ATPase and Multidrug Transport Activities of the Overexpressed Yeast ABC Protein Yor1p*

(Received for publication, October 24, 1997, and in revised form, March 12, 1998)

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The Saccharomyces cerevisiae genome encodes 15 full-size ATP binding cassette transporters (ABC), of which PDR5, SNQ2, and YOR1 are known to be regulated by the transcription factors Pdr1p and Pdr3p (pleiotropic drug resistance). We have identified two new ABC transporter-encoding genes, PDR10 and PDR15, which were up-regulated by the PDR1–3 mutation. These genes, as well as four other ABC transporter-encoding genes, were deleted in order to study the properties of Yor1p. The PDR1–3 gain-of-function mutant was then used to over-produce Yor1p up to 10% of the total plasma membrane proteins. Overexpressed Yor1p was photolabeled by [γ-32P]-2',3',5'-O-(2,4,6-trinitrophenyl)-8-azido-ATP (Kd = 45 μM) and inhibited by ATP (KD = 0.3 mM) in plasma membranes. Solubilization and partial purification on sucrose gradient allowed to detect significant Yor1p ATP hydrolysis activity (−100 nmol of Pi·min⁻¹·mg⁻¹). This activity was phospholipid-dependent and sensitive to low concentrations of vanadate (I50 = 0.3 mM) and oligomycin (I50 = 8.5 μg/ml).

In vivo, we observed a correlation between the amount of Yor1p in the plasma membrane and the level of resistance to oligomycin. We also demonstrated that Yor1p drives an energy-dependent, proton uncoupler-insensitive, cellular extrusion of rhodamine B. Furthermore, cells lacking both Yor1p and Pdr5p (but not Snq2p) showed increased accumulation of the fluorescence derivative of 1-myristoyl-2-[6-(NBD)aminocaproyl]phosphatidylethanolamine.

Despite their different topologies, both Yor1p and Pdr5p mediated the ATP-dependent translocation of similar drugs and phospholipids across the yeast cell membrane. Both ABC transporters exhibit ATP hydrolysis in vitro, but Pdr5p ATPase activity is about 15 times higher than that of Yor1p, which may indicate mechanistic or regulatory differences between the two enzymes.

The yeast YOR1 gene confers oligomycin resistance on over-expression in a 2-μm plasmid (1). Its nucleotide sequence reveals an ORF of 1477 amino acids encoding an ABC protein highly homologous to mammalian transporters such as the multidrug resistance-conferring enzyme MRP (BLAST (see Ref. 2) sequence homology score: p = e⁻⁰²²⁰), the organic anion transporter cMOAT (p = e⁻²¹⁶), the sulfotransferase receptor (p = e⁻¹⁶⁴), and the cystic fibrosis transmembrane conductance regulator CFTR (p = e⁻¹³²). Yor1p is a “full-size” ABC transporter with the topology (TM-NBF)2 (3, 4). It consists of two homologous halves, with each containing a putative ATP-binding domain (NBF) and a transmembrane domain of six membrane spans (TM). Cui et al. (5) showed that Yor1p confers resistance to a series of drugs including reveromycin A and suggested that Yor1p may be involved in the cellular efflux of organic anions including the fluorescent dye rhodamine B. They also showed that incubation with reveromycin A increases the YOR1 mRNA level. The transcription of YOR1 is controlled by the homologous pair of transcription factors Pdr1p/Pdr3p. The level of YOR1 transcription is decreased by the deletion of either PDR1 or PDR3 and increased in the presence of the gain-of-function PDR1 alleles (1).

In this paper, we have investigated the transport activity of Yor1p. Building on previous studies, which indicated that the (TM-NBF)2-type Yor1p, together with the (NBF-TM)2-type Pdr5p and Snq2p ABC transporters, are overexpressed in the PDR1–3 mutant plasma membrane (6–8), the PDR1–3 mutant has been used as a tool that enhances the Yor1p protein level. As another investigative tool, we constructed a set of isogenic strains, in the PDR1–3 mutant, with multiple deletions of homologous ABC genes since, in situations where two or more proteins located in the same subcellular compartment share a common substrate, a clear phenotype is only seen when all the corresponding genes are deleted, as illustrated by the work of Mahé et al. (9), who showed that Pdr5p and Snq2p have an overlapping transport capacity for steroids. We deleted the yeast ABC transporter-encoding genes known or suspected to

* This work was supported by grants from the Service des Affaires Scientifiques, Techniques et Culturelles, Pôles d’Attraction Interuniversitaires, the Fonds National de la Recherche Scientifique, and the Foundation for Research Development of South Africa, and by NATO Collaborative Exchange Research Grant CRC940493 and National Institutes of Health Grant GM52410 (to J. W. N.) and a National Institutes of Health minority predoctoral fellowship (to A. M. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.

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1 The abbreviations used are: YOR1, yeast oligomycin resistance; ORF, open reading frame; ABC, ATP-binding cassette; PDR, pleiotropic drug resistance; Pma1p, H⁺-plasma membrane ATPase; PDRE, Pdr1p/Pdr5p response element; M-C₅-NBD-P-E, 1-myristoyl-2-[6-(NBD)aminocaproyl]phosphatidylethanolamine; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; SDC, synthetic complete glucose medium; YD, rich glucose medium; YM, rich glycerol medium; MES, 2-(N-morpholino)ethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; TNP, trinitrophenyl; TNP-8-azido-ATP, 2',3'-O-(2,4,6-trinitrophenyl)8-azido-adenosine triphosphate; MOPS, 3-(N-morpholino)propanesulfonic acid; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase pair(s); TM, transmembrane; NBF, nucleotide-binding fold; CFTR, cystic fibrosis transmembrane regulator.

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be controlled by the transcription factors Pdr1p and Pdr3p. The YCF1 gene, which encodes a glutathione S-conjugate pump (10), was also deleted. The multiply deleted mutants have allowed the demonstration that Yor1p and Pdr5p share several substrates, which include fluorescent phosphatidylethanolamine (10), was also deleted. The multiply deleted mutants have allowed the demonstration that Yor1p and Pdr5p share several substrates, which include fluorescent phosphatidylethanolamine.

