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Overexpression of a Cloned IMP Dehydrogenase Gene of *Candida albicans* Confers Resistance to the Specific Inhibitor Mycophenolic Acid

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An IMP dehydrogenase gene was isolated from *Candida albicans* on a ~2.9-kb *Xba*I genomic DNA fragment. The putative *Candida* IMP dehydrogenase gene (*IMH3*) encodes a protein of 521 amino acids with extensive sequence similarity to the IMP dehydrogenases of *Saccharomyces cerevisiae* and various other organisms. Like the *S. cerevisiae IMH3* sequence characterized in the genome sequencing project, the open reading frame of the *C. albicans IMH3* gene is interrupted by a small intron (248 bp) with typical exon-intron boundaries and a consensus *S. cerevisiae* branchpoint sequence. IMP dehydrogenase mRNAs are detected in both the yeast and hyphal forms of *C. albicans* as judged by Northern hybridization. Growth of wild-type (sensitive) *C. albicans* cells is inhibited at 1 μg of mycophenolic acid (MPA), a specific inhibitor of IMP dehydrogenases, per ml, whereas transformants hosting a plasmid with the *IMH3* gene are resistant to MPA levels of up to at least 40 μg/ml. The resistance of cells to MPA is gene dosage dependent and suggests that *IMH3* can be used as a dominant selection marker in *C. albicans*.

IMP dehydrogenase (EC 1.1.1.205) is the key enzyme in the de novo biosynthesis of GMP, catalyzing the NAD-dependent oxidation of IMP to XMP. This conversion of IMP to XMP is the rate-limiting step in the biosynthetic pathway of guanine nucleotides, at least in mammals (26). While genes encoding IMP dehydrogenases have been isolated from archaea (11), protozoa (5, 54, 55), bacteria (2, 3, 16, 31, 36, 47, 49), plants (12), invertebrates (46), and mammals (10, 13, 39, 50), the only fungal sequences known are those encoding the putative IMP dehydrogenase genes sequenced in the *Saccharomyces cerevisiae* genome project (14, 27, 28). The polymorphic fungus *Candida albicans* is an opportunistic pathogen in immunocompromised individuals, causing oral and vaginal candidiasis as well as disseminated infections in neutropenic patients. Genetic manipulations of this diploid microorganism have been hampered by the apparent absence of a haploid phase and the dependence of marker systems on the spontaneous occurrence of auxotrophic mutants. Thus, the identification of the *IMH3* gene in *C. albicans* suggested to us that mycophenolic acid (MPA), a specific inhibitor of IMP dehydrogenases, might be used to develop a dominant selectable marker system in *Candida*. In several other organisms, it has been shown that amplification of an IMP dehydrogenase gene can confer MPA resistance in a gene-dosage-dependent manner by overexpression of the enzyme (9, 20, 23, 25, 54). We report the cloning and sequencing of a DNA fragment of *C. albicans* with a coding region for a protein with high amino acid similarity and identity to known IMP dehydrogenases. Transformation of *C. albicans* by using a multicopy plasmid vector yielded transformants resistant to the specific IMP dehydrogenase inhibitor MPA. Culturing of transformant cells in the presence of MPA stabilized replicative plasmids, suggesting the feasibility of using *IMH3* as a dominant selection marker for genetic studies in *C. albicans*.

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MATERIALS AND METHODS

Bacterial and yeast strains, plasmids, and growth conditions. *Escherichia coli* DH5α was used for maintenance of plasmids, and *E. coli* XL1-Blue MRA (P2) was used in screening the *C. albicans* genomic library. *C. albicans* SS is a clinical isolate provided by Remo Morelli (San Francisco State University, San Francisco, Calif.), and strains 3153a (ATCC 28367) and WO-1 were generously provided by David Soll (University of Iowa). The *ura3* auxotrophic strain *C. albicans* CaI4, obtained from William Fonzi (Department of Microbiology and Immunology, Georgetown University Medicine Center, Washington, D.C.), was used as the transformation host. The library of partially *Sau*3A-digested genomic DNA of *C. albicans* SS was constructed in Lambda FixII (Stratagene, La Jolla, Calif.). The yeast expression plasmid pRC2312 (7) was provided by Richard D. Cannon (Department of Oral Biology and Oral Pathology, University of Otago, Dunedin, New Zealand). Plasmid pTA (Invitrogen, San Diego, Calif.) was used for cloning PCR products, and pBluescript SK (Stratagene) was used for subcloning in *E. coli* and sequencing.

Bacteria were grown at 37°C in Luria-Bertani medium. Yeast strains were maintained by weekly passages on 1% yeast extract–2% Bacto Peptone–2% glucose (YPD) plates at 25°C. Medium for *C. albicans* CaI4 was supplemented with 50 μg of uridine per ml to enhance growth. To analyze expression in yeast and hyphal cells, *C. albicans* was grown in Lee medium (33) at the appropriate temperatures and pHs for yeast (25°C, pH 4.5) and hyphal (37°C, pH 6.7) growth. Selective medium for yeast transformants contained 6.7 g of yeast nitrogen base (YNB) without amino acids (Difco Laboratories, Detroit, Mich.) per liter, 2% glucose, and 0.77 g of complete supplement medium without uracil (CSM-URA; Bio101, San Diego, Calif.) per liter. For exclusive selection with MPA, the CSM-URA was supplemented with 100 μg of uridine per ml. MPA (Sigma Chemical Co., St. Louis, Mo.) was added from a 20-mg/ml stock solution in pure ethanol.

Isolation and subcloning of *IMH3*. Total RNA and poly(A)$^+$ RNA were isolated from *C. albicans* by standard procedures (42). Reverse transcription-PCR (RT-PCR) was carried out with avian myeloblastosis virus reverse transcriptase (Promega, Madison, Wis.) as described previously (41), using a set of highly degenerate primers designed to clone putative *Candida* β1 integrin-like sequences. RT-PCR products were cloned by using the TA Cloning Kit (Invitrogen). One of many RT-PCR fragments amplified by using these primers and then sequenced revealed a high degree of sequence similarity with IMP dehydrogenases according to data bank searches with BLAST (1). By using the RT-PCR

fragment of *IMH3* as a probe, the entire coding region of the gene was isolated from a library of partial *Sau*3A digests of genomic DNA from *C. albicans* SS constructed in Lambda FixII (Stratagene). For subcloning and sequencing in *E. coli*, the plasmid pBluescript (Stratagene) was used.

