig. 1A). The cellular accumulation of cholesterol is strongly dependent on esterification, such that in the absence of Are1 and Are2, the total amount of internalized cholesterol is low (32).

In contrast to cholesterol, exogenously supplied ergosterol is poorly esterified since it remains localized to the PM (see Discussion) (32).

Because the accumulation of cholesterol requires its transport from the PM to the ER, we chose a tritium suicide selection strategy to isolate mutants with defects in sterol transport. *Upc2-1* cells were mutagenized with two different pools (pools 24 and 28) of the mTk3 LacZ/Leu2 transposon library (42) to create insertions in genomic DNA and to disrupt numerous different ORFs. Roughly 15,000 colonies from each library pool were collected and subjected to suicide selection. An exponentially growing culture was loaded with [3H]cholesterol for 60 min at 37°C, during which time the culture internalized ~10 cpm/cell. The culture was washed, divided into aliquots, and frozen at −80°C. A parallel sample was loaded with unlabeled cholesterol and treated similarly. During cold storage, cells that internalize a significant amount of the [3H]cholesterol are predicted to accumulate radiation-induced DNA damage and die. In contrast, mutants with defects in sterol accumulation, including cells with defects in PM–ER sterol transport, are likely to survive. To check the survival of the culture, aliquots of cells were removed periodically, and the number of survivors was determined by plating serial dilutions. The proportion of viable cells in the control culture remained relatively constant, at ~30% (Fig. 1B). As predicted, the [3H]cholesterol-loaded sample showed a dramatic decrease in survival, with only ~0.05% of the cells remaining after 80 days (Fig. 1B).

At this point, the remaining aliquots of the loaded culture were removed from the freezer and plated on YPD; 4,000 survivors were recovered from an initial load of 8 × 10⁶ cells.

The survivors were screened for the ability to incorporate exogenous cholesterol. Colonies were transferred from solid medium to a nitrocellulose disc that was then overlaid onto a [14C]cholesterol-containing plate; after a period of incubation,
Suicide selection survivors show reduced incorporation of exogenous sterols. The 23 genes that we identified in the suicide selection fell into a number of broad categories, and many did not have an assigned function according to the Saccharomyces Genome Database (http://www.yeastgenome.org). In our initial analyses, we chose to focus on the 14 genes in the categories “regulation of protein signaling” and “unknown” listed in Table 3 since, based on available information, they appeared most likely to be involved in an aspect of steroid transport.

To confirm that the 14 mutants corresponding to these two categories were defective in accumulating exogenously supplied steroid, we determined cholesterol uptake using suspension-grown cells. This provides a quantitative complement to the more qualitative filter uptake assay used in our initial screen. Exponentially growing cultures of the 14 mutants were incubated for 60 min with 1 μM cholesterol and a trace amount of [3H]cholesterol. Relative to the parent Upc2-1 strain, all mutants had a decreased ability to accumulate exogenous sterols (Fig. 2A). Six of the mutants, those with inserts in AIM44, HOF1, RAM1, TVP18, SCY1, and the YDR051c gene (which we henceforth refer to as DET1 [decreased ergosterol transport]), had significantly reduced accumulation that approached the level of a wild-type strain (BY4741) that lacks the ability to take up exogenous sterols because of aerobic sterol exclusion. Of the small amount ofsterol that was taken up by these six mutants, a significant portion (50 to 90%) in all strains was esterified to cholesteryl ester, as determined by separating and quantitating the incorporated [3H]cholesterol by TLC (Fig. 2B). These data suggest that the poor accumulation of [3H]cholesterol in the six mutants was not due to defective esterification.