**EXPERIMENTAL PROCEDURES**

Chemicals—n-Dodecyl β-o-maltoside was purchased from Boehringer Mannheim; bovine serum albumin, 2-oxy-glucose, and oligomycin were from Sigma; o-nitrophenyl-β- D-galactopyranosidase and rhodamine B were from Merck; molecular weight markers (range 53,000–212,000 Da) and Taq polymerase were from Amersham Pharmacia Biotech; yeast extract was purchased from both KAT and Difco. M-C_\text{2}-NBD-PE, dioleoylphosphatidylcholine, and N-rhamidine-dioleoylphosphatidylethanolamine were from Avanti Polar Lipids Inc. (Alabaster, AL). TNP-8-azido-ATP and the v-23 species were synthesized as described previously (18, 19). All other reagents were of analytical grade.

**Yeast Strains**—The Saccharomyces cerevisiae strains used in this study are listed in Table I. Multiple deletions were performed sequentially in the US50–18C PDR1–3 strain by repeated use of the hisG-URA3-hisG cassette followed by selection of the ura3 auxotrophic marker with 5-fluoroorotic acid (20). The plasmids for the deletion of PDR5 (12), SNQ2, and YOR1 (1) genes were kindly provided by W. S. Moei-Rawley (Department of Physiology and Biophysics, University of Iowa, Iowa City, IA). For the deletion of PDR10, PDR11, PDR15, YCF1 genes, we amplified fragments of the promoter and the ORF 3′-end of each gene (Table II). The gene promoters were cloned into the EcoRI/BamHI sites of pSK, the ORF ends were cloned into the BamHI/XbaI sites of pEGH452 cosmid (kind gift of Herve Tettelin). Linearized fragments of the plasmids were used to transform the yeast for deletion of PDR5 (BamHI, SalI), SNQ2 (ScaI), YOR1 (SacI/BamHI), PDR10 (EcoRI/NotI), PDR11 (KpnI/NotI), PDR15 (EcoRI/NotI), YCF1 (EcoRI/NotI), and PDR3 (EcoRI/NotI). The deletion of PDR5, SNQ2, and YOR1 genes was checked by Southern blotting analysis. Deletion of the other genes was checked by PCR screening: yeast cells from a 1.5-ml overnight rich glucose medium (YD: 2% yeast extract, 5.8% glucose) culture were washed, resuspended in 200 μl of 10 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 1 mM EDTA and broken by vortexing for 2 min with glass beads and 300 μl of phenol-chloroform (50:50). After a second phenol-chloroform extraction and a washing with 1 volume of ether, 2 μl of cell extract were used for the PCR analysis. Recovery of the ura3 marker was monitored by plating onto 5-fluoroorotic acid 10^5 cells of an overnight YD culture. The resistant cells were screened by PCR as described above. The deletions of PDR5, SNQ2, and YOR1 were monitored by Southern analysis and PCR as described above. The deletions of PDR5, SNQ2, and YOR1 were monitored by Southern analysis and PCR as described above.

The SUPERYOR strain was constructed as follows: 1129 bp of the PDR5 promoter (from position 1141 to 12) and 505 bp of the PDR5 ORF end (from +4206 to +4831) were amplified by PCR using the following primer sequences: 5′-CCCAGCATTTACCTGCGGCCGG-3′ and 5′-GGTCAGCCTCGACCCAGG-3′. The PCR fragments were cut by either Clal and SmaI (PDR5 promoter) or XbaI and SmaI (PDR5 end) and cloned into Clal-XbaI-cleaved pSK (pSK: PDR5/PHOS-PDR5STOP). The YOR1 DNA was prepared as follows. The pEGH452 cosmid (kind gift of Herve Tettelin) containing 35.4 kb of S. cerevisiae chromosome VII was cut by MinI. The 5.7-kb fragment, containing the YOR1 ORF, was blunted with Klenow

**TABLE I**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>II.125–2B</td>
<td>MATa, PDR1, his1, parental of the DRI9-T8 PDR1–3 mutant</td>
<td>Balzi et al. (24)</td>
</tr>
<tr>
<td>2229–5C</td>
<td>MATa, PDR1, ura3</td>
<td>Balzi et al. (24)</td>
</tr>
<tr>
<td>US50–18C</td>
<td>MATa, PDR1–3, ura3, his1, obtained by cross between DRI9-T8 and 2229–5C</td>
<td>Balzi et al. (24)</td>
</tr>
<tr>
<td>D1–3/3</td>
<td>US50–18C disrupted by Δpdr1::URA3</td>
<td>Balzi et al. (24)</td>
</tr>
<tr>
<td>AD1*</td>
<td>Δyor1::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>AD2*</td>
<td>Δyor1::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>AD3*</td>
<td>Δyor1::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>AD4*</td>
<td>Δpdr10::hisG, Δpdr5::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>AD5*</td>
<td>Δpdr11::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>AD6*</td>
<td>Δpdrf1::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>AD12*</td>
<td>Δyor1::hisG, Δpdr5::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>AD13*</td>
<td>Δyor1::hisG, Δpdr5::hisG</td>
<td>This study</td>
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<td>AD123*</td>
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<td>AD234*</td>
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<td>This study</td>
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<tr>
<td>AD123456*</td>
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<td>AD124567*</td>
<td>Δyor1::hisG, Δpdr5::hisG, Δpdr11::hisG, Δpdr10::hisG, Δpdr12::hisG, Δpdrf1::hisG, Δpdr3::hisG</td>
<td>This study</td>
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<tr>
<td>AD1234567*</td>
<td>Δyor1::hisG, Δpdr5::hisG, Δpdr11::hisG, Δpdr10::hisG, Δpdr12::hisG, Δpdrf1::hisG, Δpdr3::hisG</td>
<td>This study</td>
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<td>AD1234568*</td>
<td>Δyor1::hisG, Δpdr5::hisG, Δpdr11::hisG, Δpdr10::hisG, Δpdr12::hisG, Δpdrf1::hisG, Δpdr3::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>FY1679–28C</td>
<td>MATa, PDR1, PDR3, ura3–52, trpl1, trp1, his1, Δ1, his3–Δ200, GAL2</td>
<td>C. Fairhead, B. Dujon</td>
</tr>
<tr>
<td>FY1679–28C/EC</td>
<td>FY1679–28C disrupted by Δpdr1::TRP1</td>
<td>Delaveau et al. (44)</td>
</tr>
<tr>
<td>FY1679–28C/AD</td>
<td>FY1679–28C disrupted by Δpdr3::HIS3</td>
<td>Delaveau et al. (44)</td>
</tr>
<tr>
<td>FY1679–28C/AD</td>
<td>FY1679–28C/AD disrupted by Δpdr1::TRP1</td>
<td>Delaveau et al. (44)</td>
</tr>
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</table>