DNA sequencing and RNA mapping. The nucleotide sequence of a 2,908-bp *Xba*I-fragment harboring *IMH3* and its flanking regions was determined by the dideoxy sequencing method with the Taq Cycle Sequencing Kit and a 370A sequencer (Applied Biosystems, Inc., Foster City, Calif.) or the Sequenase Version 2.0 protocol (U.S. Biochemical Corp., Cleveland, Ohio) with 5′-[α-^{32}P]dATP. The 5′ end of the *IMH3* mRNA was mapped by primer extension analysis with avian myeloblastosis virus reverse transcriptase and the oligonucleotide 5′-GGGAGAAATGGGAATGGGTAGAAGG-3′ as described previously (51).

Southern and Northern hybridizations. Southern and Northern blot hybridizations were carried out by standard methods (42) with digoxigenin-labeled (Genius System; Boehringer Mannheim, Indianapolis, Ind.) and 5′-[α-^{32}P]dATP-labeled nucleic acid probes, respectively. For *IMH3* expression analysis by Northern hybridization with the 2,908-bp *Xba*I-fragment, total RNA was isolated from wild-type *C. albicans* cells grown in Lee medium (33) under the respective conditions for yeast and hyphal forms (25°C and pH 4.5 or 37°C and pH 6.7) with controls at 25°C and pH 6.7 and at 37°C and pH 4.5. *IMH3* mRNA levels in *C. albicans* CaI4 and plasmid transformants were detected in total RNA isolated from cells grown to an optical density at 600 nm of 1 in synthetic broth with 100 μg of uridine (CaI4) per ml, no uridine (Ura selection), or 100 μg of uridine per ml and 10 μg of MPA per ml (MPA selection).

Construction of plasmid pGKIII. To construct plasmid pGKIII, the 2.9-kb *Xba*I fragment containing the entire coding region of *IMH3* and its 5′ and 3′ flanking regions was inserted into the *Hin*dIII site of plasmid pRC2312 (7), following two-base fill-in reactions with the Klenow fragment of DNA polymerase I (42). Like the parent plasmid pRC2312, the derivative plasmid pGKIII also carried a *URA3* gene and a *LEU2* gene for selection in *ura3* and *leu2* cells, respectively, and a *C. albicans* autonomously replicating sequence. For selection and replication in *E. coli*, both plasmids contained an ampicillin resistance gene (*bla*) and an origin of replication (*ori*).

Transformation of *C. albicans* by electroporation. *C. albicans* cells were transformed by electroporation by using methods derived from an *S. cerevisiae* protocol (6). Briefly, *C. albicans* CaI4 cells were grown in 200 ml of YPD plus 50 μg of uridine per ml at 30°C to a density of approximately 10^8 cells/ml, as determined by turbidity. The cells were pelleted at 5,000 × *g* and suspended in 40 ml of water. To this were added 5 ml of 10× TE (100 mM Tris-HCl, 10 mM EDTA, pH 8.0) and 5 ml of 10× lithium acetate (1 M lithium acetate, adjusted to pH 7.5 with dilute acetic acid), and the suspension was incubated in a rotory shaker at 150 rpm for 45 min at 30°C. After addition of 1.25 ml of 1 M dithiothreitol, the suspension was kept in the shaker for 15 min more. Following this incubation, 200 ml of water was added to wash the cells. Next, the cells were pelleted at 5,000 × *g* and washed once in 125 ml of ice-cold water. Finally, cells were washed with 20 ml of ice-cold 1 M sorbitol and suspended in 250 μl of 1 M sorbitol. For each electroporation experiment, 40 μl of electrocompetent cells was used. The cells were transformed with 0.1 to 10 μg of plasmid DNA in a Bio-Rad Gene Pulser (0.2-cm cuvette, 1.6 kV, 200 Ω, 25 μF) with a Bio-Rad Pulse Controller included in the circuit and were subsequently plated on selective medium. Plates were incubated at 30°C for 3 to 8 days.

MPA susceptibility testing. A microtiter plate assay was used for quantitative determination of the susceptibility of *C. albicans* strains and transformants to MPA. This assay was a modified version of the method described by Sanglard et al. (43). Briefly, six serial dilutions of MPA in synthetic broth were prepared, with the concentrations ranging from 1.25 to 40 μg/ml. In one row of a 96-well flat-bottom microtiter plate, 200 μl of synthetic broth (YNB without amino acids, 2% glucose, and the appropriate supplements) per well with no MPA was pipetted, and in another row only 100 μl per well was pipetted. The latter was used as a blank in the optical density measurements. To each of the remaining six rows, 100 μl of a serial MPA dilution per well was transferred. An inoculum was prepared by diluting an overnight culture of a *C. albicans* strain or transformant to a cell density of 1.5×10^4 cells per ml in synthetic broth. To each well with 100 μl, an inoculum of 100 μl was added, yielding final MPA concentrations of 0 to 40 μg/ml. Each strain was tested in duplicate. The plates were sealed with Parafilm and incubated at 25°C for 48 h. The optical densities were determined by using a Bio-Rad model 450 microplate reader with a 570-nm filter.

Protein extracts and SDS-PAGE. For protein extracts, transformant *C. albicans* cells were grown in synthetic broth with no uridine (Ura selection) or with 100 μg of uridine per ml and 10 μg of MPA per ml (MPA selection). For strain CaI4, synthetic medium supplemented with 100 μg of uridine per ml was used. Protein was isolated from cells grown in 10 ml of synthetic broth to an optical density at 600 nm of 1 as described for *S. cerevisiae* (58). Protein extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie brilliant blue R-250 staining by standard protocols (42).