Mot3 regulates the expression of Aus1 and Pdr11. We repeatedly recovered mutants with transposon insertions in TVP18. Although TVP18 encodes a Golgi membrane protein of unknown function (Table 3) that could potentially play a role in sterol transport and accumulation, we considered a different possibility. We noticed that the TVP18 locus is located adjacent to the MOT3 locus in the yeast genome. Mot3 is a transcription factor that is known to repress numerous anaerobic/hypoxic genes under aerobic conditions (19, 26, 27, 47). We hypothesized that insertion of a transposon into the TVP18 locus could have an indirect effect on sterol uptake by increasing the transcription of MOT3. To test this possibility, we used reverse transcription-QPCR to quantitate levels of MOT3, AUS1, and PDR11 mRNAs in the tvp18::mTn3 mutant as well as the Upc2-1 strain. Figure 3 shows that the level of MOT3 mRNA was significantly increased in the tvp18::mTn3 mutant compared to the Upc2-1 cells, while AUS1 and PDR11 transcript levels were reduced. To investigate this further, we overexpressed Mot3 in the Upc2-1 strain; as expected, MOT3 mRNA levels were increased, while the levels of AUS1 and PDR11 were reduced. Consistent with the reduction in AUS1 and PDR11 transcript levels, exogenous sterol incorporation in the Mot3-overexpressing strain was reduced 60% ± 2% (at room temperature; incorporation was reduced ~70% at 37°C) relative to that in the Upc2-1 strain. Our results suggest that the insertion of a transposon into the TVP18 locus increased the level of MOT3 mRNA, resulting in a transcriptionally mediated decrease in ABC transporter expression and a concom-
itant decrease in sterol uptake. Indeed, analysis of the promoter regions of both AUS1 and PDR11 indicates numerous putative Mot3 binding sites and suggests that AUS1 and PDR11 can be included in the list of known anaerobic genes that are repressed by Mot3.

Expression and trafficking of Aus1 and Pdr11 are defective in some survivors. Since the suicide selection approach relies on efficient uptake of exogenous sterols, we anticipated that a number of the mutants that we recovered would have defects in the expression and trafficking of Aus1 and Pdr11 (40, 53). To examine this, Aus1-GFP and Pdr11-GFP fusion proteins (32) were expressed from their endogenous promoters in the mutants with transposon insertions in HOF1, SCY1, RAM1, AIM44, and DET1; Upc2-1 cells expressing Aus1-GFP and Pdr11-GFP were used as a control. The localization and relative expression levels of the constructs were examined using fluorescence microscopy. Figure 4 shows that both Aus1-GFP and Pdr11-GFP were localized primarily to the PM in Upc2-1 cells, consistent with their function in exogenous sterol uptake. However, the fluorescence of both Aus1-GFP and Pdr11-GFP was significantly reduced in all mutant strains examined except C209 (det1::mTn3), and in some strains, a large fraction of the

TABLE 3. Genes involved in transport-dependent accumulation of exogenous sterols

<table>
<thead>
<tr>
<th>Cellular process</th>
<th>ORF</th>
<th>Gene</th>
<th>Function†</th>
<th>No. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene regulation and chromosome maintenance</td>
<td>YDR213w</td>
<td>UPC2</td>
<td>Transcription factor</td>
<td>14</td>
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<tr>
<td></td>
<td>YKL160w</td>
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<td></td>
<td>YMR106c</td>
<td>YKU80</td>
<td>Telomere length maintenance</td>
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<td></td>
<td>YDR448w</td>
<td>ADA2</td>
<td>Histone acetyltransferase subunit</td>
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</tr>
<tr>
<td>Protein biosynthesis</td>
<td>RDN25-1</td>
<td>RDN25</td>
<td>Encodes rRNA</td>
<td>21</td>
</tr>
<tr>
<td>Regulation of protein signaling</td>
<td>YMR032w</td>
<td>HOF1</td>
<td>Regulator of cytokinesis</td>
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<td></td>
<td>YNL098c</td>
<td>RAS2</td>
<td>GTPase, regulator of cell growth</td>
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<td></td>
<td>YDR436w</td>
<td>PPZ2</td>
<td>Phospho-Ser/Thr protein phosphatase</td>
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<td></td>
<td>YDL090c</td>
<td>RAM1</td>
<td>Farnesyltransferase subunit</td>
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<td></td>
<td>YER155c</td>
<td>BEM2</td>
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<td>Unknown function</td>
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<td>Similarity to phosphoglycerate mutase</td>
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<td>YMR071c</td>
<td>TVP18</td>
<td>Membrane protein localized to late Golgi vesicles</td>
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<td></td>
<td>YPL158c</td>
<td>AIM44</td>
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<tr>
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<td>YFL040w</td>
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<td>Putative transporter, member of the sugar porter family</td>
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<td>YKL027w</td>
<td></td>
<td>Localized to mitochondrial outer membrane</td>
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<td>YNR075w</td>
<td>COS10</td>
<td>DUP380 family member</td>
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<td></td>
<td>YGL183w</td>
<td>SCY1</td>
<td>Putative kinase</td>
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<td></td>
<td>YHR080c</td>
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<td>May interact with ribosomes, copurifies with mitochondrial</td>
<td>1</td>
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</tbody>
</table>