* In this study, the deletions have been done in the PDR1–3 US50–18C strain.
Yeast ABC Transporter Yor1p

TABLE II

<table>
<thead>
<tr>
<th>Gene</th>
<th>First primer&lt;sup&gt;a,b&lt;/sup&gt;</th>
<th>Second primer</th>
<th>Restriction sites&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Size&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDR10</td>
<td>P: 5'-GGAATTCCTGCGCAACTTACAGAATC</td>
<td>5'-GGGATCCCGGACTTTCAGGAGG</td>
<td>EcoRI/BamHI</td>
<td>709 bp</td>
</tr>
<tr>
<td></td>
<td>S: 5'-GGAATTCCTGCGCAATTGAGGAGGAG</td>
<td>5'-GGTACACACATGCTGTAATCC</td>
<td>BamHI/NotI</td>
<td>912 bp</td>
</tr>
<tr>
<td>PDR15</td>
<td>P: 5'-GGAATTCCTGCGCAACTTACAGAATC</td>
<td>5'-GGGATCCCGGACTTTCAGGAGG</td>
<td>EcoRI/BamHI</td>
<td>952 bp</td>
</tr>
<tr>
<td></td>
<td>S: 5'-GGAATTCCTGCGCAATTGAGGAGGAG</td>
<td>5'-GGTACACACATGCTGTAATCC</td>
<td>BamHI/NotI</td>
<td>845 bp</td>
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<tr>
<td>PDR11</td>
<td>P: 5'-GGAATTCCTGCGCAACTTACAGAATC</td>
<td>5'-GGGATCCCGGACTTTCAGGAGG</td>
<td>EcoRI/EcoRI</td>
<td>1137 bp</td>
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<tr>
<td></td>
<td>S: 5'-GGAATTCCTGCGCAATTGAGGAGGAG</td>
<td>5'-GGTACACACATGCTGTAATCC</td>
<td>BamHI/NotI</td>
<td>827 bp</td>
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<tr>
<td>YCF1</td>
<td>P: 5'-GGAATTCCTGCGCAACTTACAGAATC</td>
<td>5'-GGGATCCCGGACTTTCAGGAGG</td>
<td>EcoRI/BamHI</td>
<td>1041 bp</td>
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<tr>
<td></td>
<td>S: 5'-GGAATTCCTGCGCAATTGAGGAGGAG</td>
<td>5'-GGTACACACATGCTGTAATCC</td>
<td>BamHI/NotI</td>
<td>523 bp</td>
</tr>
<tr>
<td>PDR3</td>
<td>P: 5'-GGAATTCCTGCGCAACTTACAGAATC</td>
<td>5'-GGGATCCCGGACTTTCAGGAGG</td>
<td>EcoRI/BamHI</td>
<td>907 bp</td>
</tr>
</tbody>
</table>

<sup>a</sup> The primer sequences for the PCR amplifications were chosen in the promoter (P) and in the ORF 3'-end (S) sequence of each gene.

<sup>b</sup> Restriction sites were added for the cloning (underlined sequences).

<sup>c</sup> Size of the PCR-amplified DNA fragments.

and cloned into EcoRV-cleaved pSK. The pSK:YOR1 plasmid was cut by NheI and ShoBI to generate a 5-kb fragment, which was purified, blunted with Klenow, and cloned into SmaI-cleaved-pSK:: PDR5PROM-PDR5STOP. The pSK::PDR5PROM-YOR1-PDR5STOP resulting plasmid was cut by ClaI and NdeI and used to transform the AD124567 strain. The transformed yeasts were plated on 0.75 μg/ml oligomycin. Replacement of the PDR5 chromosomal allele by the modified YOR1 gene was checked by PCR using primer sequences corresponding to both the YOR1 and the PDR5 ORFs.

Transcriptional Activity of the Yeast ABC Genes—Transcriptional activity of the yeast ABC genes was measured after transformation of the FY1679–28C/EC (pdr1Δ) strain with 2 centromeric plasmids. The first plasmid carries neither an allele (pRS315), or the wild type (pRS315::PDR1), or the mutant allele (pRS315::PDR1–3) of the PDR1 gene as described previously (21). The second plasmid (pSEY102) bears a translational fusion of the ABC gene promoters and the E. coli lacZ gene. The PDR5–lacZ fusion (12), the SNG2-lacZ fusion (8), and the YOR1-lacZ construct (1) have been described previously. For the construction of the PDR11-lacZ fusion, 687 bp of the PDR11 gene, including 633 bp of the promoter, were amplified by PCR using the primers 5'-GGAATTCGCCATGATCGAGTACAGAGG and 5'-GGAATTCCCTTATAGTACCTTG. The resulting plasmid was cut by ClaI and NdeI and used to transform the AD1234568 strain for testing complementation of oligomycin and rho-1 deficiency (17). Phospholipid concentrations were determined by the method of Rhee et al. (22) with modifications. Phosphatidylethanolamine (200 mg/ml) and phosphatidylglycerol (200 mg/ml) were prepared as described previously (8). Rhodamine B Fluorescence Measurements in Intact Cells—Four-m1 YD cultures were inoculated with ~50 × 10^6 cells from an overnight preculture and incubated for 3 h at 30°C. Culture aliquots (250 μl) were removed at the indicated times, filtered through a 0.45-μm filter, and washed three times with buffer A (50 mM Hepes-NaOH, pH 7.0), resuspended in 2 ml of buffer A containing 100 μg/ml rhodamine B and either 10 mM t-glucose or 5 mM 2-deoxy-d-glucose and incubated for 1 h at 28–30°C. A 1.5-ml sample was pelleted and washed three times with buffer A. The cell pellet resuspended in 800 μl of water was maintained on ice until cell fluorescence was measured using an SLM Aminco 48000 S spectrophotofluorimeter. The excitation wavelength was 545 nm (emission width of 4 nm), and the emission wavelength was 575 nm (slit of 4 nm).