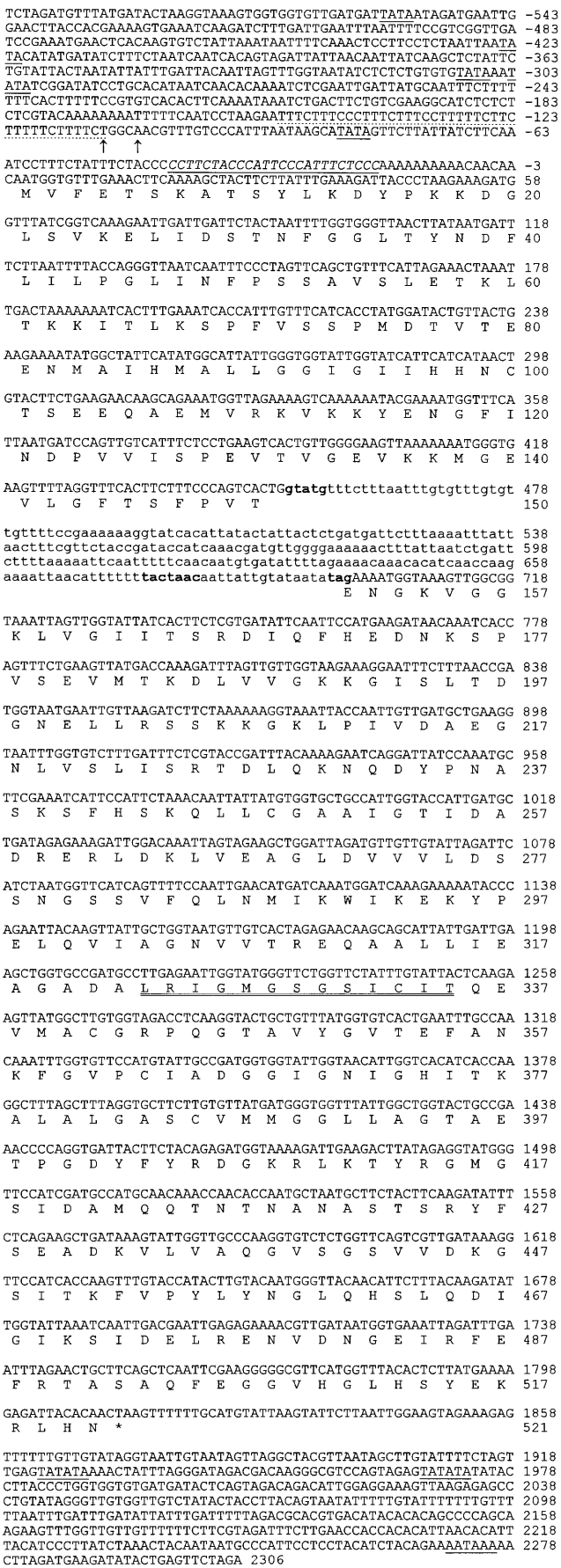
Nucleotide sequence accession number. The DNA sequence of *IMH3* was deposited in the GenBank database under accession number U85049.

RESULTS

Isolation of the *IMH3* gene. We performed RT-PCR experiments using *C. albicans* poly(A)$^+$ RNA and a set of degenerate oligonucleotides complementary to mammalian and avian β1 integrins. One of these oligonucleotides (5′-TCNACRAA NGANCCRAANCC-3′) yielded a 160-bp DNA fragment with an open reading frame (ORF) which displayed a high degree of amino acid sequence similarity to known IMP dehydrogenases upon BLAST searches of GenBank sequences (1). The entire coding region of the putative IMP dehydrogenase gene was isolated by screening of a genomic library of *C. albicans* with the cloned RT-PCR product as probe. Restriction fragments giving positive signals were subcloned in pBluescript (Stratagene), and one clone containing a 2,908-bp *Xba*I fragment was further sequenced. Analysis of this sequence indicated that it harbored the entire coding region of a putative IMP dehydrogenase gene (*IMH3*) of *C. albicans*. Southern analysis of genomic DNA cut with *Xba*I (data not shown) revealed that while *C. albicans* CaI4 and WO-1 show one band, strains 3153a and SS show two bands hybridizing with an *IMH3*-specific probe, which may represent two gene loci or just two alleles.

Sequence characteristics of the *C. albicans IMH3* gene and flanking regions. The clone containing the 2.9-kb *Xba*I fragment was sequenced in its entirety. It encodes a 1.6-kb ORF which is interrupted by a 248-bp intron starting at base 452 of the nucleic acid sequence shown in Fig. 1. The intervening sequence is delineated by a typical yeast 5′ splice site (GTAT GT) and 3′ splice site (TAG) and contains a yeast branchpoint consensus sequence (TACTAAC) (30) 22 nucleotides upstream of the 3′ splice site. Assuming splicing of the intron at these sites, the resulting ORF encodes a protein of 521 amino acids with a calculated M_r of 56,239 and a relative isoelectric point of 6.52. The gene was further analyzed by sequencing of 602 bp of the 5′ flanking region and 492 bp of the 3′ flanking region. The assignment of the predicted translation start codon at nucleic acid position 1 (Fig. 1) is supported by the 5′ mapping of the *IMH3* transcription unit and by the amino acid alignment shown in Fig. 2. Primer extension analysis using an oligonucleotide complementary to the 5′ sequence (positions −43 to −19) proximal to the ATG revealed two transcription start sites at nucleotides −111 and −107, respectively. The major transcription start site is located at position −111 about 110 bp upstream of the start codon; no other ATG codons are found in this region. Neither are there any in-frame ATG codons for more than 200 bases downstream of the ATG codon at position 1. Furthermore, a sequence which is similar to that used by *S. cerevisiae* for efficient translation initiation is also found in close proximity to the putative translational start of the *C. albicans IMH3* gene (24). The promoter region of *IMH3* is very AT rich, like many intergenic regions in *C. albicans*, making the identification of promoter elements tentative. Notable sequence characteristics of the 5′ flanking region are a CT-rich tract immediately 5′ of the transcription start site and the relatively long distance between the first upstream TATA box (192 nucleotides) and the start site. In *S. cerevisiae*, TATA elements for transcription initiation are usually found between 40 to 120 nucleotides from the start site (35). In the 3′ flanking region, sequence elements like TATATA (at nucleotide positions 1923 and 1968) and/or AATAAA (position 2271) might be involved in 3′ end formation if nascent mRNA polyadenylation in *C. albicans* resembles that in *S. cerevisiae* (22).

Several sequence characteristics contribute to the identification of the sequence described above as encoding IMP de-



