

Identification of a *Cryptococcus neoformans* Gene That Directs Expression of the Cryptic *Saccharomyces cerevisiae* Mannitol Dehydrogenase Gene

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The *Mtl* gene from *Cryptococcus neoformans*, which confers the ability of *Saccharomyces cerevisiae* *Sc41 YJO* to grow on mannitol with substantial NAD-dependent mannitol dehydrogenase activity, was identified. Purifications and characterizations of this enzyme show that it is found in polyploid strain BB1, and the peptide sequence of the enzyme helped identify the *Saccharomyces* gene encoding this mannitol dehydrogenase activity. On the other hand, the *Mtl* gene of *C. neoformans* encodes a 346-amino-acid protein which is not mannitol dehydrogenase but a regulatory element which is active in a heterologous fungus.

Cryptococcus neoformans is a significant fungal pathogen in patients with AIDS or cancer and those receiving corticosteroid therapy (13). Despite amphotericin B, flucytosine, and fluconazole treatments, mortality and relapse rates remain high (21). One approach to the development of new antifungal therapies is to identify crucial receptors or products required by the yeast to produce disease. In a search for these virulence factors in *C. neoformans*, several phenotypic characteristics have been identified. These factors include phenoloxidase production, the presence of a capsule, and the ability to grow at 37°C (8, 9, 18). Another potential virulence factor is *C. neoformans* mannitol metabolism. D-Mannitol has been detected in the cerebrospinal fluid of rabbits (27) and in random cerebrospinal fluid specimens from AIDS patients with cryptococcal meningitis (26a). The D-mannitol concentration in the cerebrospinal fluid correlates with the fungal burden of organisms in rabbits and will predict the eventual outcome of infection (27). The consequences of the presence of this solute in or around tissues of the central nervous system remain uncertain, but theoretically, mannitol has the potential to increase tonicity, edema, and intracranial pressure in brain tissue. Increased intracranial pressure has been identified as a poor prognostic complication in cryptococcal meningitis (5). Also, excretion of D-mannitol may aid *C. neoformans* survival in the host by its ability to scavenge hydroxyl radicals. Since phagocytic cells and their oxidative products have been shown to kill *C. neoformans* in vitro (7), the production and excretion of mannitol may contribute to *C. neoformans* survival by interference with oxidative killing mechanisms (3). Recently, a *C. neoformans* mutant hypoproducing mannitol was found to be less virulent than the wild-type parental strain (2). Although this mutant was similar to its parent with respect to all known virulence factors, it was hypersusceptible to osmotic and heat

stresses and also to oxidative killing by normal human neutrophils and cell-free oxidants. On the other hand, mannitol metabolism is not limited to *C. neoformans*, since we have previously studied such metabolism in *Aspergillus* species, including purification of NADP-dependent D-mannitol dehydrogenase activity (11).

To understand the importance of mannitol in pathobiology of *C. neoformans*, it will be necessary to identify and characterize genes that catalyze key steps within the mannitol cycle. We initially identified *C. neoformans* NAD- and NADP-dependent mannitol dehydrogenase catalytic activities. Because of the known ability of *Saccharomyces cerevisiae* to express *C. neoformans* genes (17), it was used as a surrogate to identify a *C. neoformans* mannitol dehydrogenase gene. Haploid strains of *S. cerevisiae* grow well on media containing fructose as a sole carbon source but very poorly on mannitol because of very low levels of mannitol dehydrogenase activity (19). These factors enabled a strategy to isolate and characterize a cryptococcal gene (*Mtl-1*) which possesses the ability to directly increase levels of active mannitol dehydrogenase in *S. cerevisiae*. The isolated gene was initially proposed as encoding cryptococcal mannitol dehydrogenase (12, 16) but was later proven to promote increased levels of endogenous mannitol dehydrogenase in the *S. cerevisiae* strain.

MATERIALS AND METHODS

Enzymes and chemicals. Lysing enzymes (catalog no. L 2265) and β -glucuronidase (catalog no. G 2887) were supplied by Sigma Chemical Company (St. Louis, Mo.). Restriction enzymes and T4 DNA ligase were from New England Biolabs (Beverly, Mass.) and International Biotechnologies, Inc. (New Haven, Conn.). A calf alkaline intestinal phosphatase and oligolabeling kit was from Pharmacia LKB (Piscataway, N.J.). Nytran nylon membranes were obtained from Schleicher and Schuell, Inc. (Keene, N.H.). The radiolabeled nucleoside triphosphates [α -³²P]dCTP and α -³⁵S-dATP were supplied by New England Nuclear (Boston, Mass.). Autoradiography was performed with XAR 5 film (Eastman Kodak Co.). Coenzymes and buffers were obtained from Sigma. Ion-exchange matrices, Q Sepharose and S Sepharose, Fast Flow, and Phenyl Sepharose were from Pharmacia, and Matrex Gel Blue A was from Amicon. All other chemicals were of reagent grade.