In rhodamine B extraction experiments, cells from 3.5 ml of YD culture (~40 × 10^6 cells/ml) were washed three times with buffer A, incubated in 2 ml of buffer A containing 100 μg/ml rhodamine B and 5 mM 2-deoxy-d-glucose for 2 h at 28–30°C and then washed three times with buffer A and resuspended in 1.5 ml of buffer B. Rhodamine B fluorescence was measured in response to either 10 mM t-glucose, 4% ethanol, or no carbon source (control). At the indicated times, the fluorescence of 300 μl of both the cell-free supernatant and the cell pellet were measured.

Yeast Cell Labeling with NBD-phosphatidylethanolamine—Lipid vesicles including 50 μM total lipids comprising M-C2-NBD-PE (40 mol%), dioleoylphosphatidylcholine (58 mol%), and N-rhodamine-dioleoylphosphatidylethanolamine (2 mol%) were prepared as described previously (17). Phospholipid concentrations were determined by the lipid phosphorus assay (26). For internalization of M-C2-NBD-PE, yeast cells were grown overnight in SDC at 30°C, diluted, and allowed to grow to an A_o of 0.2–0.3. Donor vesicles containing the fluorescent lipids were added to the yeast cells and incubated for 30 min at 37°C. Cells were washed three times with ice-cold SCNaC (SDC lacking glucose but containing 2% sorbitol and 20 mM sodium azide) prior to analysis by fluorescence microscopy and flow cytometry.

Fluorescence Microscopy—Fluorescence microscopy was performed on a Zeiss Axiovert microscope equipped with barrier filters that allowed no detectable crossover of NBD and rhodamine fluorescence. The fluorescence image was enhanced with a VE1000-SIT image-intensi-
ing camera (DAGE-MTI Inc., Michigan City, IN), digitized, and stored. Image manipulation and editing were performed with Metamorph software (Universal Imaging Corp., West Chester, PA).

Flow Cytometry—Flow cytometric analysis of the M-C6-NBD-PE labeled cells was performed with a FACScan cytometer (Becton-Dickinson Immunocytochemistry, San Jose, CA) equipped with an argon laser operating at 488 nm. Ten μl of a 50 μg/ml stock solution of propidium iodide was added to approximately 4 × 10⁶ cells in 200 μl of SCNAn immediately prior to dilution (~3 times) and flow cytometric analysis. Ten thousand cells were analyzed without gating during acquisition. Analysis was performed with Lysis II (Becton-Dickinson Immunocytochemistry Systems) software. A dot plot of forward scatter versus the red fluorescence channel (propidium iodide) was used to set a gate that excluded dead cells from the analysis. The remaining live cells were plotted on a histogram with the green fluorescence (M-C6-NBD-PE) plotted on a log scale, and the mean (F₅₇₅⁶) and standard deviation of the fluorescence intensity of the live cells calculated.

Other Methods—The protein content was measured as described by Lowry et al. (27) with bovine serum albumin as the standard. The protein samples were electrophoresed on SDS-polyacrylamide gel according to Laemmli (28) and stained with either Coomassie Blue or silver. The yeast cells were transformed as described by Kuo and Campbell (29), and bacteria (DH5α strain) transformation was performed by electroporation using a Bio-Rad Gene Pulser apparatus, following the manufacturer’s instructions.

RESULTS

Overexpression of Yor1p and Genetic Purification—The low level of expression of YOR1 in wild-type yeast (1) precludes characterization of its properties. Traditional overexpression of plasma membrane proteins using strong promoters and multicopy vectors often causes mistargeting and stimulates accumulation of intracellular membranes (30). These problems have been overcome in a new approach, which has allowed dramatically enhanced overexpression of Pdr5p and Snq2p ABC transporters in the yeast plasma membrane (6–8). The method takes advantage of point mutations in the transcription factor-encoding genes PDR1/PDR3, which activate the transcription of their target genes. The target gene promoters contain typical binding sequences called PDREs (for PDR response elements) which correspond to the 5'-TCCGCA/GA-3' consensus sequence (12, 31, 32) (Table III). An inherent problem to this approach is the simultaneous overexpression of several Pdr1p-regulated proteins, including other ABC transporters. It was therefore necessary to identify all potentially interfering proteins and eliminate them by gene deletion. Systematic sequencing of the yeast genome has revealed new ORFs, which encode a total of 15 full-size ABC transporters (3) including the PDR10 (33), PDR15 (34), and PDR11 (35) genes whose promoters display at least one putative PDRE. Table III shows the transcription activity mediated by the PDR5, YOR1, SNQ2, PDR10, PDR15, and PDR11 promoters in the presence of PDR1 wild-type-, null, or mutated PDR1–3 allele as measured by the β-galactosidase activity of fusion constructs. The PDR11 promoter-mediated transcription activity was weak and not significantly affected by Pdr1p, possibly because the 5'-TCCGCA/GA-3' sequence in the promoter was insufficient for Pdr1p/Pdr3p recognition. The PDR1–3 mutation slightly increased the efficiency of the PDR15 gene promoter despite the presence of a perfect Pdr1p/Pdr3p-binding site. In contrast, the PDR1–3 allele increased the efficiency of the PDR10 promoter 11-fold. Note, however, that the putative PDREs of the PDR10, PDR15 and PDR11 gene promoters have yet to be verified experimentally. In the presence of the wild-type allele of PDR3, the PDR1–3 mutation increased expression of β-galactosidase 25 times for the YOR1 promoter, 17 times for the PDR5 promoter, and 5 times for the SNQ2 promoter.