hydrogenase. Overall, there is a high degree of amino acid sequence identity to IMP dehydrogenases from other organisms (Fig. 2). Alignments of the deduced amino acid sequence of the *C. albicans* enzyme were made with the sequences of IMP dehydrogenases from 21 other organisms (in Fig. 2 only six are shown), and the clustering relationships of the sequences were compared in a dendrogram (data not shown). The *C. albicans IMH3* protein compares favorably with all three of the putative IMP dehydrogenases of *S. cerevisiae* (14, 27, 28) derived from sequences determined in the *S. cerevisiae* genome project (ScIMH3p [Swiss protein accession number P50094], 75.4%; ScIMH2p [P50095], 74.3%; and ScIMH1p [P38697], 72.0%) and forms a cluster with these proteins when compared to other nonfungal sequences. The DNA sequences (not shown) of these IMP dehydrogenases also resemble that of *IMH3*, at 74.1% (*S. cerevisiae IMH3*), 72.7% (*S. cerevisiae IMH2*), and 70.1% (*S. cerevisiae IMH1*). The *C. albicans* gene has been designated *IMH3*, not only because it has the highest degree of DNA and protein sequence identity with the *S. cerevisiae IMH3* (14) but also because both genes are interrupted by an intron, while the two other genes in *S. cerevisiae* are intronless. The introns of both the *C. albicans* and *S. cerevisiae IMH3* genes (248 and 408 bp, respectively) interrupt the gene-protein sequence at the same amino acid site (Fig. 1 and 2). The *C. albicans* IMH3 protein also displays a high degree of amino acid identity to other eukaryotic (e.g., human IMP dehydrogenase 2 [64.7%]), prokaryotic (e.g., *E. coli* [41.1%]), and archaeal (e.g., *Pyrococcus furiosus* [33.9%]) IMP dehydrogenases. The vertebrate (10, 39, 50) and invertebrate (46) IMP dehydrogenases are more similar to one another than to fungal, protozoan (5, 54, 55), plant (12), bacterial (2, 3, 16, 31, 36, 47, 49) and archaeal (11) IMP dehydrogenases. In general, phylogenetic groupings seem to be well represented.

Analysis of the putative *C. albicans* IMP dehydrogenase amino acid sequence with the MOTIFS program of the Genetics Computer Group Sequence Analysis Software Package (19) in the PROSITE Dictionary of Protein Sites and Patterns (Amos Bairoch, University of Geneva, Geneva, Switzerland) revealed an amino acid consensus pattern for IMP dehydrogenase and GMP reductase: (L, I, V, M)(R, K)(L, I, V, M)G (L, I, V, M)GXGS(L, I, V, M)CXT. The signature sequence for IMP dehydrogenase (LRIGMGSGSICIT) is located at amino acid positions 323 to 335 (Fig. 1) in the *C. albicans IMH3* product. The amino acid alignment showed that the signature sequences of the IMP dehydrogenases of other organisms are similarly placed (Fig. 2).

Expression of *IMH3*. Northern hybridization with total RNA isolated from cells grown under conditions favoring either yeast or hyphal growth (see Materials and Methods) was used to assess the levels of *IMH3* mRNA during growth and morphogenesis. Equivalent amounts of RNA obtained from yeast forms grown in Lee medium at pH 4.5 and 25°C were compared with RNA obtained from hyphae grown in Lee medium at pH 6.8 and 37°C. RNA isolated from cells grown at pH 6.8 and 4.5 and at 25 and 37°C, respectively, were included as controls. All samples were hybridized with the 2.9-kb *Xba*I