Media. Yeast extract-peptone-dextrose broth or agar was used to propagate or store all yeasts. Uracil-negative, mannitol-positive plates contained 0.5% mannitol, which replaced dextrose and all necessary amino acids except uracil. These plates were used for selection of Ura⁺, mannitol-positive *Sc41 YJO* transformants. For enzyme isolation and characterization, *S. cerevisiae* strains were grown to stationary phase at 28°C with shaking in 2.8-liter Fernbach flasks

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TABLE 1. Cytoplasmic polyol dehydrogenase activities in cells grown in yeast extract-peptone with mannitol

Substrate	Activity ($\mu\text{mol}/\text{mg}$ of protein/min) in:											
	<i>C. neoformans</i> H99				<i>Sc41 YJO</i>				<i>Sc41 YJO</i> with <i>Mtl-1</i>			
	NAD	NADP	NADH	NADPH	NAD	NADP	NADH	NADPH	NAD	NADP	NADH	NADPH
Mannitol	0.105	0.154			0.039	0.0056			0.66	0.018		
L-Arabinitol	0.002	<0.001			<0.001	<0.001			<0.001	0.002		
Ribitol	0.021	0.007			<0.001	<0.001			0.024	0.001		
Fructose			0.018	0.050			0.089	0.049			3.27	1.73
Mannose			<0.001	<0.001			ND ^a	ND			0.10	<0.001

^a ND, not done.

containing 1 liter of medium consisting of 2% peptone, 1% yeast extract, 100 mM mannitol, 30 mM $(\text{NH}_4)_2\text{SO}_4$, and 2 mM adenosine.

Strains. *C. neoformans* H99 is a serotype A, α mating type isolate from a human infection. Bacterial strains were *Escherichia coli* K-12 TG-1 and XL1-Blue. *S. cerevisiae Sc41 YJO* (*gal4 gal80 ura3-52 leu2-3 his3 ade2-101 trp1*) was used as the recipient yeast strain. *S. cerevisiae* BB1 from the Bass Research collection was received from David Quain.

***C. neoformans* DNA isolation and purification.** (i) **Genomic.** An overnight culture of H99 was diluted 1:300 in yeast extract-peptone-dextrose broth and grown at 30°C for 16 to 18 h. This cell culture yields approximately 5×10^7 cells per ml. The cells were pelleted, washed three times in 50 ml of 0.5 M NaCl–50 mM EDTA (pH 7.4), resuspended in 10 ml of H_2O containing 5% β -mercaptoethanol, and incubated for 1 h at 37°C with occasional shaking. The cells were then pelleted and resuspended in 5 ml of spheroplasting solution (1 M sorbitol, 0.1 M sodium citrate [pH 5.8], 0.01 M EDTA). Lysing enzymes were added to the cell suspension at a final concentration of 5 mg/ml, and the suspension was incubated for 1 h at 37°C with occasional shaking. Spheroplasts were washed twice and lysed with a solution containing 4% sodium dodecyl sulfate (SDS) and 50 mM EDTA (pH 7.4), and nucleic acids were extracted and purified by standard methods. For cloning procedures, the DNA was further purified with CsCl gradients because of contaminating polysaccharides.

(ii) **Chromosomal.** Chromosomes from serotypes A (H99), B, C, and D were prepared as previously reported (15). Chromosomes were separated by using a contour-clamped homogeneous electric field pulsed-field electrophoresis unit (Bio-Rad, Richmond, Calif.) at settings of 150 V with a 120-s switch for 24 h and then 150 V with a 180-s switch for 18 h.

Library construction. CsCl-purified *C. neoformans* DNA was partially digested with *Hind*III and size fractionated by gel electrophoresis, and the 4- to 20-kb fragments were isolated by electroelution and ligated into Yep352. The genomic library contained 50 to 80,000 recombinants with an average insertion size of 5,000 bp.

Yeast transformation. *Sc41 YJO* was transformed with 50 μg of the *C. neoformans* library by standard spheroplasting techniques with modifications. Aliquots of the transformed spheroplasts were resuspended in molten top agar (1 M sorbitol, 0.67% yeast nitrogen base with appropriate amino acids added, 2% glucose, 3% agar) precooled to 49°C and poured onto prewarmed (37°C) uracil-negative plates to select for transformants. Uracil-positive yeast cells were collected, washed three times in sterile phosphate-buffered saline, and plated on uracil-negative, mannitol-positive plates.

DNA techniques. A *C. neoformans Hind*III fragment (1,800 bp) conferring to *Sc41 YJO* the ability to grow on mannitol was subcloned into vectors pBluescript SK– and SK+ to generate a single-stranded template for DNA sequence determination by the dideoxy-chain termination method of Sanger et al. (22). Analysis of the DNA sequence and comparison of the amino acid sequence with known genes from GenBank and SwissProt were performed by using the Genetics Computer Group Sequence Analysis software package on a Microvax 3600 and a Genetics Computer Group motif search program (6).