† Obtained from the Saccharomyces Genome Database (http://www.yeastgenome.org/).

FIG. 2. Quantitative assay of sterol incorporation by a subset of suicide selection survivors. (A) Logarithmically growing cultures of isolates from the suicide selection were incubated in YPD with 1 μM cholesterol (containing 10 μCi [3H]cholesterol) in 0.5% (wt/vol) ethanol–0.5% (wt/vol) Tween 80 for 60 min at 37°C. Cells were recovered, washed, and lysed. The incorporation of [3H]cholesterol was determined by scintillation counting and extrapolated to the amount of bulk cholesterol uptake in the culture. The graph shows the averages and variances for two independent experiments. (B) Lysed cells from panel A were taken for lipid extraction; free cholesterol and cholesteryl esters in the extract were resolved by TLC and quantitated as described in Materials and Methods.
fluorescence appeared to be localized to internal structures. Although it is not clear how these gene products regulate Aus1 and Pdr11 expression and trafficking, the decreased incorporation of exogenous sterols in strains carrying transposon insertions in SCY1, AIM44, RAM1, and HOF1 is likely due to improperly sorted or expressed Aus1 and Pdr11 and not a result of a defect in sterol transport from the PM to the ER. In det1::mTn3 cells, Aus1-GFP and Pdr11-GFP were detected in a pattern similar to that seen in Upc2-1 cells. We conclude that the PM-localized sterol uptake machinery is normal in det1::mTn3 cells. Together with the observation that sterol esterification is essentially unaffected when DET1 is disrupted (Fig. 2B), this suggests that Det1 is likely to be involved in the transport of sterols from the PM to the ER.

ER-to-PM sterol transport is decreased in det1Δ cells. Since ergosterol transport between the ER and the PM occurs via a bidirectional process (3), we hypothesized that cells lacking DET1 would be defective in biosynthetic sterol transport. To test this, we assayed transport of newly synthesized ergosterol from the ER to the PM in det1Δ cells obtained from the yeast nonessential gene deletion collection (54). Ergosterol was pulse labeled in the ER, using [3H]methylmethionine, and then chased with medium containing unlabeled methionine. Arrival of [3H]ergosterol at the PM was measured by withdrawing an aliquot of cells and sampling PM sterol by extraction with MβCD. Ergosterol in both the MβCD extract and the corresponding cells was quantitated using scintillation counting to determine the SR in the sample. The RSR of ergosterol in the MβCD extract (the ratio of the SR in the MβCD extract to SR in the corresponding cell sample) was plotted against time to generate a transport curve (Fig. 5). ER-to-PM transport was significantly reduced in det1Δ cells: the initial rate of transport (determined from the linear portion [0 to 5 min] of a monoexponential fit to the data) was reduced threefold in det1Δ cells relative to that in the parent strain BY4741. The cumulative data indicate that Det1 is involved, either directly or indirectly, in the bidirectional transport of sterols between the ER and the PM.

