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Molecular Cloning and Analysis of the *NAG1* cDNA Coding for Glucosamine-6-phosphate Deaminase from *Candida albicans**

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Candida albicans **and other pathogenic** ***Candida*** **species can use** ***N*****-acetylglucosamine as a sole carbon source for growth. GlcNAc induces the enzymes of GlcNAc catabolic pathway; besides, under certain conditions, GlcNAc also induces a change from the yeast to germ tube morphology. Glucosamine-6-phosphate deaminase (EC 5.3.1.10) is the terminal enzyme of the GlcNAc catabolic pathway. We have purified the deaminase from** ***C. albicans*** **and studied its characteristics. The size of the deaminase estimated from SDS-polyacrylamide gel electrophoresis is 28 kDa.** ***N*****-Acetylglucosamine 6-phosphate, an allosteric activator of the** ***Escherichia coli*** **deaminase, has no effect on the activity of the** ***C. albicans*** **enzyme. The deaminase is induced over 100-fold by GlcNAc and its level is about 0.3–0.5% of the proteins in crude extract. Three cDNA clones were obtained from a λgt11 expression library by immunoscreening with deaminase antiserum.** ***C. albicans*** **genomic DNA blot hybridization revealed that the** ***NAG1*** **gene, encoding the glucosamine-6-phosphate deaminase, is present in a single copy. Hybrid-selected translation and immunoprecipitation experiments revealed that the purified deaminase and the protein encoded by the clones were similar in size and in their antigenicity. DNA sequencing revealed that the largest cDNA clone contained the complete open reading frame, which can code for a 27.5-kDa protein. The NH_2-terminal sequence (35 residues) determined from the purified deaminase was identical to the sequence of the deduced protein. The Nag1 protein has about 47% identity with the sequence of the** ***E. coli*** **glucosamine-6-phosphate deaminase. Furthermore, RNA blot hybridization showed that GlcNAc induces the expression of** ***NAG1*** **gene.**

Several cellular functions are controlled by coordinated action of genes which constitute various metabolic pathways. Such genes, especially if they are inducible, attract particular attention. *N*-Acetylglucosamine catabolic pathway is one such example. GlcNAc can support a high growth rate of *Candida albicans*; the cell growth is accompanied by the induction of enzymes of the GlcNAc catabolic pathway. *C. albicans* is a diploid yeast-like fungus, and is being actively studied because of its pathogenicity to humans and other vertebrates. It is an opportunistic pathogen and causes candidiasis of either the harmless superficial-type or the more serious systemic variety.

In *C. albicans*, GlcNAc is transported into cells by GlcNAc permease and then by the sequential action of GlcNAc kinase, GlcNAc-6-phosphate deacetylase, and glucosamine-6-phosphate deaminase, GlcNAc is converted to fructose 6-phosphate (Singh and Datta, 1979a; Shepherd *et al.*, 1980; Gopal *et al.*, 1982; Datta *et al.*, 1989) which is metabolized through the Embden-Meyerhoff pathway. *N*-Acetylmannosamine is converted to GlcNAc presumably by the action of *N*-acetylmannosamine epimerase (Biswas *et al.*, 1979); the GlcNAc that is formed is catabolized by the enzymes of the GlcNAc catabolic pathway. GlcN is converted to GlcN-6-P (Corner *et al.*, 1986), which is the substrate for glucosamine-6-phosphate deaminase. Amino sugars are catabolized by this route at least in the few well studied organisms, namely *Escherichia coli* (Peri *et al.*, 1990; Rogers *et al.*, 1988; White, 1968), *C. albicans* (Singh and Datta, 1979a; Datta *et al.*, 1989) and *Klebsiella pneumoniae* (Vogler and Lengeler, 1989). However, in the bacterium *Pseudomonas fluorescens*, GlcN is catabolized by a different set of enzymes, namely glucose dehydrogenase and glucosaminate dehydratase (Iwamoto and Imanaga, 1991). The end product of this route is glyceraldehyde 3-phosphate, which is further metabolized by the Entner-Doudoroff pathway. It is not clear if this alternate pathway is widespread in occurrence.

Glucosamine-6-phosphate deaminase (EC 5.3.1.10) catalyzes the reversible conversion of GlcN-6-P to Fru-6-P. The forward reaction involves an aldo-keto isomerization coupled with an amination-deamination process (Noltmann, 1972). No cofactors are required for the deaminase activity. In the backward reaction, GlcN-6-P is produced from Fru-6-P and ammonia. However, in *E. coli* (Calcagno *et al.*, 1984) and in *C. albicans*, the backward reaction requires very high substrate concentrations, suggesting that it may not occur under physiological conditions.

Molecular aspects of the GlcNAc catabolic pathway has been reported from the prokaryotes *E. coli* (Peri *et al.*, 1990; Rogers *et al.*, 1988) and *K. pneumoniae* (Vogler and Lengeler, 1989). In this paper, we describe the purification, cDNA cloning, and characterization of glucosamine-6-phosphate deaminase from *C. albicans*, an eukaryote. Studies from this laboratory (Singh and Datta, 1979c) have suggested that nonpathogenic *Candida* species and other yeasts such as *Saccharomyces cerevisiae* cannot utilize GlcNAc. An understanding of the regulation of the pathway might uncover certain important differences between pathogenic and nonpathogenic *Candida* species, which might pave the way for a molecular approach to control candidiasis.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L07558.