Enzyme assays and purification. Two mannitol dehydrogenase assays were used. The standard assay mixture contained 100 mM mannitol and 0.36 mM NAD in 25 mM Na 2-(*N*-cyclohexylamino)ethanesulfonic (CHES) buffer, pH 9. Reactions were initiated with enzyme, and NADH formation was monitored at 340 nm by using a Gilford spectrophotometer. Alternatively, we employed the coupled assay with phenazine methosulfate and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The enzyme activity in the supernatant from 10^8 yeast cells broken by vortexing with glass beads was measured over time at 578 nm after addition of 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 9), 5 mM MTT, 25 mM phenazine methosulfate, 25 mM NAD or NADP, and 50 mM polyol. The results were expressed as micromoles of NAD or NADP utilized per milligram of protein per minute.

Transformed *Sc41 YJO* cells (40 g) were suspended in 250 ml of buffer A (20 mM K phosphate [pH 7.5], 1 mM EDTA, 1 mM thioglycol, 2 mM MgCl_2) and disrupted with a Bead Beater (Biospec Products, Bartlesville, Okla.). Cell debris was removed by centrifugation, and the crude extract was clarified by addition of streptomycin sulfate to 1% and centrifugation at $16,000 \times g$ for 15 min. To this

supernatant fluid was added solid $(\text{NH}_4)_2\text{SO}_4$ to 45% saturation, and the resulting precipitate was removed by centrifugation and discarded. The supernatant fluid was brought to 80% saturation with solid $(\text{NH}_4)_2\text{SO}_4$, and the precipitate, containing mannitol dehydrogenase, was collected by centrifugation, redissolved in 50 ml of buffer A, and buffer exchanged to buffer A on a column of Sephadex G25-300. The pH of the sample was adjusted to 8.0 with KOH, and the sample was applied to a column (2.5 by 10 cm) of Q Sepharose equilibrated with buffer A at pH 8.0. The column was washed extensively with buffer, and mannitol dehydrogenase was eluted isocratically with 50 mM $(\text{NH}_4)_2\text{SO}_4$ in buffer A, pH 6.8. Solid $(\text{NH}_4)_2\text{SO}_4$ was added to 1 M, and the sample was applied to a column (1.6 by 12 cm) of Phenyl Sepharose equilibrated with 1.0 M $(\text{NH}_4)_2\text{SO}_4$ in buffer A, pH 6.8. The column was washed with a linear gradient of 40 ml each of 1.0 M $(\text{NH}_4)_2\text{SO}_4$ and 0.5 M $(\text{NH}_4)_2\text{SO}_4$ in buffer A, pH 7.5. Mannitol dehydrogenase was then eluted isocratically with buffer A containing 0.5 M $(\text{NH}_4)_2\text{SO}_4$ plus 0.2 M mannitol. The pooled active fractions were buffer exchanged to 10 mM histidine HCl, pH 5.5, on Sephadex G25-300. This fraction was applied to a column (1.6 by 5 cm) of Matrex Gel Blue A, which did not bind mannitol dehydrogenase but did remove several contaminating proteins. The preparation, about 75% pure at this point, was applied to a column (1.6 by 10 cm) of S Sepharose equilibrated with the histidine buffer. The column was washed extensively with buffer, and enzyme was eluted with a linear gradient formed from 40 ml each of 10 mM and 150 mM histidine HCl, pH 5.5. This purification was repeated several times, yielding homogeneous enzyme with overall recoveries ranging from 30 to 50%.

Western immunoblotting. Purified mannitol dehydrogenase from transformed *Sc41 YJO* cells was mixed with Freund's complete adjuvant to raise antibodies in rabbits according to standard procedures. Cell lysates and purified enzymes were solubilized in 2% SDS at 95°C for 5 min and separated by SDS-polyacrylamide gel electrophoresis (PAGE) according to the method of Laemmli (10) using a 10% gel (Mini-PROTEAN II; Bio-Rad). The separated components were transferred onto nitrocellulose membrane in a transblot cell (Bio-Rad). The membrane was blocked for 2 h with Tris-buffered saline (TBS; 20 mM Tris, 500 mM NaCl [pH 7.5]) containing 2% skim milk. The membrane was then incubated for 2 h with rabbit anti-mannitol dehydrogenase serum (1:2,000) diluted in TBS. After washings with TBS–0.05% Tween 20, the membrane was incubated for 2 h with goat anti-rabbit immunoglobulin G-alkaline phosphate conjugate (1:2,000) diluted in TBS. Nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate were used as substrates in detection of immunoreactive bands.

Peptide sequence determinations. The partial N-terminal peptide sequence of the mannitol dehydrogenase purified from transformed *Sc41 YJO* cells was determined with an Applied Biosystems 477A protein sequencer. In addition, purified mannitol dehydrogenase protein was digested with endoproteinase LysC, the resulting fragments were separated by reverse-phase high-pressure liquid chromatography (HPLC), and a well-resolved peptide fragment was sequenced with the automated microsequencer.