Preliminary characterization of cells lacking Det1. Compared to the parent WPY361 strain, the C209 det1::mTn3 mutant did not display increased sensitivity or resistance to nystatin (5 to 50 U/ml on solid YPD), nor did it have any significant difference in sensitivity during growth with 0.5 M NaCl or 1.0 M KCl. However, C209 was about 10² times more temperature sensitive at 37°C and 10² times more cold resistant at 16°C than WPY361, as scored from serial dilutions spotted onto YPD plates (not shown). In liquid YPD medium, the C209 mutant had a doubling time of 99 min at 30°C, which is only slightly longer than that of the parent strain (88 min), while it grew slower at 37°C (doubling time of 138 min for the mutant and 96 min for the parent). Similarly, det1Δ cells grew slightly more slowly than the BY4741 parent strain but otherwise appeared normal, as judged by (i) transmission electron microscopy of negatively stained samples, (ii) calcofluor white staining (to detect cell wall defects), and (iii) uptake of the fluorescent lipophilic dye FM4-64 (to assess bulk endocytosis) (not shown). Since a sterol transport defect could lead to
terol and ergosterol are efficiently taken up by the uptake and intracellular transport of sterols. Both cholesterol bacterial and yeast mutants with defects in lipid biosynthesis that in the parent strain, with free (nonesterified) form in cells (32). Cholesterol partitions ergosterol in the PM and is consequently found mostly in the free (nonesterified) form in cells (32). Cholesterol partitions less well into the PM, possibly because it is excluded from privileged interactions with yeast sphingolipids (or other lipids with saturated acyl chains) when ergosterol is present. Consequently, exogenously supplied cholesterol transits through the PM and moves to the ER, where it is esterified by the acylcoenzyme A cholesterol acyl transferase enzymes Are1 and Are2. We exploited this transport-dependent accumulation of exogenous cholesterol to perform [3H]cholesterol suicide selection on Upc2-1 cells mutagenized with a transposon library. We expected to recover mutants with defects in the uptake, transport, and esterification processes. From the survivors of the selection, 23 genes were identified; disruption of these genes by transposon insertion resulted in a defect in cholesterol uptake. Numerous genes were isolated only once (Table 3), indicating that coverage of the library was incomplete and that more mutants would likely be uncovered by extending or repeating the selection process. Of the genes we identified, HOF1 and genes encoding components of the Ras signaling pathway (RAS2 and RAM1) were also identified in a recent genomewide screen for genes involved in neutral lipid storage (11). Although the role of these gene products in sterol homeostasis is unclear, their repeated isolation not only validates both approaches—the published genomewide screen (11) and our suicide selection—for identifying new mutants but also confirms the importance of these proteins in intracellular sterol regulation. Interestingly, neither approach yielded mutants of OSH3 or OSH5, which would be expected to display a reduced rate of cholesterol accumulation (39). Also, none of the genes that we identified were uncovered in a screen of anaerobic inviable mutants (40), even though this screen found numerous genes involved in the trafficking of Aus1 and Pdr11 to the PM. This lack of overlap is likely due to the incomplete coverage of the genome in our suicide selection process; by continuing the selection process, we would undoubtedly uncover a number of the reported anaerobic inviable mutants as well as mutants with transposon insertions in OSH3 and OSH5.

Figure 1A envisages three steps in the accumulation of exogenously supplied cholesterol. C209 (det1::mTn3) cells esterify cholesterol similarly to parental Upc2-1 cells and display a normal pattern of expression of Aus1 and Pdr11 (Fig. 4), suggesting that the defect in these cells is in the step of sterol transport between the ER and PM rather than in sterol uptake at the PM or sterol esterification. In support of this, assays of biosynthetic sterol transport in det1Δ cells revealed that transport of newly synthesized ergosterol from the ER to the PM was slowed threefold compared to that in parental BY4741 cells (Fig. 5).

Sequence analysis of the det1::mTn3 mutant showed that transposon insertion occurred between chromosomal coordinates 557748 and 557749 on chromosome IV, allowing for the production of a truncated protein corresponding to the first 104 amino acids of Det1. Preliminary results indicated that this truncated protein acts in a dominant-negative way to inhibit sterol uptake (not shown), potentially accounting for the severity of the defect (~20-fold) in the rate of sterol accumulation in C209 cells (Fig. 2) compared with the milder defect (~3-fold reduction) in the rate of biosynthetic sterol transport in det1Δ cells (Fig. 5).