FIG. 1. Nucleotide and predicted amino acid sequences of the *C. albicans IMH3* gene. The sequence of the single intron is in lowercase letters, and its 5′ splice site (gtatgt), the branchpoint sequence (tactaac), and the 3′ splice site (tag) are in boldface. The amino acid sequence is shown with the IMP dehydrogenase signature sequence (double underlined) and the TAA stop codon (asterisk). The 5′ noncoding region includes TATA elements (underlined), the CT-rich sequence (dotted line), and the 25-mer oligonucleotide primer (underlined in italics) used in primer extension to map the transcription start sites (arrows). In the 3′ flanking region, putative termination signals are underlined.

```
          1                                                                                                  90
CaIMH3p   ...MVFETSK ATSYLKDYPK KDGLSVKELI DSTNFGGLTY NDFLILPGLI NFPSSAVSLE TKLTKKITLK SPFVSSPMDT VTEENMAIHM
ScIMH3p   MSAAPLDYKK ALEHLKTYSS KDGLSVQELM DSTTRGGLTY NDFLVLPGLV NFPSSAVSLQ TKLTKKITLN TPFVSSPMDT VTEADMAIYM
 Human2   ..MADYLISG GTSYVPD... .DGLTAQQLF NCG..DGLTY NDFLILPGYI DFTADQVDLT SALTKKITLK TPLVSSPMDT VTEAGMAIAM
  Leido   .....MATNN ANYRIKTI.. KDGCTAEELF RG...DGLTY NDFIILPGFI DFGAADVNIS GQFTKRIRLH IPIVSSPMDT ITENEMAKTM
  Arath   .......... ......MSTL EDGFPADKLF AQGY..SYTY DDVIFLPHFI DFSTDAVSLS TRLSRRVPLS IPCVSSPMDT VSESHMAAAM
  Ecoli   .......... .......... .........M LRIAKEALTF DDVLLVPAHS TVLPNTADLS TQLTKTIRLN IPMLSAAMDT VTEARLAIAL
  Pyrfu   .......... .......... ...MGKFVEK LENAIRGYTF DDVLLIPQPT EVEPKDVDVS TQITPNVKLN IPILSAAMDT VTEWEMAVAM
   Cons   ---------- ---------- ---------- --------T- -D----P--- ---------- --------L- -P--S--MDT --E---A---

          91                                                  *                                          180
CaIMH3p   ALLGGIGIIH HNCTSEEQAE MVRKVKKYEN GFINDPVVIS PEVTVGEVKK MGEVLGFTSF PVTE..NGKV GGKLVGIITS RDIQFH..ED
ScIMH3p   ALLGGIGFIH HNCTPKEQAS MVKKVKMFEN GFINSPIVIS PTTTVGEVKV MKRKFGFSGF PVTE..DGKC PGKLVGLVTS RDIQFL..ED
 Human2   ALTGGIGFIH HNCTPEFQAN EVRKVKKYEQ GFITDPVVLS PKDRVRDVFE AKARHGFCGI PITD..TGRM GSRLVGIISS RDIDFLKEEE
  Leido   ALMGGVGVLH NNCTVERQVE MVKSVKAYRN GFISKPKSVP PNTPISNIIR IKEEKGISGI LVTE..NGDP HGKLLGIVCT KDIDYVKNKD
  Arath   ASLGGIGIVH YNCGIAAQAS IIRQAKSLKH PIASDAGVKF PEYEITSL.. ..DAFGPSSF VFVEQTGTMT TPKLLGYVTK SQWKRMNYEQ
  Ecoli   AQEGGIGFIH KNMSIERQAE EVRRVKKHES GVVTDPQTVL PTTTLREVKE LTERNGFAGY PVVTEEN... ..ELVGIITG RDVRFVTDLN
  Pyrfu   AREGGLGVIH RNMSIEEQVE QVKRVKRAER FIVEDVITIA PDETIDYALF LMEKHGIDGL PVV.EED... ..RVVGIITK KDI..AAREG
   Cons   A--GG-G--H -N-----Q-- -----K---- ---------- P--------- -----G---- ---------- -----G---- ----------

          181                                                                                                270
CaIMH3p   NKSPVSEVMT K..DLV.VGK KGISLTDGNE LLRSSKKGKL PIVDAEGNLV SLISRTDLQK NQDYPN..AS KSFHSKQLLC GAAIGTIDAD
ScIMH3p   DSLVVSEVMT K..NPV.TGI KGITLKEGNE ILKQTKKGKL LIVDDNGNLV SMLSRADLMK NQNYPL..AS KSATTKQLLC GAAIGTIEAD
 Human2   HDCFLEEIMT KREDLV.VAP AGITLKEANE ILQRSKKGKL PIVNEDDELV AIIARTDLKK NRDYPL..AS KDA.KKQLLC GAAIGTHEDD
  Leido   ..TPVSAVMT RREKMT.VER APIQLEEAMD VLNRSRYGYL PIVNENDEVV NLCSRRDAVR ARDYPH...S TLDKSGRLIC AAATSTRPED
  Arath   REMKIYDYMK SCDSSDYCVP WEIDFEKLEF VLEDKQKG.F VVLERDGETV NVVTKDDIQR VKGYPKSGPG TVGPDGEWMV GAAIGTRESD
  Ecoli   Q..PVSVYMT PKERLVTV.R EGEAREVVLA KMHEKRVEKA LVVDDEFHLI GMITVKDFQK AEAKPN...A CKDEQGRLRV GAAVGAGAGN
  Pyrfu   R..TVKELMT RE..VITV.P ESVDVEEALK IMMENRIDRL PVVNEDGKLV GLITMSDLVA RKKYKN...A VRNEKGELLV AAAV..SPFD
   Cons   --------M- ---------- ---------- ---------- ---------- ------D--- ---------- ---------- -AA-------

          271                                                                                                360
CaIMH3p   RERLDKLVEA GLDVVVLDSS NGSSVFQLNM IKWIKEKYPE LQVIAGNVVT REQAALLIEA GADA**LRIGMG SGSICIT**QEV MACGRPQGTA
ScIMH3p   KERLRLLVEA GLDVVILDSS QGNSVFQLNM IKWIKETFPD LEIIAGNVAT REQAANLIAA GADG**LRIGMG SGSICIT**QEV MACGRPQGTA
 Human2   KYRLDLLAQA GVDVVVLDSS QGNSIFQINM IKYIKDKYPN LQVIGGNVVT AAQAKNLIDA GVDA**LRVGMG SGSICIT**QEV LACGRPQATA
  Leido   KRRVAALADV GVDVLVLDSS QGNTIYQIAF IKWVKSTYPH LEVVAGNVVT QDQAKNLIDA GADG**IRIGMG SGSICIT**QEV LACGRPQGTA
  Arath   KERLEHLVNV GVNAVVLDSS QGNSIYQLEM IKYVKKTYPE LDVIGGNVVT MYQAQNLIQA GVDG**LRVGMG SGSICTT**QEV CAVGRGQATA
  Ecoli   EERVDALVAA GVDVLLIDSS HGHSEGVLQR IRETRAKYPD LQIIGGNVAT AAGARALAEA GCSA**VKVGIG PGSICTT**RIV TGVGVPQITA
  Pyrfu   LRRAIELDRA GVDVIVVDTA HAHNLKAIKA MKEMRQKVSA .DFIVGNIAN PKAVDDLTFA D..A**VKVGIG PGSICTT**RIV AGVGVPQITA
   Cons   --R---L--- G------D-- ---------- ---------- -----GN--- ------L--A -------**G-G -GSIC-T**--V ---G--Q-TA

          361                                                                                                450
CaIMH3p   VYGVTEFANK FGVPCIADGG IGNIGHITKA LALGASCVMM GGLLAGTAET PGDYFYRDGK RLKTYRGMGS IDAMQQTNTN ANASTSRYFS
ScIMH3p   VYNVCQFANQ FGVPCMADGG VQNIGHITKA LALGSSTVMM GGMLAGTTES PGEYFYKDGK RLKAYRGMGS IDAMQKTGNK GNASTSRYFS
 Human2   VYKVSEYARR FGVPVIADGG IQNVGHIAKA LALGASTVMM GSLLAATTEA PGEYFFSDGI RLKKYRGMGS LDAMDKHLSS QN....RYFS
  Leido   VYKVAQYCAS RGVPCTADGG LRQVGDICKA LAIGANCAML GGMLSGTTET PGEYFFKGGV RLKVYRGMGS LEAM....SQ GKESGKRYLS
  Arath   VYKVCSIAAQ SGIPVIADGG ISNSGHIVKA LVLGASTVMM GSFLAGSTEA PGGYEYTNGK RIKKYRGMGS LEAM..... TKGSDQRYLG
  Ecoli   VADAVEALEG TGIPVIADGG IRFSGDIAKA IAAGASAVMV GSMLAGTEES PGEIELYQGR SYKSYRGMGS LGAM..... SKGSSDRYFQ
  Pyrfu   IAMVADRAQE YGLYVIADGG IKYSGDIVKA IAAGADAVML GNLLAGTKEA PGKEVIINGR KYKQYRGMGS LGAM..... MKGGAERYYQ
   Cons   ---------- -G----ADGG ----G-I-KA ---G----M- G--L----E- PG------G- --K-YRGMGS --AM------ ------RY--

          451                                                                                                540