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EXPERIMENTAL PROCEDURES

Media and Growth Conditions

C. albicans SC5314 strain (D. R. Kirsch, The Squibb Institute for Medical Research, NJ) was used in this work.

Media—GPK medium consisted of 0.5% glucose, 0.5% peptone, and 0.3% KH_2PO_4. The NPK medium used was 0.5% GlcNAc, 0.5% peptone, and 0.3% KH_2PO_4. The YNB + GlcNAc medium was 0.67% yeast nitrogen base without amino acids (Difco Laboratories, Michigan) plus 0.5% GlcNAc; this medium was used for growth of cells for RNA isolation.

Growth Conditions—A preculture (GPK medium) inoculated with a loopful of cells from a slant was grown for 17 h at 30 °C with shaking at 200 rpm. It was then diluted 100-fold in fresh GPK or NPK media. For Western blotting experiments and RNA isolation, cells were grown at 30 °C to a density of 10^8 cells/ml. For deaminase purification, cells were grown in GPK medium (15 liters) to 10^8 cells/ml in a fermenter (BioEngineering, Switzerland). Cells were harvested using a continuous flow centrifuge (New Brunswick Scientific Co., NJ) and then induced in 3 liters of induction medium (0.5% GlcNAc, 0.3% KH_2PO_4) for 3.5 h at 30 °C with shaking at 200 rpm. The cells were chilled, quickly harvested, and the pellet was stored at −70 °C until use.

Purification of Glucosamine-6-phosphate Deaminase

Buffers—Cell breakage buffer was 50 mM potassium phosphate, pH 7.6, 1 mM each of EDTA, β-mercaptoethanol, and phenylmethylsulfonyl fluoride; TSEM, pH 5.7, at 4 °C, was 27 mM Trizma (Tris base), 15 mM succinic acid, 1 mM EDTA, 1 mM β-mercaptoethanol; and TEM, pH 8.9, at 4 °C, was 20 mM Tris-HCl, 1 mM EDTA, 1 mM β-mercaptoethanol.

Step 1: Crude Extract—GlcNAc-induced *C. albicans* cells (35 g), were suspended in 35 ml of breakage buffer. The cell suspension was distributed into 10–15-ml parts, and about 15 g of glass beads (0.45-mm diameter) were added. Cells were homogenized by vortexing at full speed for 5 min in 1-min cycles with intermittent cooling on ice. The sample was centrifuged at 5,000 × *g* for 10 min. The pellet was then resuspended in another 35 ml of buffer and centrifuged as above. The supernatants were combined and spun at 25,000 × *g* for 20 min.

Step 2: Isoelectric Precipitation—The pH of the supernatant from the above step, pH 6.9 at 0–2 °C, was lowered to pH 4.8 using acetic acid (4 M stock preadjusted to pH 3.5 with KOH) and incubated on ice for 15 min. The precipitated proteins were removed by centrifugation at 20,000 × *g* for 15 min and the supernatant was recovered.

Step 3: Polyethylene Glycol Treatment—To the step 2 supernatant, 11 ml of polyethylene glycol 8000 (Sigma; 50% (w/v) stock in TSEM) was added to a final concentration of 5%, mixed, and incubated for 30 min on ice. The sample was spun at 20,000 × *g* for 15 min. To the supernatant more polyethylene glycol 8000 solution (see above) was added to take the final concentration of polyethylene glycol to 25%, incubated on ice for 30 min, and centrifuged as above. The supernatant was discarded, and the pellet was gently resuspended in TSEM buffer, and the deaminase activity was recovered. After 30 min at room temperature, the sample was clarified by centrifugation at 10,000 × *g* for 10 min. Supernatant was collected, and the pellet was re-extracted with more TSEM and centrifuged as above. The supernatants were combined to give a final volume of 28 ml.

Step 4: CM-cellulose Chromatography—CM-cellulose (fibrous form; Sigma) was pretreated using the precycling procedure, involving alternate incubations in 0.5 M NaOH and 0.5 M HCl, as described (Ion Exchange Cellulose Information booklet No. IL-6; Whatman Biosystems Ltd., United Kingdom). The matrix was rinsed initially with 10 × TSEM for faster equilibration, then resuspended in 1 × TSEM, packed in a column (2.6 × 17 cm), and equilibrated with 500 ml of TSEM. The sample from step 3 was loaded at 15 ml/h and the column was washed with 300 ml of TSEM. The bound deaminase was eluted with a linear salt gradient (0–0.2 M KCl in TSEM; 200 ml each) at a flow rate of 20 ml/h. Five-milliliter fractions were collected and absorbance was checked at 280 nm. Deaminase was assayed in the fractions and those containing activity were pooled; the deaminase eluted at about 75 mM KCl.