Table III also shows that the number of putative PDREs per promoter did not correlate with the extent of the induction of β-galactosidase activity mediated by Pdr1p. Although the PDR5 and SNQ2 promoters each have three PDREs (12, 31, 32), PDR5-lacZ expression was enhanced 5 times by the wild-type Pdr1p while the expression of the SNQ2-lacZ fusion was increased only 1.4 times upon the addition of the wild-type PDR1 allele. Under the same conditions, the induction of the other translational lacZ fusions showing 1 or 2 putative PDREs varied between 1.4 (YOR1-lacZ) and 2.2 (PDR10-lacZ). A further important point is that the β-galactosidase activities of PDR5-, SNQ2-, and YOR1-lacZ fusions reported here in the presence of wild type Pdr3p are quite different to when Pdr3p is absent (21).

In agreement with the results of Table III, Fig. 1 shows that the PDR1–3 mutant plasma membrane (compared with that from either wild type or pdr1Δ strains) dramatically overexpressed a 160-kDa Coomassie Blue-stained band, which mainly comprised Pdr5p, Snq2p, and Yor1p. In order to free Yor1p from membrane contaminants of similar size and possibly overlapping function, we deleted PDR5, SNQ2, PDR10, and PDR15 yeast ABC transporter-encoding genes whose expression was also induced by the PDR1–3 mutation. The PDR11 gene whose product is a constituent of the 160-kDa overexpressed band (8) was also deleted. YCF1 was deleted because this gene is involved in drug resistance (36, 37).

Yor1p Binds TNP-8-azido-ATP and Shows ATPase Activity—Using the AD234567 multiply deleted strain overexpressing Yor1p, we started to investigate the Yor1p potential NTP hydrolysis activity. Pdr5p has an NTPase activity, which was distinguished from that of the H⁺-pump Pma1p by its
broader nucleotide specificity and pH dependence (7, 8). With similar protein levels in the plasma membrane, the UTPase activity of Pdr5p was up to 10-fold higher than that of Pma1p (Fig. 2), which is the major ATPase in the yeast plasma membrane. The situation with Yor1p-enriched plasma membrane (AD234567) was very different since no significant difference in the NTPase activity was detected compared with the Yor1p-depleted strain (AD1234567). The same results were obtained with the AD234567/AD1234567 strains. However, in the SUPERYOR strain obtained by fusion of the PDR5 promoter to the YOR1 ORF, a survey of pH from 5.0 to 8.5 revealed that some very low ATPase activity could be associated with SuperYor1p above pH 7.0 (data not shown).

Partial purification of SuperYor1p by centrifugation on sucrose gradient, separated it from H\(^+\)-ATPase (Fig. 3A). This allowed us to detect ATPase activity associated with the SuperYor1p-enriched fractions (fractions 6–12) after subtraction of the contaminating ATPase activity (75 nmol of Pi/min · mg\(^{-1}\)) measured in the corresponding fractions of the AD1234567 Yor1p-depleted strain (Fig. 3B). Detection of the ATPase activity required the presence of 150 \(\mu\)g/ml asolectin, which was found to be optimal concentration (data not shown).

By scanning the SDS-polyacrylamide gel, we found good correlation between the SuperYor1p band intensity and its ATPase activity (Fig. 3B). The enzyme was able to hydrolyze ATP from pH 7.0 to 8.5 with similar efficiency (Fig. 3C). ATPase activity was strongly sensitive to vanadate (150 \(\mu\)M) and inhibited by oligomycin concentrations above 3 \(\mu\)g/ml (Fig. 3, D and E).

Recently, the fluorescent ATP analogue, TNP-ATP, was shown to bind tightly to several proteins including the purified recombinant NBD1 and NBD2 of mouse MDR P-glycoprotein (38, 39), a synthetic NBD1 of human CFTR (40), and the Chinese hamster P-glycoprotein (41). Photolabeling of plasma membranes enriched in Yor1p (AD234567) and Pdr5p (AD124567) with \(\gamma\)\(^{32}\)P-TNP-8-azido-ATP is shown in Fig. 4A. The labeling of Pma1p is given for comparison. The two ABC transporters exhibited a very similar dependence on nucleotide concentration for photolabeling (\(K_0.5\) = 45 and 46 \(\mu\)M for Yor1p and Pdr5p, respectively), whereas Pma1p required a slightly

![Fig. 1. Pdr5p, Snq2p, and Yor1p are overexpressed in the PDR1–3 mutant plasma membrane. Plasma membrane-enriched fractions were separated onto 7–14% SDS-PAGE and stained with Coomassie Blue. From left to right: US50–18C (PDR1–3), AD3 (PDR1–3, pdr5Δ), AD23 (PDR1–3, pdr5Δ, snq2Δ), AD123 (PDR1–3, pdr5Δ, snq2Δ, yor1Δ), 2229–5C (PDR1), and D1–3/3 (pdr1Δ). Each lane contains 150 \(\mu\)g of protein.](http://www.jbc.org/)