CaIMH3p   E...ADKVLV AQGVSGSVVD KGSITKFVPY LYNGLQHSLQ DIGIKSIDEL RENVDNGEIR FEFRTASAQF EGGVHGLHSY EKRLHN....
ScIMH3p   E...SDSVLV AQGVSGAVVD KGSIKKFIPY LYNGLQHSCQ DIGCESLTSL KENVQNGEVR FEFRTASAQL EGGVHNLHSY EKRLYN....
 Human2   E...ADKIKV AQGVSGAVQD KGSIHKFVPY LIAGIQHSCQ DIGAKSLTQV RAMMYSGELK FEKRTSSAQV EGGVHSLHSY EKRLF.....
  Leido   E...NEAVQV AQGVSGNVVD KGSAAKLIAY VSKGLQQSAQ DIGEISFDAI REKMYAGQVL FSRRSPTAQG EGGVHSLHSY EKKLFAAKM.
  Arath   D...QTKLKI AQGVVGAVAD KGSVLKLIPY TMHAVKQGFQ DLGASSLQSA HGLLRSNILR LEARTGAAQV EGGVHGLVSY EKKSF.....
  Ecoli   SDN.AADKLV PEGIEGRVAY KGRLKEIIHQ QMGGLRSCMG LTGCGTIDEL RTKAE..... FVRISGAGIQ ESHVHDVTIT KESPNYRLGS
  Pyrfu   GGYMKTRKFV PEGVEGVVPY RGTVSEVLYQ LVGGLKAGMG YVGARNIKEL KEKGE..... FVIITSAGLR ESHPHDIIIT NEAPNYPLER
   Cons   ---------- --G--G-V-- -G-------- ---------- --G-------- --------- ---------- E---H----- ----------
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FIG. 2. Alignment of the deduced amino acid sequences of the *C. albicans IMH3* gene (CaIMH3p) with the amino acid sequence of an IMP dehydrogenase of *S. cerevisiae* (ScIMH3p; Swiss-Prot accession number P50094) (14), *Homo sapiens* (Human2; P12268) (39), *L. donovani* (Leido; P21620) (54), *Arabidopsis thaliana* (Arath; P47996) (12), *E. coli* (Ecoli; P06981) (49), and *P. furiosus* (Pyrfu; P42851) (11). The predicted amino acid sequences were aligned by the PILEUP program of the Wisconsin Package, version 8, using default parameters (19). The consensus sequence (Cons) created by the PRETTY program (19) shows the amino acid residues conserved in all sequences. The IMP dehydrogenase signature pattern (see text) is in boldface in the sequences. The location of the last coded amino acid 5′ of the intron in *C. albicans IMH3* and *S. cerevisiae IMH3* is indicated by an asterisk.

DNA fragment containing the *IMH3* coding region (Fig. 3), and in each a transcript of approximately 1.8 kb was detected. The size of this mRNA suggests a relatively short 3′ untranslated region, considering 1,563 bases of coding sequence and 110 bases of 5′ untranslated region, and is consistent with the removal of the 248-bp intron by RNA splicing. Hybridization with actin mRNA was used as an internal control of mRNA levels (for details, see reference 52). The relative signals obtained from yeast and hyphal forms are essentially equivalent (Fig. 3, first and last lanes), indicating that the levels of *IMH3* mRNA do not differ markedly in yeast and hyphal forms. The equivalence of *IMH3* mRNA levels throughout morphogenesis further supported the potential utility of employing *IMH3* as a dominant selectable marker in *C. albicans.*

Expression of *IMH3* on a plasmid. MPA is synthesized by *Penicillium* spp. (8). This compound specifically inhibits the IMP dehydrogenases of several organisms, including *C. albicans*, where growth inhibition occurs at relatively low concentrations of MPA (1 μg/ml) (18, 40). In a microdilution assay, we found strain-specific differences in the sensitivity to MPA.

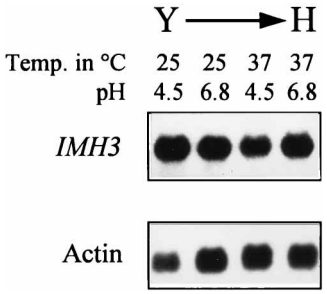

FIG. 3. Expression of the *IMH3* gene in yeast and hyphal forms of *C. albicans*. Total RNA was prepared from cells grown in Lee medium (33) under conditions favoring the yeast-to-hyphal transition as indicated by Y→H. At 25°C and pH 4.5, cells grow exclusively as yeast, whereas at 25°C and pH 6.7, 37°C and pH 4.5, and 37°C and pH 6.7, cells increasingly form germ tubes and hyphae. An oligonucleotide specific to *C. albicans* actin was used to indicate mRNA levels (52).

A concentration of 5 μg of MPA per ml yielded more than 90% growth inhibition in *C. albicans* WO-1 (white and opaque forms were equally sensitive) (Fig. 4A) and CaI4 (Fig. 4B), while strains SS and 3153a were somewhat more resistant (Fig. 4A), showing more than 90% inhibition at 10 μg of MPA per ml. Coincidentally, strains 3153a and SS also differ from CaI4 and WO-1 in their banding patterns in Southern analysis (see above).

In the majority of systems investigated, the levels of resistance to MPA are elevated as the gene dosage of IMP dehydrogenase increases (9, 20, 23, 25, 54). To determine the effects of increased gene dosage and/or overexpression of *IMH3* on the sensitivity of *C. albicans* to MPA, we constructed the plasmid pGKIII by inserting the entire *IMH3* gene and flanking sequences into the replicative plasmid pRC2312 (7) as described in Materials and Methods.

C. albicans CaI4 (17) was transformed with the parent plasmid pRC2312 and with pGKIII. Transformants containing either plasmid were selected by plating on Ura$^-$ medium. Resistance to MPA was subsequently tested by plating Ura$^+$ transformants on medium containing MPA at concentrations of from 0 to 10 μg/ml. Transformants containing the pGKIII plasmid grew on medium containing up to 10 μg of MPA per ml, the highest concentration tested, whereas cells transformed with the parent plasmid pRC2312 remained sensitive to 1 μg of MPA per ml. Further MPA susceptibility testing of replicative transformants in a microtiter plate assay revealed that pGKIII transformants still grow considerably at 40 μg of MPA per ml (Fig. 4B), suggesting that resistance is probably due to overexpression of *IMH3*. Overexpression was confirmed by Northern analysis and SDS-PAGE of whole-cell protein extracts of pGKIII transformants (Fig. 5). *IMH3* mRNA levels are highly elevated in cells harboring pGKIII during Ura selection and further increase during MPA selection. On the protein level, a predominant band at approximately 56 kDa could be detected in extracts of cells grown during MPA selection. The molecular size of this protein correlates well with the calculated molecular weight of the *IMH3* gene product.