Step 5: DEAE-Sepharose Chromatography—The pooled sample from the CM-cellulose step (65 ml) was desalted and the buffer was exchanged by two successive dialysis against 1 liter of TEM for about 8–10 h each. The pH of the dialyzed sample was about 9.0. DEAE-Sepharose CL-6B (Pharmacia) was pre-equilibrated with 10 × TEM, resuspended in 1 × TEM, packed in a column (2.6 × 17-cm), and equilibrated with about 500 ml of TEM. The sample (70 ml) was loaded at 15 ml/h and the column was washed with 400 ml of TEM at 25 ml/h. Deaminase was eluted with a linear salt gradient (0–0.2 M KCl in TEM; 200 ml each) at a flow rate of 15 ml/h, and 5-ml fractions were collected. The fractions were assayed and those containing activity were pooled; the pH was readjusted to 5.6 and chromatographed through a 2-ml CM-cellulose column (see step 4 for details). The bound enzyme was eluted with 0.2 M KCl (in TSEM) and the sample was desalted and concentrated using Centricon-10. Purified deaminase was stored in TSEM at 4 °C without significant loss of activity for about 3–4 months.

Protein Estimation and Gel Electrophoresis—Protein was estimated using Bio-Rad reagent (Bradford, 1976) with bovine γ-globulin as the standard. SDS-polyacrylamide gel electrophoresis was done using the discontinuous buffer system (Laemmli, 1970) but without SDS in the gel as suggested by Chrambach (1985). However, the tank buffer contained 0.1% SDS.

Enzymology

Coupled Assay—Deaminase was assayed by modification of the method described by White and Pasternak (1975). Each assay (1 ml) contained 0.2 mM NADP, 8 units of phosphoglucose isomerase, 3 units of glucose-6-phosphate dehydrogenase, 1 mM glucosamine 6-phosphate, and either 40 mM potassium phosphate, pH 7.6, or 50 mM Tris-HCl, pH 7.6. The reaction was started by adding the enzyme sample and was incubated at 30 °C and absorbance changes at 340 nm were recorded every minute. Potassium phosphate buffer was used in the assays during purification, but studies with purified enzyme revealed that deaminase activity was higher in Tris-HCl buffer.

Colorimetric Assay—This assay was used for enzyme characterization and was performed essentially as reported (Calcagno *et al.*, 1984). The Fru-6-P formed during the reaction was estimated by the resorcinol method (Davis and Gander, 1967), except that the absorbance was measured at 510 nm.

Backward Reaction—The reaction conditions were as reported earlier (Calcagno *et al.*, 1984), and the GlcN-6-P produced was estimated by the method of Levvy and McAllan (1959).

Antibody Production, Affinity Purification, and Western Blotting

Polyclonal antibody against purified deaminase was made in rabbit as per standard procedures (Garvey *et al.*, 1977; Harlow and Lane, 1988). From the crude antiserum, deaminase-specific antibody was obtained by affinity purification essentially as described in Elledge and Davis (1987). Crude antiserum was used at 1:5000 and affinity purified antibody at 1:1000. Alkaline phosphatase-linked anti-rabbit IgG and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Promega) were used for immunodetection.

Isolation of cDNA Clones

RNA Isolation—Total RNA was isolated from 1 g of *C. albicans* cells using the guanidine thiocyanate method (Chomczynski and Sacchi, 1987), as per Ganesan *et al.* (1991) with one modification. Instead of sodium acetate, lithium chloride was used as described by Puissant and Houdebine (1990). Yield of total RNA was about 4 mg/g of cells. Poly(A)$^+$ RNA was purified from total RNA by two passages through oligo(dT)-cellulose as described (Okayama *et al.*, 1987).

cDNA Cloning—Poly(A)$^+$ RNA, from cells grown in YNB + GlcNAc medium (see section on "Media and Growth Conditions"), was used as template for cDNA synthesis using oligo(dT)$_{12-18}$ as primer. A λgt11 cDNA expression library (Young and Davis, 1983) was constructed using a commercial cDNA synthesis and cloning kit (Amersham International). Plaques were screened using deaminase antiserum (1:5000).

Isolation of Plasmid and Phage DNA—Plasmid DNA was isolated by the alkaline lysis method (Birnboim and Doly, 1979) and further purified by polyethylene glycol precipitation (Promega Protocols and Applications Guide). Phage DNA was isolated from liquid lysates (Miller, 1987) as described (Manfioletti and Schneider, 1988). Phage stocks were prepared as follows: phage plaques were resuspended in 0.5 ml of SM buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM magnesium sulfate, and 0.01% gelatin). Ten-microliters of the suspension, appropriately diluted to give about 100 phages/μl, were spotted on top of agar overlay containing *E. coli* Y1090 host cells. After incubation at 42 °C, confluent lysis was obtained. The cleared area termed macroplaque was collected, and resuspended in 1 ml of SM buffer. The macroplaque was titered and used as phage stock. By

this macroplaque technique, several clones could be easily amplified on a single plate to about 2–3 orders of magnitude (10^{10}–10^{11} plaque forming units/ml).

Southern Blotting and Hybridization

Genomic DNA from *C. albicans*, isolated by the method of Holm *et al.* (1986) with some modifications (Ganesan *et al.*, 1991), was a gift from A. Banerjee and K. Ganesan. Two micrograms of DNA were digested to completion with *Bam*HI, *Eco*RI, *Hae*III, *Hin*dIII, *Pst*I, *Ssp*I, and *Xba*I. They were resolved in a 1.2% agarose gel and Southern blotted to GeneScreen Plus membrane using 10 × SSC (1 ×SSC: 0.015 M sodium citrate, 0.15 M NaCl). The *Eco*RI insert (800 bp)[1] from the plasmid pD6 was purified from gel using an electroelution device (IBI-Kodak, USA). The purified fragment was radiolabeled by the random primer method (Feinberg and Vogelstein, 1984) to a specific activity of 6.3×10^8 cpm/μg and used in the Southern or Northern hybridization. The hybridization solution contained 50% formamide, 1 M NaCl, 1% SDS, and 10% dextran sulfate at 42 °C and the probe concentration was 2×10^5 cpm/ml. The blot was washed in 2 × SSC, 1% SDS at 65 °C (GeneScreen Plus membrane protocols, Du Pont-New England Nuclear).