![Fig. 2. The level of Yor1p in the plasma membrane is enhanced by fusing the PDR5 promoter to the YOR1 ORF. One hundred \(\mu\)g of plasma membrane-enriched fractions were separated onto 7%–SDS-PAGE and stained with Coomassie Blue. From left to right: AD234567 (yor1Δ, snq2Δ, pdr5Δ, pdr10Δ, pdr11Δ, ycf1Δ, pdr3Δ), AD234567 (snq2Δ, pdr5Δ, pdr10Δ, pdr11Δ, ycf1Δ, pdr3Δ), SUPERYOR (yor1Δ, snq2Δ, pdr5;PDR5PROM-YOR1-PDR5STOP, pdr10Δ, pdr11Δ, ycf1Δ, pdr3Δ), AD124567 (yor1Δ, snq2Δ, pdr10Δ, pdr11Δ, ycf1Δ, pdr3Δ). The nature of the 160-kDa overexpressed protein is given in each case. The maximum non-inhibitory concentrations of drug for growth on either oligomycin or cycloheximide are shown. We could not determine the corresponding oligomycin concentration for the SUPERYOR strain because of its poor solubility in rich glycerol medium (YG: 2% yeast extract, 4% glycerol) plates. The UTPase activities of the plasma membrane-enriched fractions at pH 6.3 are given in the last row.](http://www.jbc.org/)
higher concentration range ($K_{D,5} = 93 \, \mu M$). The effect of competitor ATP on the photolabeling is shown in Fig. 4B. The concentration of TNP-nucleotide chosen was close to the $K_{D,5}$ value for the ABC transporters. It has been shown previously that the labeling reaction in the presence of ATP approximates an equilibrium situation and the "true" dissociation constants for ATP ($K_{D,5}(ATP)$) can be derived from the observed inhibition curves ($K_{D,5}(ATP)$) using the equation: $K_{D,5}(ATP) = K_{D,5}(ATP)/(1 + ([TNP-8-azido-ATP]/[TNP-8-azido-ATP]))$ (19). The labeling of all three pumps is inhibited by ATP in the millimolar concentration range, yielding $K_{D}$ values of 0.3 mM for both the ABC transporters and the proton pump. This value is in good agreement with the $K_{D}$ for activation of proton transport (42). Note, however, that the TNP-8-azido-ATP binding on Pdr5p and Yor1p proteins has yet to be verified experimentally.

**Overexpressed Yor1p Confers Increased Resistance to Oligomycin in Vivo**—Overexpressed Yor1p has been reported to confer resistance to oligomycin (1). The analysis of the multiple deletions shows that oligomycin resistance of the PDR1–3 mutants is always dependent on the presence of Pdr5p. Unlike AD1 (yor1Δ) and AD12 (yor1Δsnq2Δ) strains, the AD13 (yor1Δpdr5Δ) strain does not grow on plates containing 0.25 μg/ml oligomycin (Fig. 5). Previous studies showed unmodified oligomycin sensitivity in single PDR5 deletants (11, 12) but Kolaczkowski et al. (43) showed that oligomycin is a competitive inhibitor of Pdr5p-mediated transport of rhodamine 6G. As also shown by Fig. 5, Pdr1p more strongly influences oligomycin resistance than its homolog Pdr3p (compare the FY1679/EC (pdr1Δ) and FY1679/AD (pdr3Δ) strains). This is in agreement with previous observations (12) even though no difference in oligomycin resistance between pdr1Δ and pdr3Δ strains was observed by Delaveau et al. (44). As also shown by Fig. 2, oligomycin resistance was increased 40 times in the strain AD234567 which overexpresses Yor1p and was further increased by a factor of more than 8 in the SUPERYOR strain. These data indicate that, at the cell level, the drug transport properties of Yor1p are conserved during overexpression.
mediate rhodamine B and oligomycin resistance through both Pdr5p and Yor1p. Notice that Yor1p and Pdr5p share other common substrates, including the fungicide miconazole (data not shown).

The involvement of Yor1p in rhodamine B and oligomycin resistance of pdr5Δ strains was confirmed by complementation of the AD13 (yor1Δ pdr5Δ) and the AD1245678 (yor1Δ, snq2Δ, pdr5Δ, pdr10Δ, pdr11Δ, ycf1Δ, pdr3Δ, pdr15Δ) strains with the YOR1 gene. Transformation of both strains with the YOR1 gene on centromeric plasmid allowed recovery of growth on both 0.5 mg/ml oligomycin and 250 mg/ml rhodamine B as shown for the AD3 and AD234568 strains (data not shown).

The Efflux of Rhodamine B in Yeast Cells Overexpressing Yor1p Is Energy-dependent—We therefore used the Yor1p-overexpressing strain AD2345678 (snq2Δ, pdr5Δ, pdr10Δ, pdr11Δ, ycf1Δ, pdr3Δ, pdr15Δ) and its isogenic control, deleted in YOR1, AD12345678 (yor1Δ, snq2Δ, pdr5Δ, pdr10Δ, pdr11Δ, ycf1Δ, pdr3Δ, pdr15Δ) to demonstrate the involvement of Yor1p in the rhodamine B cell content. In the yor1Δ strain, rhodamine B accumulation was slightly lowered (82%) by deoxyglucose compared with glucose (100%). However, in the presence of overexpressed Yor1p, rhodamine B accumulation was much higher (58%) in energy-starved cells (deoxyglucose), while glucose caused a drastic reduction (to 8%) in the cellular rhodamine B content (data not shown). Fig. 6A shows the Yor1p-mediated energy-dependent extrusion of rhodamine B from pre-loaded cells incubated with deoxyglucose, which depletes the intracellular ATP (45). The addition of glucose caused rapid extrusion of rhodamine B from the Yor1p-expressing cells, while no glucose-dependent effect was observed in the yor1Δ cells. Fig. 6B shows the difference in the supernatant fluorescence (Fglucose - Fcontrol) in the same experiment and establishes the Yor1p energy-dependent extrusion of rhodamine B out of the cell. Addition of 30 μM protonophore FCCP in the presence of the respiratory substrate ethanol as the sole energy source completely abolished rhodamine B transport, while, in

\[ \text{Fig. 4. TNP-8N3-ATP photolabels Yor1p and Pdr5p and the labeling is inhibited by ATP. Photolabeling was performed with the concentrations of [γ-32P]TNP-8-azido-ATP shown (A) or with 45 or 50 μM [γ-32P]TNP-8-azido-ATP, for Yor1p (strain AD234567) or Pdr5p (AD124567) and Pma1p (AD124567), respectively, and the concentrations of ATP shown (B). Open triangles, Yor1p (AD234567); open circles, Pdr5p (AD124567); closed circles, Pma1p (AD124567). In A, the lines show the best fit to the data. The } K_{0.5}^{\text{TNP-8-azido-ATP}} \text{ values are given in the text. In B, the line shows the best fit to the data obtained for Pdr5p and yielded a } K_{0.5}^{\text{ATP}} \text{ of 0.9 mM. That for Yor1p was similar. The value for Pma1p was 0.6 mM. The "true" } K_{0.5} \text{ values are given in the text. The insets show autoradiographs of the relevant gel bands.} \]
the presence of glucose, FCCP only slightly affected Yor1p-mediated rhodamine B transport (Fig. 6C). This may be explained either by the partial involvement of oxidative phosphorylation in the ATP formation of glucose-grown cells and/or by a possibly higher use of cellular ATP by the Pma1p H^+--ATPase under these conditions. In the absence of an energy source, the application of a pH gradient of 2 units (pH 7.0 inside the cell and pH 5.0 outside the cell) did not cause rhodamine B extrusion (Fig. 6C).