As originally reported by Cannon et al. for pRC2312 (7), transformation with either pRC2312 or pGKIII resulted in small, slow-growing colonies at a much higher frequency than large, fast-growing colonies. Slow- and fast-growing pGKIII transformants were further characterized by Southern hybridization with the cloned *IMH3* gene as a probe. As reported previously (7), small clones contained an autonomously replicating plasmid, whereas genomic integration in multiple copies resulted in the larger, fast-growing colonies. Ura$^+$ pGKIII transformants (large and small) were grown for 48 h in synthetic medium containing 100 μg of uridine per ml with either 0 or 5 μg of MPA per ml. Undigested and *Hin*dIII-digested total DNA isolated from each culture were compared by Southern hybridization as shown in Fig. 6. Analysis of either class of transformants showed that the plasmid is retained in the presence of MPA, while in the absence of MPA selection the autonomously replicating plasmid was lost; its integrated form, however, remained stable.

Direct selection of transformants with MPA. The results of the experiments described above suggested the use of *IMH3* as a dominant selection marker in transformation of *C. albicans*. Consequently, equal amounts of the cell suspensions after electroporation with pRC2312 or pGKIII were plated on MPA selection medium and Ura selection medium. Transformation efficiencies with Ura selection for both plasmids were in the range of 1.0×10^2 to 5.0×10^2 CFU/μg of DNA, depending on the electroporation parameters (see Materials and Methods)

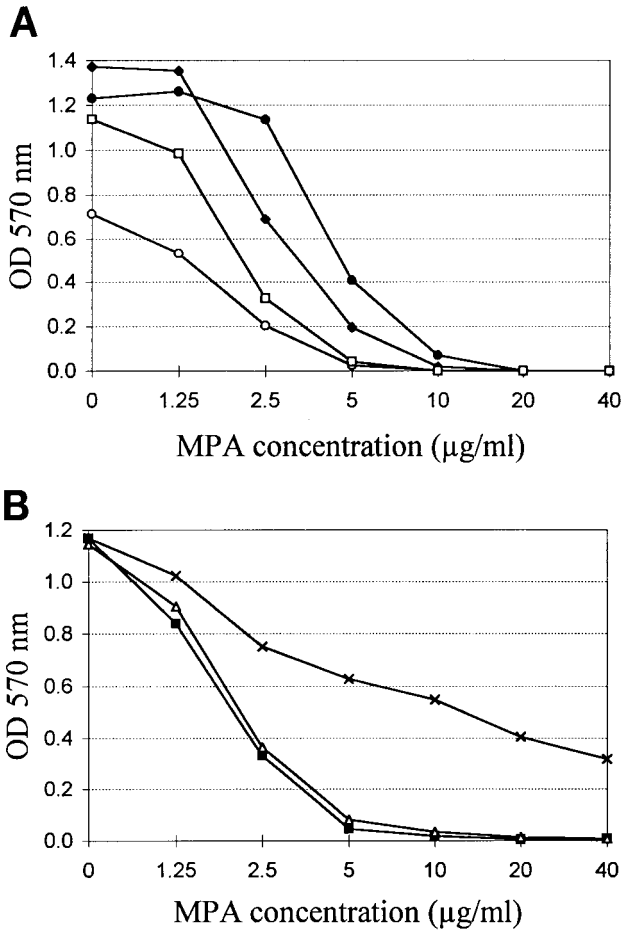

FIG. 4. Sensitivity of *C. albicans* wild-type cells and transformants to MPA. (A) Susceptibility of the prototrophic *C. albicans* strains 3153a, SS, and WO-1 (O, opaque phenotype; W, white phenotype) to MPA in the microtiter plate assay described in Materials and Methods. Assays were performed in duplicate with strains 3153a (◆), SS (●), and WO-1 opaque (○) and white (□) grown at 25°C in synthetic medium containing 100 μg of uridine per ml and the indicated MPA concentrations. The optical density at 570 nm (OD 570 nm) was determined after 48 h of growth at 25°C, and the means were plotted against their corresponding antibiotic concentrations. (B) Susceptibility of *C. albicans* CaI4 (■) and replicative transformants hosting pRC2312 (△) and pGKIII (×) to MPA as determined with the microtiter plate assay.

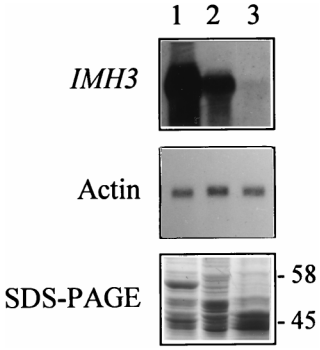

FIG. 5. Overexpression of the *C. albicans IMH3* gene. The Northern analysis shows *IMH3* overexpression in pGKIII transformants of CaI4 during MPA selection with 10 μg of mycophenolic acid per ml (lane 1) and Ura selection in uridine-deficient medium (lane 2) compared to the low-level expression in the auxotrophic strain CaI4 (lane 3). An actin-specific probe was used to indicate mRNA levels (see Fig. 3). SDS-PAGE of whole-cell extracts further reveals that MPA-selected pGKIII transformants produce very high levels of the 56-kDa *IMH3* protein. Total RNA and protein were isolated as described in Materials and Methods. Numbers on the right refer to polypeptide molecular mass markers (in kilodaltons).

used. With MPA selection following electroporation with pRC2312, no transformants could be selected above the background level of spontaneous MPA resistance ($\sim 10^{-7}$). After electroporation with pGKIII, however, transformants could be selected on MPA-containing medium. The efficiency was lower, but initial plating with a relatively low MPA concentration of 3.5 to 5 μg/ml and prolonged incubation periods (4 to 8 days) enabled us to reach transformation efficiencies of up to 75% of those with regular Ura selection. Following this selection, transformants were maintained on defined medium containing 10 μg of MPA per ml. By standard methods, plasmids from MPA-selected *C. albicans* transformants could be reisolated and retransformed into *E. coli*. Using the CaI4 electroporation protocol, we were also able to introduce pGKIII into the wild-type *C. albicans* strains SS and WO-1. The transformation efficiency, however, was lower than that with CaI4.

DISCUSSION

The *C. albicans* gene cloned in this study has been designated an IMP dehydrogenase based on its phylogenetic similarity with other IMP dehydrogenases, the presence of well-conserved sequence motifs within the gene, such as the IMP dehydrogenase signature sequence, and the ability of the 2.9-kb DNA fragment to confer resistance to the specific IMP dehydrogenase inhibitor MPA. Its further designation as the *C. albicans* cognate of *S. cerevisiae IMH3* (rather than *IMH1* or *IMH2*) is based not only on its similarity in amino acid and DNA sequences but also on the presence and position of an intervening sequence in its gene structure.

Few interrupted genes are known to exist in *C. albicans*. They include the gene which encodes actin (*ACT*) (34), the genes for β-tubulin (48), the calmodulin gene (44), and the peptide transport gene *PTR2* (4). The 248-bp intron found in the *C. albicans IMH3* gene has the hallmarks of *S. cerevisiae* introns as characterized by Kalogeropoulos (30). In the intron-containing genes in *S. cerevisiae*, there is usually only one, relatively small (<550-bp), intron per gene, with a conserved 5′ splice site (GTAYGT), a conserved 3′ splice site (YAG), and an almost invariant, and for correct splicing essential, branch-point sequence (TACTAAC). The *IMH3* intron in *C. albicans* fulfills all of these criteria (Fig. 1), thereby indicating the close phylogenetic relationship of these fungi and the functionality of the intervening sequence.