Nucleotide sequence accession number. The nucleotide sequence of the 1,038-bp intronless open reading frame of the 1,764-bp *Hind*III fragment containing *Mtl-1* has been deposited in GenBank under accession no. U20146.

RESULTS

Polyol dehydrogenases of *C. neoformans*. *C. neoformans* lysates were examined for polyol dehydrogenase catalytic activities. As shown in Table 1, *C. neoformans* H99 contains cytoplasmic NAD- and NADP-linked mannitol dehydrogenase activity which is induced by growth in mannitol. In contrast, *Sc41 YJO* cells produced only small colonies when cultured on 0.5% mannitol plates for 7 to 14 days and contained very low levels of mannitol dehydrogenase activity (Table 1).

Isolation and properties of the *C. neoformans Mtl-1* gene. To clone the *C. neoformans* gene(s) encoding mannitol dehydro-

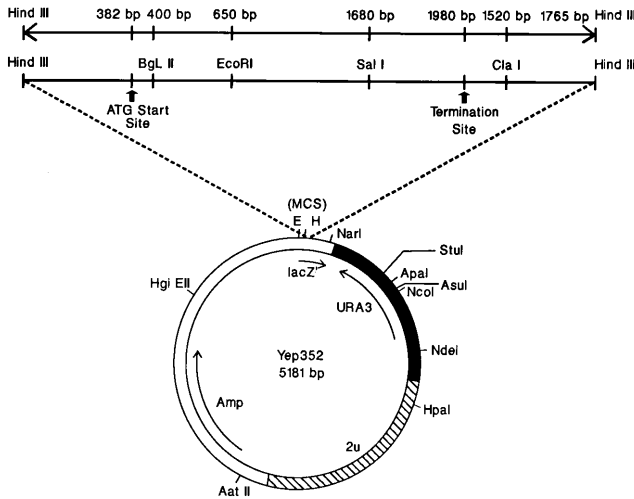


FIG. 1. Plasmid Yep352 containing the 1,764-bp *HindIII* fragment from *C. neoformans*. The open reading frame for the *Mil-1* gene is located along with several relevant restriction endonuclease sites.

genase, *Sc41 YJO* was used to express cryptococcal genes and become mannitol positive. *Sc41 YJO* was transformed with the *C. neoformans* H99 genomic library; approximately 50,000 *Ura⁺* transformants were recovered from *Ura⁻* plates, plated onto the uracil-negative, mannitol-positive plates, and incubated for 72 h. Several colonies appeared on these plates within 48 h and were purified by restreaking. Individual plasmids from mannitol-positive colonies were examined. A plasmid containing a single 1,800-bp *HindIII* fragment was shown to allow *Sc41 YJO* cells to grow vigorously on mannitol plates, and in the presence of this plasmid cells contained abundant NAD-dependent mannitol dehydrogenase activity (Table 1).

Restriction analysis of the fragment was performed (Fig. 1), and subcloning of the different fragments and retransformation demonstrated that functional activity was associated only with the *HindIII* fragment. When an internal *BglIII-SalI* fragment was used to probe *ApaI* and *XhoI* digests of *C. neoformans* genomic DNA, the *Mil-1* gene was found on single 10- and 20-kb fragments, respectively. Cryptococcal chromosomes were separated by pulsed-field electrophoresis and probed with *Mil-1*. *Mil-1* is a single-copy gene which occupies a position on a 1,100-kb chromosome in three strains, i.e., a serotype D strain and two clinical isolates of which one is known to be serotype A. In serotype B and C strains the gene was located on chromosomes significantly different in size, i.e., on a larger one (1,600 kb) and smaller one (650 kb), respectively.

Lastly, when total RNA was extracted from *C. neoformans* cells grown for 24 h in yeast extract-peptone-dextrose with either 5% mannitol or glucose, no transcript was demonstrated by Northern (RNA) hybridization probed with the 1,800-bp *HindIII* fragment.

Nucleotide sequence of the *Mil-1* gene. The nucleotide sequence of the 1,764-bp *HindIII* fragment containing *Mil-1* showed a 1,038-bp intronless open reading frame that encodes a putative 346-amino-acid polypeptide (Fig. 2). A consensus TATA box was present at position -232 from the apparent translation start site, and a CAAT box was found at position -277. There is also a possible transcription initiation site at position -42, GCTCA. The *Mil-1* gene, however, lacks a polyadenylation signal (AATAAA) typical of higher eucaryotes and yeasts.

No significant homologies were found when BLAST or

FAST-A was used to search GenBank for sequences homologous to *Mil-1* or its deduced protein product. However, the Genetics Computer Group motif search program identified three areas within the gene which showed possible ATP and/or GTP binding site motifs with only one mismatch. These motifs have been found commonly in a variety of ATP- and/or GTP-binding proteins. There is also a short amino acid sequence within the coding region which shows homology with only one mismatch to a section of the beta-transducing protein which represents a multigenic family of highly conserved G proteins of about 340 amino acid residues.