RNA Blot Analysis

Ten micrograms each of total RNA from Glc-grown (GPK medium) and GlcNAc-grown (NPK medium) cells were denatured using glyoxal/dimethyl sulfoxide (McMaster and Carmichael, 1977; Sambrook *et al.*, 1989) and resolved in an agarose gel (Sambrook *et al.*, 1989); RNA was immediately transferred to GeneScreen Plus membrane using a pressure blotting device (Stratagene) and RNA was fixed using a UV cross-linking device (Stratagene). After fixation, the blot was treated with 50 mM NaOH for 30 s and then neutralized by washing in 2 × SSC for 10 min. RNA was visualized on the blot by staining with methylene blue for 20–30 s (Herrin and Schmidt, 1988). The RNA blot was hybridized with the D6 probe as described for Southern hybridization. *C. albicans* actin gene (Losberger and Ernst, 1989) was used as a control probe to verify the RNA levels in the Glc and GlcNAc lanes.

Restriction Mapping, Subcloning, and Sequencing

Insert from the λgt11 clones was recloned into the pTZ18U vector (U. S. Biochemical Corp.) by the shotgun method (Davis *et al.*, 1986). The D6 insert was subcloned by the nested deletion method using exonuclease III (Henikoff, 1987; Hoheisel and Pohl, 1986). Plasmid DNA was sequenced by Sanger's dideoxy chain termination method (Sanger *et al.*, 1977) using the Sequenase 2.0 kit (U. S. Biochemical Corp.) and the following oligonucleotide primers: M13 universal sequencing primer (U. S. Biochemical Corp.), the M13 or the λgt11 reverse sequencing primers (New England Biolabs).

Hybrid Selected Translation and Immunoprecipitation

Hybridization Selection of mRNA—Two micrograms of plasmid DNA were denatured as described for the DNA dot blot procedure (GeneScreen Plus membrane protocols, Du Pont-New England Nuclear) and the DNA was immobilized on GeneScreen Plus membrane. The membrane strips were washed in 2 × SSC and then incubated in 1 ml of sterile water for 1 min in a boiling water bath to remove loosely bound DNA. The membrane strips were prehybridized and then hybridized in 1 ml of hybridization solution (Jagus, 1987) containing 500 μg of total RNA, at 50 °C for 4 h. The strips were recovered and washed in 1 × SSC, 0.5% SDS at 60 °C. Bound RNA was eluted twice by boiling the strips in 0.3 ml of sterile water (containing 30 μg of yeast tRNA), extracted with phenol/chloroform (1:1), and precipitated. The hybrid-selected RNA was translated in 35 μl of rabbit reticulocyte lysate reaction (Promega). [^{35}S]Methionine-labeled products were either directly analyzed or immunoprecipitated. For direct analysis, 1 μl of the reaction mixture (diluted to 10 μl in Laemmli sample buffer) was heated in a boiling water bath for 4 min and resolved by SDS-PAGE. The gel was treated with 1 M sodium salicylate (Chamberlain, 1979), dried, and exposed to a preflashed X-Omat AR film at −70 °C (Bonner, 1987).

Immunoprecipitation—Fifty microliters of affinity purified antibody (routinely used at 1:1000 dilution in immunoblots) and 125 μl of crude preimmune serum were made up to 300 μl in 0.1 M sodium phosphate, pH 8.0. The diluted antibody solutions were added to 40 mg each of Protein A-Sepharose CL-4B (Pharmacia LKB Biotechnology Inc.) that had been hydrated and washed once in 0.1 M sodium phosphate, pH 8.0, and incubated for 3–4 h at room temperature with gentle mixing. The beads were washed and resuspended in 1.5 ml of immunoprecipitation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 0.02% SDS, 5 mM EDTA, and 0.02% aprotinin). Thirty microliters from each translation mixture were processed as described by Anderson and Blobel (1983), and precleared using 120 μl of preimmune beads. The beads were sedimented in a microcentrifuge for 2 min at full speed. To the supernatant, 75 μl of immune beads was added, rotated for 4 h at room temperature, and pelleted in a microcentrifuge for 1 min at 5000 rpm. The beads were washed four times in 1 ml of immunoprecipitation buffer and finally washed once in 1 ml of sterile water. Equal volumes of 2 × Laemmli sample buffer were added to the beads incubated in a boiling water bath for 4 min and the supernatant was electrophoresed in an SDS-polyacrylamide gel. The gel was fluorographed as described under "Hybridization Selection."