Expression of the pGKIII plasmid and its maintenance in the presence of MPA is consistent with presence of transcription initiation regions within the cloned sequence. 5′ of the putative translation start site at the ATG in nucleic acid position 1 (Fig. 1), there are several potential TATA-like elements which may serve as promoters. The spacing between the closest TATA box and the experimentally determined transcription start site of *IMH3* is longer than the 40 to 120 bp found in the regulatory regions of most genes of the related yeast *S. cerevisiae* (35). However, nothing is known about spacing and other sequence requirements for TATA boxes in *C. albicans*. An interesting feature of the transcription initiation region of *IMH3* is the CT-rich stretch of nucleotides in immediate proximity to the transcription start sites. A similar pyrimidine-rich sequence was found by Mason et al. (37) in the initiation region of the highly expressed enolase gene of *C. albicans*. CT regions near start sites are indicative of strong promoters in *S. cerevisiae* (15) and appear to increase fidelity of transcription initiation in highly expressed genes (38). Our knowledge of the signals for transcription termination and polyadenylation in *C. albicans* is scarce, and *S. cerevisiae* again serves as a model system. Formation of correct 3′ ends of mRNAs in this yeast depends on several sequence elements (22), some of which can be found in the 3′ flanking region of *IMH3* (Fig. 1).

According to the expression analysis by Northern blot hybridization of total RNA isolated from cells grown under conditions favoring either the yeast or the hyphal form of *C. albicans*, *IMH3* is expressed in approximately equal levels in both forms, as one would expect for a gene with a housekeeping function. The extent to which IMP dehydrogenase may be

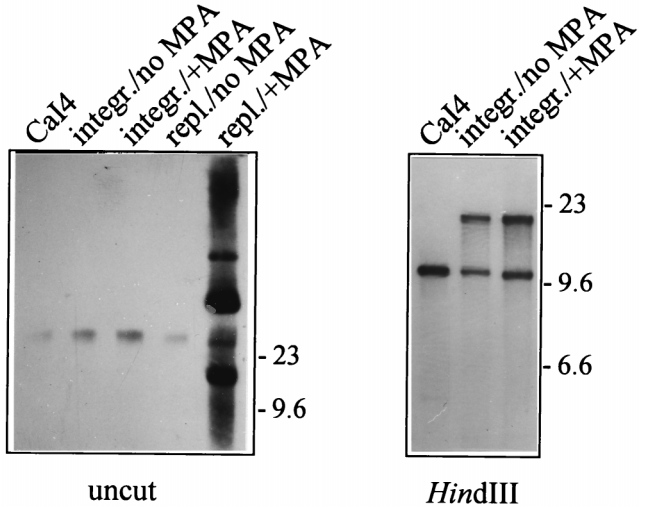

FIG. 6. Southern analysis of integrative and replicative pGKIII transformants probed with the *IMH3* gene sequence. (Left) Southern hybridization of uncut total DNA from *C. albicans* CaI4 as well as an integrative (integ.) and a replicative (repl.) pGKIII transformant with and without MPA selection. Only the replicative transformant grown under selective pressure by MPA reveals hybridization to the topoisomeric (closed and nicked circular) forms of free plasmid. The high-molecular-weight components are probably due to plasmid multimers (32). (Right) *Hin*dIII digest of total DNA isolated from *C. albicans* CaI4 and the integrative transformant with and without MPA selection. CaI4 shows only one band, whereas an additional band is visible in the hybridization pattern of the transformants. Even without selective pressure, the transformed *IMH3* gene(s) remains stably integrated. The cells were grown in liquid synthetic medium supplemented with 100 μg of uridine per ml to eliminate Ura selection with the indicated concentrations of MPA (0 or 5 μg/ml). Size markers are in kilobase pairs.

important for virulence and survival of *C. albicans* in the host during commensal or noncommensal infection is unknown. However, the availability of guanosine nucleotide precursors, which could be used in the salvage pathway for nucleotide biosynthesis bypassing the XMP oxidation by IMP dehydrogenase (18, 29), may not be optimal for growth in the various host environments. For example, the purine level in blood is so low that blood-borne pathogens depend on de novo purine synthesis for survival (36). Strain-specific diversity in levels of susceptibility to MPA (Fig. 4A) might be an indicator for differences in expression or activity of this essential enzyme and might have an influence on virulence characteristics in disseminated infection.

Specific inhibitors of IMP dehydrogenase, e.g., MPA, have been of therapeutic importance as drugs with antiviral and antitumor activities (53) and have been discussed as potential chemotherapeutic agents for protozoan infections (5). Resistance to MPA in some kinetoplastid protozoans involves interesting mechanisms of chromosomal and extrachromosomal IMP dehydrogenase gene amplification (56, 57). The increase in gene dosage renders kinetoplastids like *Trypanosoma brucei* and *Leishmania donovani* resistant to elevated levels of MPA. The sensitivity of *C. albicans* to a specific inhibitor of IMP dehydrogenase like MPA (18, 21, 40) (Fig. 4A) not only shows that the enzyme is essential but also suggests that this enzyme-inhibitor system could be employed in molecular biological experiments. *C. albicans*, as a diploid, anamorphic fungus with no known teleomorph or haploid phase (45), is not readily accessible to the powerful molecular genetic techniques routinely used with *S. cerevisiae*. Auxotrophic mutants are much rarer and more tedious to generate in *C. albicans* than in *S. cerevisiae*, so the development of dominant markers similar to those used in bacterial or mammalian cells would be of great advantage in adapting molecular genetic techniques to *C. albicans*. We showed in our experiments that increasing the gene dosage by introducing plasmids carrying the *IMH3* coding region renders the recipient *C. albicans* cells resistant to elevated concentrations of MPA due to overexpression of the drug target enzyme (Fig. 5). The plasmids can clearly be stabilized by selection with MPA (Fig. 6), and it is possible to use *IMH3* as a dominant marker in spite of the weakened recovery of transformants compared to that with the Ura selection in auxotrophic strains. The lowered transformation efficiency is probably caused by the fact that not all transformants are able to establish the plasmid copy number necessary for sufficient overexpression of *IMH3* to confer MPA resistance. We are currently working on improving the selection system with MPA by generating mutagenized MPA-resistant isoforms of IMP dehydrogenase that will allow selection independent of overexpression.

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