Properties of mannitol dehydrogenase from *Mil-1*-transformed *Sc41 YJO*. A series of biochemical and molecular studies to determine if *Mil-1* encodes mannitol dehydrogenase were performed. First, the mannitol dehydrogenase activity from *Mil-1*-transformed *Sc41 YJO* cells was purified (Table 2), and SDS-PAGE analysis indicated that the enzyme's subunit molecular mass was approximately 50 kDa. The specific activity was constant across a protein peak eluting from a Sephadex G-100 column at an elution volume corresponding to 95 kDa. Also, the specific activity and SDS-PAGE pattern were constant across the peak eluting from the S Sepharose column. This preparation gave a single Coomassie Blue-staining band in nondenaturing PAGE, which was coincident with the single band stained for mannitol dehydrogenase activity with phenazine methosulfate and MTT. These results suggested that mannitol dehydrogenase is a dimer of identical approximately 50-kDa subunits, whereas the deduced protein product of *Mil-1* was a 38-kDa protein. Moreover, the isoelectric point of the purified mannitol dehydrogenase was estimated to be 6 to 7 on the basis of ion-exchange chromatography. In contrast, the calculated isoelectric point of the deduced peptide encoded by *Mil-1* was 4.9. Lastly, polyclonal rabbit antibodies directed against mannitol dehydrogenase from *Mil-1*-transformed *Sc41*

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-331  AAGCTTCTTCAGCATCCAGCTTTCAAAAGCCGANGCTCCACCTCCAGGACTTACCAATCTCCCTAGCTGGCATCCGAGATT
-248  AGAGAGGGGCGCTTCATATAAAGACACACAGCATTCACCGGGCTTTCAGCAAGAACAATCCAGCTCCAGGGCAAGGTACTCG
-165  CGCTCGAGGACATCATCAGCATTCAGTCCGATCCCAAACTAGAGCTTAAACCACTCTTTCAGAGGGGGAGCCACAGCATTTG
-82  TTAGGCTCAGATTCGACACACACTTCCGCTTTTGTGGGGCTTCAGCTGTTTGGCTTCCTCCGAGGGTTCACATGGAGAAAC
1  M P S A T F F G G R P N H T G D G L S F G G T P V D
2  ATG CCA AGT TTT GGG AGG CCC AAT CAT ACT GGA GAT CTA TCT TTT GGC GGA ACA CCC GTT GAC
3  S V D N E Q A I G V Y F Q R S Q N K S P S T
4  AGC GTG GAC AAT GAG CAA GCG ATC GGA TAC TTT CAA CGA AGT CAA AAT AAA TCA CCG TCA ACC
5  S F P P N G A G P N I E G F H N P P Q F Q
6  TCA TTC CCC CCG AAT GCA GCA GGC CCC AAT ATC ACA GGA TTC CAT AAT CCA CCG CAG TTT CAA
7  F S S T S V F Q G L G D F M G V H R A P T
8  TTC TCA TCT ACA TCG GTA TTT CAG GCG CTG GGC GAT TTT ATG GGG GFA AGA AGG CCG CCA
9  E N G G L H L H S D A L H Q C A A G E F L T G Q
10 GBA AAT CCG CTT CAT CTG CAT AGC GAT GCA TTA CAT CAG CAA GGT GAA TTC TTG CAT GGA CAA
11 L P Y Q T Q P T G D N A I R A N G G P S T S
12 TTC CTT TAF CAA ACA CAA CCC ACC GGC GAC TCC CCG ATC CBT GCA AAT GGG CCA TCG ACC TCG
13 A M P Q L P V S I A P F Q L P R Y N Q S Q A
14 CTT ATG CCC CAA CTT CCA GTA TCA ATC CCG TTC CAA CCG CCG CCA TAT AAT CAA TCC CAG GGC
15 P R Q N N Y P Y Q G G G N I D P E P L R L R
16 CCG CCG CAA AAT TAT TAT CCA TAC CAA GGC GAC GGC AAT ATC GAC CCG GAA CCC CTG AGC CTC
17 Y S P F Q Q H P N R E S L Q V P L R T Y Q
18 TCA TCA TTT TTT CAA CAA CAC CCC AAT AGA GAA YGS CTT CAA TTA HGA ACA TAT CAA
19 G N Q Q F L L S K Y S D Y H T T A Q A N I
20 GGA AAT CAA CAG TTT TTG CTT TCC AAA TAC TAT AGC GAT TAT CAG ACC CAT CAA CCG AAC ATT
21 H P L H E L Q P Q A H S S Y E P P V P Y T
22 ATC CCT TTG CAG GTG CTG CAG CCT CAA GCA ATG TCA TCT TAT GAG CCA CCA GTC CCC TAT ACC
23 T T S W R M F K A L V A P F D W R F D P T I
24 ACG ACA TCA TGG CCG ATG TTC TAG GCT CTC GTA GCG CCC TTC CAG TGG GAA TTT GAT CTT
25 E P Q R E P R F L D S S T N I R S K R P T
26 GAG CCC CAA AGA GAG TTC CGA TTT CTC GAT TCG TCG AGC AAC ATC CCG TCT AAG CCA CCG ACA
27 L V S S T I N N L D L N V L N G S H D E
28 TTG GTA TCT TCC ACT ATC AAC AAT CTT GAT CTG AAC GTG CTC AAT GGC ACC GAT GAT GAG
29 S L N E A N D Q Y L S F D H F D Q P S F A
30 TCA TCA TAT CAG GCC AAT GAT CAG TAC CTT TCT TTC GAT CAT TTT GAT CAG CCA AGT TTT GCA
31 E Q G M H E E R P S K G A E G Q V G V T E
32 GBA CAG GCG ATG TGG CAA GAA CCG CCA AGT AAA GCG GCA GAG GAA GTT GGA CTC ACA GAA
33 E D N N R L W G Q R *
34 GBA CAG TGG AAT AGA TTA TGG GGG CAA CCA TAA TATATACATGATTTAAACCAATGTGGAGATATGAAC
1081 GTTCTCATGCTCTTFFAATCATCTATCGCTCCGCTACCTACCTAATATGACTCTCTTAATAATACATACATTCGGAGAAAGA
1164 ACTGTTTATCCGACTGATCCAGCTCCGACATCTGGGAAATACAGTACTGTTATAGCCATCTTACATGGAAGGGAACC
1247 ACCACGAAAATTAACCAATAGTGGGAGAGGAAGACTTTTACACTCGATCTGATCTCTTTCCTCCGAGCTCAATAT
1330 TTTCTATGATTTTCCGAAAGGCTCTTGGAGCTCCATGCTCCCTGCTCTCTTATCTTCCGTAACCCCTCGACATAAACATA
1413 TCTCATGCTTATAGCTT
    