These data indicate that the energy required for drug efflux
by Yor1p is not provided by the proton motive force across the plasma membrane. This is in agreement with previous studies showing the ATP requirement for drug transport by several other ABC transporters, including the yeast Pdr5p (43) and Ycf1p (10), the lactococcal MDR transporter (46), and the mammalian MRP (47) and MDR1 (48).

Yor1p and Pdr5p Are Involved in M-C₆-NBD-phosphatidylethanolamine Accumulation in Vivo—Several mammalian ABC transporters translocate phospholipids (13–16). A “flip-flop” of hydrophobic drugs from the inner leaflet to the outer one has been proposed as part of the mechanism of drug transport (49). We have tested whether overexpressed Yor1p was involved in the transport of a fluorescent phospholipid analog, M-C₆-NBD-PE. Cell fluorescence was measured by flow cytometry after the incubation of yeast cells with M-C₆-NBD-PE (Fig. 7A) and analyzed by fluorescence microscopy (Fig. 7B).

The average accumulation of M-C₆-NBD-PE in the PDR1—3 mutant strain (US50–18C) was about 13% of the PDR1 parent strains (IL125–2B and 2229–5C). In similar experiments, another mutant, PDR1—11, accumulated 1–2% of its isogenic parent.² A strain in which the PDR1 gene was deleted (D1—3/) accumulated about 70% more M-C₆-NBD-PE than the PDR1 parent strains (Fig. 7A). It therefore seems likely that Pdr1p activates the expression of genes encoding proteins that decrease the steady-state accumulation of M-C₆-NBD-PE by either increasing its efflux or decreasing its influx.

Thus, to determine if any of the seven ABC transporters included in this study were responsible for the efflux of M-C₆-NBD-PE, we analyzed the multiply deleted strains. Based on the average M-C₆-NBD-PE fluorescence intensity for 10,000 cells, the PDR1—3 strain accumulated only 8% of the pdr1Δ strain. Deletion of YOR1 (AD1) or PDR5 (AD3) in the PDR1—3 strain resulted in an increase to 13% M-C₆-NBD-PE accumulation. The fluorescence intensity of the doubly deleted strain yor1Δ pdr5Δ (AD13), was increased to 36% of the pdr1Δ strain (60% of the PDR1 wild-type strain), a value slightly higher than if the two effects were additive. Single deletions of SNQ2 (AD2), PDR10 (AD4), PDR11 (AD5), YCF1 (AD6), or PDR15 (AD8) had no effect on M-C₆-NBD-PE accumulation. Thus, of the ABC transporters tested, only Pdr5p and Yor1p appeared to transport M-C₆-NBD-PE.

DISCUSSION

This study provides some new information on the control of YOR1 and other yeast ABC genes by the transcription factors Pdr1p and Pdr3p. The expression of lacZ gene fusions with PDR10, PDR15, and PDR11 gene promoters containing putative PDREs reveals that PDR10 may be a target of Pdr1p/Pdr3p transcription factors. However, its level of expression was very low compared with that of PDR5, SNQ2, and YOR1, whose transcription was greatly enhanced by the PDR1—3 mutation. Transcription of the PDR15 gene, despite the presence of one perfect Pdr1p/Pdr3p-binding site (PDRE) in its promoter, was only increased 2 fold by the PDR1—3 mutation. The transcription level of the PDR11 gene was not modified upon addition of either the wild type PDR1 or the mutated PDR1—3 allele, possibly because the PDRE sequence in its promoter is not sufficient for Pdr1p/Pdr3p binding (TCCGCAGA instead of TCCGG/CCG).

In the PDR1—3 gain-of-function mutant, the PDR5 gene promoter gave the highest absolute β-galactosidase activity among the fusion products with five different Pdr1p-regulated gene promoters. This is consistent with the SDS-PAGE analysis of plasma membrane-enriched fractions of multiply deleted strains. The latter analysis also confirmed that Pdr5p, Snq2p,

² A. M. Grant and J. W. Nichols, unpublished observations.

FIG. 7. Yor1p and Pdr5p are involved in M-C₆-NBD-PE accumulation in vivo. After M-C₆-NBD-PE internalization, cells were submitted to both flow cytometric analysis and fluorescence microscopy. A, flow cytometric analysis of the M-C₆-NBD-PE-labeled cells. Cell fluorescence was measured by flow cytometry after the incubation of yeast cells with M-C₆-NBD-PE (Fig. 7A) and analyzed by fluorescence microscopy (Fig. 7B). The average accumulation of M-C₆-NBD-PE in the PDR1—3 mutant strain (US50–18C) was about 13% of the PDR1 parent strains (IL125–2B and 2229–5C). In similar experiments, another mutant, PDR1—11, accumulated 1–2% of its isogenic parent.² A strain in which the PDR1 gene was deleted (D1—3/) accumulated about 70% more M-C₆-NBD-PE than the PDR1 parent strains (Fig. 7A). It therefore seems likely that Pdr1p activates the expression of genes encoding proteins that decrease the steady-state accumulation of M-C₆-NBD-PE by either increasing its efflux or decreasing its influx.

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transports without impairing growth.

Membrane-bound Yor1p was labeled with TNP-8-azido-ATP in vitro in a saturable manner and the labeling was inhibited by ATP. Interestingly, the $K_{0.5}$ for TNP-8-azido-ATP that we found for Yor1p (about 45 $\mu$M) is very close to the value found by Liu and Sharam for TNP-ATP binding to human P-glycoprotein (43 $\mu$M) (50). The nucleotide binding properties of Pdr5p and Yor1p appeared very comparable.