RESULTS

Purification and Characterization of Deaminase

Purity and Size Estimate—GlcN-6-P deaminase was purified from crude extract as shown in Table I. The specific activity of the deaminase in the crude extract was 0.07 units/mg protein; after about 485-fold purification, the enzyme had a specific activity of 34 units/mg protein, with a net yield of 20% (Table I). To analyze the purity, doubling dilutions of the purified enzyme from 8000 to 8 ng was resolved on a 10% SDS-polyacrylamide gel (Fig. 1). Only the deaminase band was seen in the lane containing 8000 ng of deaminase, although the sensitivity of detection is about 64 ng (125-fold dilution), suggesting that this preparation is >99% pure. The molecular weight of the denatured form of deaminase is about 28,000 (Fig. 1) and the native size was about 43,000 as determined by gel permeation chromatography using the Superose-12 column (data not shown). However, the native size of *C. albicans* deaminase is markedly different from that of the *E. coli* deaminase, which is made of six identical subunits of 29.7 kDa, to form a native enzyme of 178 kDa (Calcagno *et al.*, 1984).

Enzymology—Using a coupled enzyme assay, the V_{max} for the deamination reaction was determined to be 60 nmol/min/mg protein and the K_m for GlcN-6-P as substrate was about 100 μM. The pH stability of deaminase was determined by preincubating the enzyme in buffers of different pH values for 5 h at 30 °C. Between pH 7.6 and 8.1, the enzyme was completely stable and at pH 5.5 and 9.8, the enzyme had about 80% activity. The optimal pH for the deamination reaction was between 7.5 and 7.8. It was also observed that the enzyme activity was higher in Tris-HCl buffer than in potassium phosphate buffer. However, ionic strength *per se* does not affect the activity of deaminase (data not shown). The enzyme is stable at 40 °C for at least 30 min, but was rapidly inactivated (within 5 min) at 50 °C, suggesting that there is a sharp temperature stability. The enzyme was unstable to freeze-thaw. Iodoacetamide (10 mM) and *p*-chloromercuriphenyl sulfonic acid (10 mM) reduced the enzyme activity to 28 and 6%, respectively, suggesting that sulfhydryl groups are required for enzyme activity.

The purified protein was resolved in a polyacrylamide gel under nondenaturing conditions, using the Laemmli (1970) buffer system. The protein eluted from the gel showed deaminase activity confirming its identity as GlcN-6-P deaminase. Glucose, glucose 6-phosphate, GlcNAc, GlcNAc-6-P, GlcN, and GlcN-6-P were tested individually as substrates (1 mM each), using the colorimetric assay; it was found that only GlcN-6-P could function as a substrate in the forward reac-

[1] The abbreviations used are: bp, base pair(s); kb, kilobase pair(s); PAGE, polyacrylamide gel electrophoresis.

TABLE I
Summary of purification

Purification step	Fraction volume	Total protein	Total enzyme	Specific activity	Purification factor	Yield
	ml	*mg*	*units*[a]	*units/mg*	*–fold*	*%*
Crude extract	110	4950	330	0.07	–	100
Isoelectric precipitation	100	1400	300	0.21	3	90
5% Polyethylene glycol	105	1260	315	0.25	4	95
25% Polyethylene glycol	28	840	280	0.33	5	85
CM-cellulose	65	50	260	5.0	70	79
DEAE-Sepharose and concentration	0.45	2	69	34	485	20

[a] One unit of enzyme catalyzes the formation of 1 μmol of NADPH per min under defined conditions.

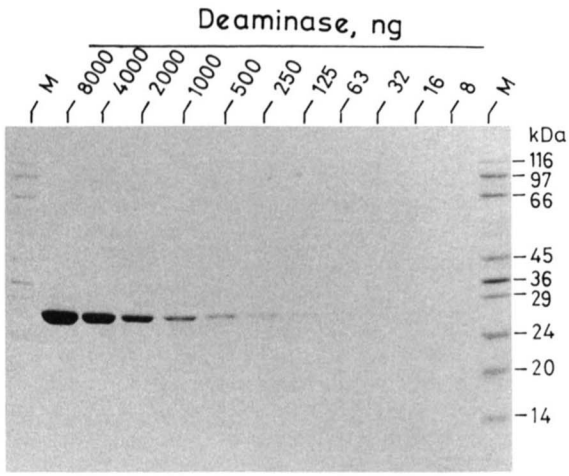

FIG. 1. **Estimation of purity and size of glucosamine-6-phosphate deaminase.** Purified deaminase was serially diluted (2-fold increments) and resolved in a 10% SDS-polyacrylamide gel. The amount of deaminase loaded is shown on *top* of the lanes; the values have been rounded off to whole numbers. A mixture of marker proteins (*lane M*; Sigma) was also run in parallel to estimate the size of deaminase.

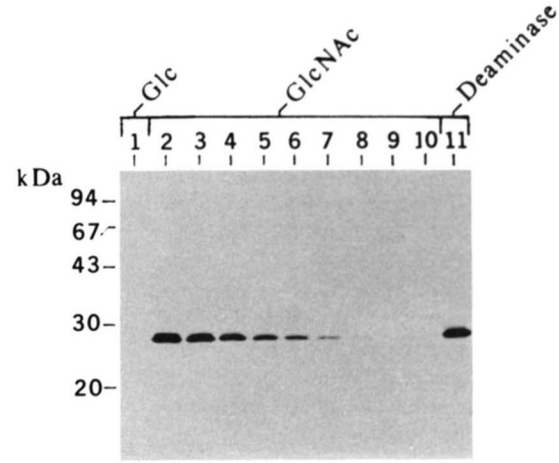

FIG. 2. **Immunoblot analysis to study the induction of glucosamine-6-phosphate deaminase.** Different amounts of *C. albicans* crude extracts (Glc- or GlcNAc-grown) and purified deaminase were resolved in a 10% SDS-PAGE and electroblotted to nitrocellulose membrane. The blot was first treated with affinity-purified antideaminase antibody (1:1000) and then with goat anti-rabbit IgG conjugated with alkaline phosphatase (1:7500; Promega). Deaminase was visualized by alkaline phosphatase activity using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as substrate. *Lane 1*, 20 μg of Glc-grown crude extract; *lanes 2–10*, GlcNAc-grown crude extracts, 20, 10, 5, 2.5, 1.25, 0.6, 0.3, 0.15, and 0.075 μg, respectively; *lane 11*, 0.05 μg of purified deaminase.