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FIG. 2. Nucleotide sequence of *Mil-1*.

TABLE 2. Purification of mannitol dehydrogenase^a

Step	Vol (ml)	Total U	Amt of protein (mg)	Sp act (U/mg)	Purification (fold)	Recovery (%)
Strep. SO ₄ ^b	600	4,700	35,000	0.13	1.0	100
(NH ₄) ₂ SO ₄ -G-25	230	4,600	14,000	0.33	2.5	100
Q Sepharose	85	4,600	2,500	1.8	14	100
Phenyl Sepharose	80	3,600	1,500	2.4	18	75
Matrex Blue	90	3,600	450	8.0	62	75
S Sepharose	38	2,500	230	11	84	50

^a Enzyme was purified from 75 g (wet weight) of *S. cerevisiae* Sc41 YJO transformed with the recombinant vector.

^b Strep. SO₄, streptomycin sulfate.

YJO cells did not react with a *C. neoformans* cell extract (Fig. 3).

Properties of mannitol dehydrogenase from *S. cerevisiae* BB1. Quain and Boulton (19) have reported that some polyploid strains of *S. cerevisiae* can utilize mannitol, and we confirmed that BB1 grew well on mannitol media. Therefore, mannitol dehydrogenase from strain BB1 was purified and its physical and catalytic properties were compared with those of the enzyme from *Mtl-1*-transformed Sc41 YJO cells. The two enzymes' subunit molecular weights were identical, as judged by SDS-PAGE. Cell extracts obtained from *Mtl-1*-transformed Sc41 YJO and BB1 gave similar patterns upon activity staining of nondenaturing polyacrylamide gels. Polyclonal rabbit antibodies to the mannitol dehydrogenase purified from *Mtl-1*-transformed Sc41 YJO also recognized mannitol dehydrogenase purified from strain BB1 (Fig. 3).

In addition, the kinetic properties of the mannitol dehydrogenases from *Mtl-1*-transformed Sc41 YJO cells and from strain BB1 were virtually identical (Table 3). Cleland's equations were used to generate values for V_{max} s and K_m s for NAD and mannitol from data that generated intersecting initial velocity patterns. The two enzyme preparations were inhibited to the same extent by high concentrations of zinc ion, exhibiting 73 and 72% inhibition, respectively, at 100 μ M ZnCl₂. By contrast, the K_i for inhibition of the *Aspergillus parasiticus* mannitol dehydrogenase was 1 μ M. The enzymes exhibited the same thermolability; the half-lives for inactivation at 60°C were 10 and 11 min, respectively, for the enzymes from Sc41 YJO and BB1. The enzymes were inactivated by *N*-ethylmaleimide at the same rate, the half-lives for first-order inactivation by 10 mM *N*-ethylmaleimide (pH 7.5) being 13 and 15 min, respectively. The two enzyme preparations also exhibited the same substrate specificity. With 100 μ M nucleotide, relative rates of reduction were as follows: NAD, 100; nicotinamide, 1; *N*-6-ethenoadenine adenine dinucleotide, 60; acetylpyridine adenine dinucleotide, 20; thionicotinamide adenine dinucleotide, 10; pyridine acryloamide adenine dinucleotide, 5; and NADP, <0.1 (NAD analogs were a generous gift of Bruce Anderson). The two enzymes exhibited absolute specificity for mannitol as the polyol substrate. No activity was found with the following polyols at 100 mM: glycerol, erythritol, ribitol, xylitol, D-arabinitol, D-galactitol, perseitol (D-mannoheptitol), and glucitol.