How might Det1 play a role in nonvesicular sterol transport? Det1 is an ~39-kDa soluble protein with a predicted abun-

**DISCUSSION**

Tritium suicide selection has been used successfully to isolate bacterial and yeast mutants with defects in lipid biosynthesis, protein N-glycosylation, and vesicular transport (8, 21, 22, 35, 37). We used this approach to identify genes involved in the uptake and intracellular transport of sterols. Both cholesterol and ergosterol are efficiently taken up by Upc2-1 cells. Exogenously supplied ergosterol equilibrates with endogenous ergosterol in the PM and is consequently found mostly in the free (nonesterified) form in cells (32). Cholesterol partitions changes in sterol content and intracellular distribution, we examined these properties of det1Δ cells (5, 11). Nile red staining showed a normal distribution of lipid droplets (Fig. 6A), with 2.6 ± 1.1 and 2.5 ± 1.2 lipid droplets per cell for det1Δ and BY4741 cells, respectively (mean ± standard deviation [SD]; >300 cells from each strain were examined). Similar results were obtained in a comparison of C209 and Upc2-1 cells. Lipid analysis of cells grown to mid-logarithmic phase showed the same ergosterol/phospholipid molar ratio in det1Δ and BY4741 cells (0.187 ± 0.004 and 0.191 ± 0.019 for det1Δ and BY4741 cells, respectively [mean ± SD of duplicate measurements from two independent experiments]). We analyzed the subcellular distribution of ergosterol by using RenoCal gradients to separate the PM from other intracellular membranes, and fractions 15 to 14, corresponding to the PM, were pooled and taken for lipid analysis. (C) Percentages of total ergosterol recovered in PM pools isolated from BY4741 and det1Δ cells.

**FIG. 6.** Lipid droplets and subcellular distribution of ergosterol in det1Δ cells. (A) Lipid droplets in BY4741 (parent) and det1Δ cells were visualized by fluorescence microscopy after Nile red staining. Bar = 10 μm. (B) Fractionation of a BY4741 cell homogenate on a discontinuous RenoCal gradient as described in Materials and Methods. Fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis immunoblotting, using antibodies against the PM (Gas1), vacuole (Vph1), ER (sec61), and Golgi complex/endosome (Pep12). Fractions 1 to 8, corresponding to intracellular membranes, and fractions 9 to 14, corresponding to the PM, were pooled and taken for lipid analysis. (C) Percentages of total ergosterol recovered in PM pools isolated from BY4741 and det1Δ cells.
dance of ~3,000 molecules per cell (20). GFP-tagged Det1 is localized primarily to the cytoplasm (12, 23), consistent with a role in sterol transport across the cytoplasm. Sequence analysis shows that Det1 has significant similarity to phosphoglycerate mutases. However, Det1 lacks a number of residues shown to be critical for catalysis (51, 52), and this activity (as performed by Gpm1, Gpm2, and/or Gpm3) in S. cerevisiae has been well characterized (17, 41). Other than the predicted similarity to phosphoglycerate mutases, sequence analysis reveals that Det1 has a number of close homologs in other fungi (i.e., phosphoglycerate mutases, sequence analysis reveals that Det1 has a number of close homologs in other fungi (i.e., KLA0F18810g in Klyveromyces lactis and ca594 in Candida albicans) but no clear homologs in mammals. Perhaps Det1 is involved in a yeast-specific or ergosterol-specific aspect of sterol transport or significant sequence divergence has occurred in the course of evolution of the more complex cholesterol regulatory mechanisms in higher eukaryotes. While the identification of Det1 as an important player in intracellular sterol transport opens the door to future studies on this poorly understood process, more analyses are required in order to determine the exact nature and mechanism of its involvement.

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