tion. In the backward reaction, deaminase converted Fru-6-P (using ammonium chloride as the ammonia donor) to GlcN-6-P. This enzyme could not use L-glutamine as ammonia donor to convert Fru-6-P to GlcN-6-P reiterating that it is an authentic glucosamine-6-phosphate deaminase (EC 5.3.1.10). An important feature of *E. coli* deaminase is that both the forward and backward reactions are allosterically activated by GlcNAc-6-P (Calcagno *et al.*, 1984). However, GlcNAc-6-P did not activate the *C. albicans* deaminase (data not shown).

Deaminase Is an Inducible Enzyme

Earlier studies (Singh and Datta, 1979a, 1979b) using enzyme assays had suggested that GlcNAc induces the GlcNAc catabolic pathway enzymes. We have used affinity-purified antibody to study the inducibility of deaminase using the Western blotting technique. *C. albicans* cells were grown in a medium containing either Glc or GlcNAc as carbon source. Crude extracts from Glc- (20 μg) and GlcNAc-grown cells (2-fold increments from 75 ng to 20 μg) were resolved in SDS-PAGE and transferred to nitrocellulose membrane. In a parallel lane, 50 ng of purified deaminase was also loaded. Deaminase was immunodetected using affinity-purified deaminase antibody (Fig. 2). Although the deaminase is seen in GlcNAc-grown cell extract even after 128-fold dilution (0.15 μg; *lane 9*), it is absent even in undiluted Glc-grown crude extract (20 μg of proteins, *lane 1*). This indicates that deaminase is induced more than 100-fold when GlcNAc is used as carbon source. From this experiment and from a similar immunoblot experiment that had different amounts of pure deaminase and Glc and GlcNAc extracts (data not shown), the level of deaminase in GlcNAc-grown cells was estimated to be about 0.3–0.5% of the total proteins in crude extract.

Isolation of NAG1 cDNA Clones

A λgt11 cDNA expression library (Young and Davis, 1983) was prepared using oligo(dT) primers and poly(A)$^+$ RNA from GlcNAc-grown *C. albicans*. About 110,000 plaques from the primary library were screened, using deaminase crude antiserum and seven immunoreactive clones were obtained and were plaque purified. Phages from these clones were lysogenized in *E. coli* (Huynh *et al.*, 1985), and fusion proteins were analyzed by immunoblotting using affinity-purified deaminase antibody. Clones 1, 2, and 4 (D1, D2, and D4) gave immunoreactive fusion proteins of 124, 132, and 142 kDa, respectively (data not shown). Fusion protein from clones D1 and D2 had very strong immunoreactivity, whereas the protein from clone D4 reacted poorly. Other clones, D3, D5, D6, and D7, and control λgt11 did not produce a detectable fusion protein in immunoblots (data not shown).

To study the relatedness, phage DNA from these clones were digested with *Eco*RI, Southern blotted, and cross-hybridized sequentially with D1, D2, D4, and D6 probes. To our surprise, clones D1, D2, and D6 were related but not D4, when D6 was used as probe. Besides, D1 and D2 did not

hybridize to each other; presumably D1 and D2 have similarity to different regions of D6 insert. The clones D1, D2, and D6 had inserts of 206, 297, and 800 bp, respectively. These three clones were used for further studies, since D1 and D2 fusion proteins had strong immunoreactivity, and the D6 clone was related to the D1 and D2 clones. Although D4 is not related as seen by high stringency hybridization, the immunoreactivity of D4 protein to the affinity-purified antibody suggests that at least some of the epitopes are common between deaminase and this protein.

Identification of Polypeptide Encoded by the Clones

Hybrid-selected translation technique was used to identify the nature of polypeptides encoded by the deaminase clones. Plasmids containing D1, D2, and D6 inserts, and vector pTZ18U DNA as a control, were immobilized onto GeneScreen Plus membranes, and were used to purify specific mRNA from total RNA isolated from GlcNAc-grown cells. The eluted mRNA was translated in a reticulocyte lysate system and a part of the translation reaction mixture was analyzed by SDS-PAGE (Fig. 3*A*). Apparently all three clones, D1, D2, and D6, had hybridized to the same species of mRNA encoding a protein of 28 kDa. This protein co-migrated with the purified deaminase as seen by Coomassie Blue staining of the gel (Fig. 3*A*, *right*). A 50-kDa band common to all lanes is an artifactual band. The identity of the minor 19-kDa band in D1, D2, and D6 translation reaction mixtures (Fig. 3*A*) is not known; however, this band is not seen in immunoprecipitates (Fig. 3*B*). Some proteins are also seen along with dye front but they are common to all lanes (Fig. 3*A*).