Peptide sequence of mannitol dehydrogenase. The N-terminal amino acid sequence of mannitol dehydrogenase from *Mtl-1*-transformed Sc41 YJO cells was TKSDETTATSLNAKT LKSFE, and the sequence of the HPLC-purified endoprotease LysC peptide was EFVAQTHSLETVYDQK. Neither of these sequences was present in the deduced protein encoded by *Mtl-1* (Fig. 2). However, the N-terminal sequence was identical to amino acids 2 to 21 in the hypothetical *S. cerevisiae* protein YEIO_YEAST (SwissProt sequence P39941), which is the deduced product of the *S. cerevisiae* YEL070W gene (nucleotides

17112 to 18620 in GenBank sequence U18795). Also, 16 of 16 amino acids in the endoprotease LysC peptide sequence were identical to amino acids 474 to 492 of hypothetical protein YEIO_YEAST when a gap of 1 amino acid was introduced into each sequence. Hypothetical *S. cerevisiae* protein YEIO_YEAST consists of 502 amino acids, its calculated molecular mass is 56,470 Da, and its isoelectric point is 5.78. The functions of theoretical protein YEIO_YEAST are not known, but amino acid residues 232 to 243 conform to the consensus core sequence that defines the mannitol 2-dehydrogenase enzyme family (23) and it possesses a possible NAD-binding domain in its sequence.

DISCUSSION

Our strategy for cloning *Mtl-1* of *C. neoformans* used Sc41 YJO as a surrogate to produce cryptococcal mannitol dehydrogenase and thus isolate the cryptococcal gene for this enzyme. The level of endogenous activity of this enzyme in Sc41 YJO was very low in our assays, and this was reflected in the organism's very poor growth on mannitol plates and low level of enzyme activity. Sc41 YJO containing *Mtl-1* showed an increase in cytoplasmic polyol dehydrogenase activity which was specific for mannitol and fructose. However, *Mtl-1* proved not to encode the cryptococcal mannitol dehydrogenase gene but actually directed the cryptic expression of the *S. cerevisiae* mannitol dehydrogenase in this strain. In fact, during this investigation the putative *S. cerevisiae* gene for mannitol dehydrogenase was identified as an open reading frame with a deduced 502-amino-

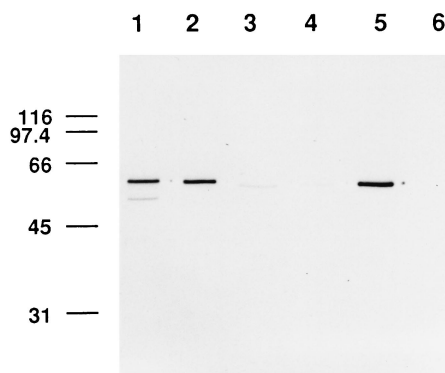


FIG. 3. Immunoblot of mannitol dehydrogenase detected with a 2,000-fold dilution of anti-mannitol dehydrogenase serum. Lane 1, purified mannitol dehydrogenase from transformed Sc41 YJO; lane 2, purified mannitol dehydrogenase from *S. cerevisiae* BB1; lane 3, cell extract from *S. cerevisiae* BB1; lane 4, cell extract from Sc41 YJO; lane 5, cell extract from transformed Sc41 YJO; lane 6, cell extract from *C. neoformans* H99 (amounts of proteins loaded were 100 ng [lanes 1 and 2] and 2 μ g [lanes 3 to 6]).

TABLE 3. Comparison of kinetic properties of mannitol dehydrogenase

Property	Value for:	
	<i>Sc41 YJO</i>	BB1
K_m of mannitol (mM) ^a	2.8	2.1
K_m of NAD (μ M) ^a	17	11
K_m of fructose (mM) ^b	29	33
V_{max} forward (U/mg) ^c	20	9
V_{max} reverse (U/mg) ^d	44	19
V_f/V_r ratio ^e	2.2	2.1
V_{max} at pH 8/ V_{max} at pH 6.5	4.0	4.0
% Inhibition by 100 μ M ZnCl ₂	73	72

^a Measured at pH 9.0.

^b Measured at pH 7.5.

^c Mannitol + NAD \rightarrow fructose + NADH. This observed variation in V_{max} was not greater than those observed between various enzyme preparations from the same strain.

^d Fructose + NADH \rightarrow mannitol + NADH.

^e V_f , V_{max} forward; V_r , V_{max} reverse.

acid protein (YEIO_YEAST) found during the yeast genome sequencing project.