Investigation of the drug transport properties of intact cells overexpressing Yor1p showed that the Yor1p enzyme was active in vivo and required energy. The oligomycin resistance was increased 40 times in the strain overexpressing Yor1p and further increased by about 8-fold in the SUPERYOR strain, indicating that overexpression of Yor1p does not change its oligomycin transport capability in vivo. Absence of Yor1p or Pdr5p caused increased sensitivity to both oligomycin and rhodamine B. These effects were additive when both proteins were missing. A requirement for energy was demonstrated by the strong efflux of rhodamine B from Yor1p-enriched energystarved cells on glucose addition. The energy required for this process appears to be provided by ATP rather than $\Delta$PH. Finally, absence of either Yor1p or Pdr5p resulted in increased accumulation of a fluorescent phosphatidylethanolamine.

Again, the effect was more pronounced when both ABC transporters were deleted, indicating that the transporters may act independently. Conversely, none of the 5 other ABC transporters, including the overexpressed Snq2p transporter, exhibited this activity.

In plasma membranes from the AD234567 Yor1p-expressing strain, it was difficult to measure ATPase activity distinct from that of Pma1p. Enhancing the level of Yor1p by fusing the YOR1 ORF to the PDR5 promoter in the PDR1–3 mutant (SUPERYOR strain) allowed us, however, to detect ATPase activity in solubilized and partially purified SuperYor1p fractions. The SuperYor1p activity of $-100$ nmol of P$_i$ per min per mg was sensitive to both vanadate and oligomycin, establishing that we are not dealing with either P-type or F-type ATPase contaminants. The use of the PDR1–3 gain-of-function allele of PDR1 has already allowed characterization of the Pdr5p and Snq2p ATPase activities (7, 8). Surprisingly, Pdr5p, Snq2p, and (Super)Yor1p ATPase activities show distinct characteristics; Pdr5p hydrolyzes all Mg-NTPs over a broad pH range, whereas Snq2p and Yor1p hydrolyze ATP preferentially. The pH profile of Yor1p ATPase activity is also very broad (with an optimum at pH 7.5), while it is much sharper for Snq2p (pH 6.3). Only Pdr5p and Yor1p ATPase activities are oligomycin-sensitive, while vanadate was shown to inhibit all three enzyme ATPase activities. Finally, taking into account the relative amount of each transporter in the plasma membrane, one can estimate that the Yor1p ATPase activity is more or less 15 times lower than that of Pdr5p or Snq2p.

As the pumping capacity (and specificity) of the Yor1p and Pdr5p transporters appears similar in vivo, the low ATPase activity of Yor1p ($-0.1$ nmol of P$_i$ per min per mg) compared with that of Pdr5p ($-1.5$ nmol of P$_i$ per min per mg) in similar conditions may indicate that the purified Yor1p transporter is a more highly coupled pump than Pdr5p. The high ATPase activity and apparently low pumping capacity of ABC drug transporters (51–54) has been a puzzling feature. For instance, Pdr5p ATPase activity is very high in the apparent absence of drugs or other substrates, slightly stimulated by substrates, and shows a broad nucleotide specificity despite an in vivo requirement for ATP for transport (43). The poor NTPase activity of Yor1p might be due to the fact that an activation factor required for ATPase activity is lost during the plasma membrane preparation or that some phospholipids block the Yor1p ATPase activity when tested in membranes. Another possibility is that Yor1p, like its ortholog CFTR, has low ATPase activity (55). Very recently, the close human ortholog of Yor1p, MRPI, was purified and shown to hydrolyze ATP at a rate of $-0.3$ nmol of P$_i$ per min per mg in the presence of 6 mM Mg-ATP (56). If we consider that, in our preparations, the purified SuperYor1p amounts to 20% of the total proteins, one can estimate that SuperYor1p may exhibit an ATP hydrolysis rate of $-0.5$ nmol of P$_i$ per min per mg SuperYor1p.

The mediation of phospholipid efflux by yeast Pdr5p and Yor1p is consistent with recent reports that identified other ABC transporters as phospholipid transporters or flippases. These include mouse mdr1 (16) and mdr2 (13, 14) and human MDR1 (16) and MDR3 (15, 16). Phospholipid transport by MDR1 and mdr1 is not head-group or glycerol backbone-specific, whereas the MDR3 P-glycoprotein transports phosphatidylcholine exclusively. The specificity of Yor1p and Pdr5p for phospholipids other than phosphatidylethanolamine remains to be seen, but it could be different for the two pumps. It seems possible that the high steady state of ATP hydrolysis by Pdr5p and the low ATPase activity of Yor1p may be related to activation of the former with certain yeast phospholipids, which may not be substrates or even inhibitors of the latter. Anyway, it is intriguing that Pdr5p and Yor1p share similar phospholipid translocation properties which were not observed for Snq2p.

Finally, we wish to point out that, taking advantage of the strong PDR5 promoter associated with the gain-of-function PDR1–3 mutation, we have developed an important new tool for overexpression of ABC transporters in the yeast plasma membrane. This system allows the overexpression of functional yeast Yor1p at a level that represents more than 10% of total plasma membrane proteins. For comparison, overexpression of the human MDR1 from a high copy number expression vector in yeast by Mao and Scarborough (57) yielded protein amounts to only 0.4% of the total yeast membrane proteins. We anticipate that the PDR system, which dramatically overexpresses the functional yeast Yor1p in the plasma membrane without associated intracellular trafficking problems, and the use of the PDR5 promoter in strains deleted in the majority of the full-size ABC transporter-encoding genes provides a prototype for high level expression of orthologous ABC transporters of medical interest.

Acknowledgments—We thank W. S. Moye-Rowley for the plasmid gift and J. Nader for useful technical assistance. We acknowledge E. Balzi, B. van den Hazel, M. Kolaczkowski, M. A. do Valle-Matta, and A. Cybularz-Kolaczkowska for helpful comments. We are grateful to B. van den Hazel for sharing unpublished observations and W. S. Moye-Rowley for constant interest in our work. We acknowledge B. C. Monk for critical reading of the manuscript.

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