To study the antigenic relationship between the *in vitro* synthesized 28-kDa protein and the deaminase, affinity-purified deaminase antibody was used in an immunoprecipitation experiment. The protein obtained by immunoprecipitation was analyzed by SDS-PAGE (Fig. 3*B*). A major band, whose mobility was similar to that of the 28-kDa deaminase, and a minor band of 40 kDa are seen. The remainder of the protein bands seen in Fig. 3*A* have disappeared indicating that they are spurious bands. The true origin of the 40-kDa band (Fig. 3*B*) is not known, but a similar band has been observed in immunoprecipitates from reticulocyte lysate translation reactions. The hybrid selected translation and immunoprecipitation experiments proved that deaminase and the recombinant protein are similar in their size and antigenicity.

Genomic Southern Blot Analysis

C. albicans genomic DNA digested with different restriction enzymes was Southern blotted and hybridized with the ^{32}P-labeled insert from D6 clone (Fig. 4). All of the six-base cutting enzymes used, except *Pst*I, produced a single hybridizing band (Fig. 4). This suggests that the *NAG1* gene, encoding the glucosamine-6-phosphate deaminase, is present in a single copy per haploid genome. Two hybridizing bands of 8.6 and 3.3 kb are seen in the *Pst*I lane, indicating that there is an internal site for *Pst*I in D6. Restriction mapping and sequence analysis of the D6 clone confirmed the existence of a single *Pst*I site. A long exposure of the same blot revealed a total of three bands in the *Hae*III lane (data not shown). The single large *Eco*RI fragment (13 kb) seen here signifies that the *Eco*RI ends of the D6 insert are artificial, derived from *Eco*RI linkers added during cDNA cloning.

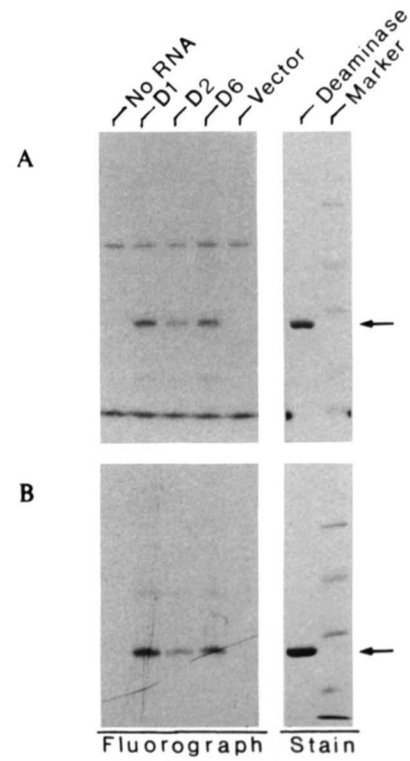

FIG. 3. **Hybrid-selected mRNA translation to study the polypeptide encoded by *NAG1* cDNA clones.** Inserts from *NAG1* cDNA clones D1, D2, and D6 recloned into pTZ18U and the vector as control, immobilized on GeneScreen Plus membranes were used to isolate specific mRNA by hybridization. The purified mRNA was translated in a rabbit reticulocyte lysate system. *A*, a part of each of the above translation reaction mixtures, a mock reaction without exogenous RNA, purified deaminase (3 μg), and marker proteins (Pharmacia) were resolved in a 10% SDS-PAGE. The gel was first stained with CBB R-250 (*right panel*) and then fluorographed (*left panel*) for 3 days at −70 °C to preflashed X-Omat AR film. *B*, the translation reaction mixtures were also immunoprecipitated using affinity-purified deaminase antibody bound to Protein A-Sepharose. The immunoprecipitates, purified deaminase, and marker proteins were resolved in a 10% SDS-PAGE, stained, and fluorographed for 3 days. The *arrow* shows the position of the 28-kDa purified deaminase. The size of the marker proteins from top to bottom are 94, 67, 43, 30, 20.1, and 14.4 kDa.

Restriction Mapping, DNA Sequencing, and Analysis of NAG1 cDNA

Restriction enzyme map of the *Eco*RI insert of clone D6 is shown in Fig. 5*A*. The 800-bp fragment has one site each for *Eco*RV, *Pst*I, and *Xba*I, and two sites for *Cla*I. The orientation of insert in the D6 λgt11 clone was also determined. For this purpose a plasmid containing the *Kpn*I-*Sac*I fragment from the D6 λ-clone (that nested the *Eco*RI insert) was sequenced using λgt11 reverse sequencing primer; the sequence data revealed that the insert in the D6 λ-clone is positioned in a direction that is opposite to that of the *lacZ* reading frame. This precludes the possibility of a *lacZ* directed expression. However, the D6 clone had produced immunoreactive protein in the plaques. Presumably the open reading frame identified in λgt11 (Moran *et al.*, 1990), in the opposite direction from the *lacZ* reading frame, is responsible for the D6 expression. However, no fusion protein was seen in D6 lysogen by immunoblot analysis. Perhaps the denaturation of the proteins from lysogen with SDS-PAGE sample buffer and subsequent blotting to nitrocellulose membrane might have destroyed the epitopes.