The *S. cerevisiae* mannitol dehydrogenase activity was found to be NAD dependent and required the presence of *Mtl-1* in *Sc41 YJO*. However, it was easily detected in a diploid strain of *S. cerevisiae* (BB1) without *Mtl-1*. D-Mannitol is the only naturally occurring polyol substrate for the *S. cerevisiae* enzyme. This finding is in sharp contrast to the rather broad substrate specificity found for mannitol dehydrogenases from other fungal sources (11, 20). In addition to being specific for mannitol, the *S. cerevisiae* enzyme also exhibits much higher affinity for mannitol than do mannitol dehydrogenases purified from other sources. This high affinity and absolute specificity for D-mannitol make *S. cerevisiae* mannitol dehydrogenase the ideal choice for quantification of mannitol in laboratory or clinical samples, using a method developed with the enzyme purified from a *Lactobacillus* sp. (1). The *S. cerevisiae* enzyme has a limit of sensitivity for mannitol detection as low as 50 μ M in a clinical sample (10a).

These biochemical studies also suggest that *C. neoformans* actually maintains at least two mannitol dehydrogenases with different cofactor dependencies. Support for the biochemical evidence that *C. neoformans* possesses at least two mannitol dehydrogenases has come from the inability to isolate *C. neoformans* mutants which cannot utilize mannitol. Screening greater than 10⁹ yeast colonies irradiated with either UV or gamma rays did not produce a single mannitol-deficient mutant, and a screen of 10⁴ colonies yielded only one hypoproducer (2). These results could be due to the possible essential feature of the locus or could reflect the fact that more than one mannitol dehydrogenase gene exists. Potential importance of possessing two enzymes for the life cycle of this yeast remains speculative. NAD-dependent activity has generally been associated with a catabolic role, while NADP-dependent processes are biosynthetic. For example, *Absidia glauca* and bacteria utilize mannitol or fructose as their main carbon source through the NAD-dependent enzyme (1, 25). On the other hand, *A. parasiticus* and other imperfect fungi utilize glucose to produce mannitol via the mannitol cycle with an NADP-dependent enzyme (11). The determination of the importance of specific mannitol dehydrogenase genes in *C. neoformans* polyol metabolism and pathobiology will await their identification.

Finally, we have isolated a *C. neoformans* gene (*Mtl-1*) which encodes a putative regulatory protein that increases the en-

dogenous mannitol dehydrogenase enzyme activity in *S. cerevisiae*. For a practical application, *Mtl-1* could be used as a dominant selection marker for vectors in *S. cerevisiae* strains such as *Sc41 YJO*. Transformants could be selected by growth on mannitol plates. On the other hand, the mechanism(s) by which the product of this gene regulates mannitol dehydrogenase in *S. cerevisiae* is not clear; it may well represent a direct inducer of the mannitol dehydrogenase gene or a derepressor of a suppressor gene for this enzyme in *Sc41 YJO*. Although there was no significant match for the entire gene in nucleic acid or protein banks, a careful search for various motifs within the predicted amino acid sequence suggested that certain sites within the gene were consistent with motifs found in some G proteins. *Mtl-1* also displayed no apparent intronic structures in a manner similar to that of *C. neoformans Trp-1*, which was also cloned by expression in *S. cerevisiae* (17). On the other hand, recently identified *C. neoformans* genes suggest that this basidiomycete contains frequent intronic structures (4). *Mtl-1* also is a single-copy gene, but the chromosomal location of *Mtl-1* varies in different strains. For example, *C. neoformans* variety *gatti* or serotype B and C contained the gene on different-sized chromosomes compared with serotype A and D strains or *C. neoformans* variety *neoformans*. It has previously been reported that there is significant genomic instability as measured by chromosome polymorphism in strains of *C. neoformans* (24). In fact, most *C. neoformans* strains have unique karyotypes (14). It has been recognized that homologous genes may be located on different-sized chromosomes in different strains, as was found with *Mtl-1* (15, 17). Some of these gene rearrangements are serotype or variety dependent (26), but there are no data to show that gene expression may be affected by this chromosomal rearrangement.

Determination of the importance of *Mtl-1* to *C. neoformans* pathobiology will require the ability to produce site-directed mutations in *Mtl-1* and the identification of the *C. neoformans* gene(s) encoding proteins for mannitol dehydrogenase activity. *Mtl-1* appears to have regulatory activity in *S. cerevisiae*, but it is uncertain whether it has similar regulatory properties in *C. neoformans*. However, *Mtl-1* does appear to be under some regulation, since the *C. neoformans* transcript of this gene was not detected after 24 h of growth in glucose- or mannitol-containing media. Further focus on the mannitol pathway with its known biochemistry and possible importance to virulence in *C. neoformans* has the potential to produce understanding at a molecular level of the effect of polyol metabolism on fungal pathobiology. *Mtl-1* could potentially be an important regulatory gene in this pathway.

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