Overlapping subclones of D6 (Fig. 5*A*) were used to deter-

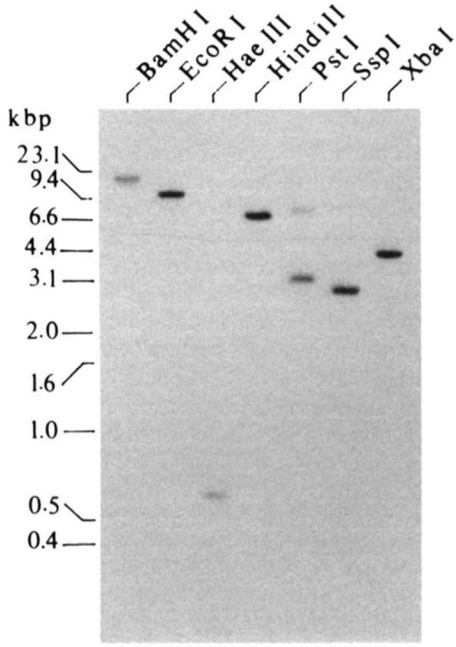

FIG. 4. **Genomic Southern blot analysis of the *NAG1* gene.** *C. albicans* SC5314 genomic DNA (1 μg each) digested to completion with different restriction enzymes was Southern blotted to GeneScreen Plus membrane. The blot was hybridized with D6 probe, washed, and exposed for 6 h to Kodak X-Omat AR film at −70 °C with one intensifying screen. The position of standards (a combination of λ *Hin*dIII and 1-kb ladder) is shown on the *left*.

mine the DNA sequence. The 800-nucleotide long sequence of D6 is shown in Fig. 5*B*. The G + C base composition of the sequence is 43%. The longest open reading frame shown in Fig. 5*B* encodes a polypeptide of 27,497 daltons, made of 248 amino acids. The predicted size of the *NAG1* product agrees with the experimentally determined value of the purified deaminase (28 kDa; Fig. 1). The deduced protein has a pI of 7.4; this value is in close agreement with the experimental value for deaminase (pI 7.5). The first 35 amino acids of the predicted protein are identical with the 35-amino acid long sequence determined from purified deaminase (Fig. 5*B*). This agreement emphasizes that the two proteins, *NAG1* product and the purified deaminase, are identical. Besides, the first methionine residue at the start of the reading frame is also the amino-terminal amino acid of deaminase.

NAG1 Is a Homologue of E. coli nagB Gene

The deduced amino acid sequence of deaminase was used to search the SWISS-PROT protein sequence data base (Release 21) using the FASTA program (Pearson and Lipman, 1988). The *E. coli* NagB sequence (Peri *et al.*, 1990; Rogers *et al.*, 1988), encoding the glucosamine-6-phosphate deaminase, had maximum identity to the Nag1 sequence. Other sequences in the data base were poorly related. The alignment of the *C. albicans* Nag1 sequence with the *E. coli* NagB protein sequence is shown in Fig. 6. Five gaps were inserted in the Nag1 sequence and two gaps in the NagB sequence to obtain a best match. About 47% of the amino acids are identical and 86% are similar between the two sequences (Fig. 6). Based on the relatedness, it is concluded that the two genes perform similar function and the bacterial and fungal proteins are well conserved.

NAG1 Transcript Is Induced by GlcNAc

To study the regulation of *NAG1* expression, mRNA level was analyzed by RNA blot. Total RNA (10 μg each) from either Glc- or GlcNAc-grown *C. albicans* were blotted to GeneScreen Plus membrane and hybridized to D6 probe. The blot result shows that the D6 probe hybridizes to a 1-kb mRNA in GlcNAc grown cells (Fig. 7). No hybridizing RNA could be seen in Glc-grown cells. The same blot was rehybridized to *C. albicans* actin gene probe, *ACT1*, and a 1.6-kb RNA is seen in both lanes. The actin gene control hybridization indicates that the signal obtained with the *NAG1* probe is not due to the presence of more RNA in the GlcNAc lane. This is also clear from the ethidium bromide staining of the RNA gel (Fig. 7), where more RNA is seen in Glc lane than in the GlcNAc lane. The Northern blot data suggests that the *NAG1* RNA is induced when GlcNAc is supplied as carbon source.

DISCUSSION

In this paper we have described the purification, characterization, cDNA cloning, and sequencing of glucosamine-6-phosphate deaminase from *C. albicans* SC5314. An earlier paper (Das and Datta, 1983) had reported the purification and some properties of this enzyme from *C. albicans* NCIM 3100. However, recent evidence indicate that the NCIM 3100 is a strain of *Candida tropicalis* and not of *C. albicans*.[2] Therefore we have used *C. albicans* SC5314, a strain used by many *Candida* researchers. Glucosamine-6-phosphate deaminase has been purified to apparent homogeneity and some characteristics have been studied. Using the deaminase antibody we have isolated cDNA clones by immunoscreening of a λgt11 cDNA library. The identity of the *NAG1* cDNA clones has been confirmed based on the following results. (i) The protein obtained by *in vitro* translation of mRNA purified by hybridization selection had the same size and antigenicity as purified deaminase. (ii) The NH_2-terminal 35 amino acids of the purified deaminase are identical with the first 35 amino acids of the deduced sequence of the Nag1 protein. Besides, the Nag1 protein sequence is similar to that of the NagB protein, which also codes for the *E. coli* glucosamine-6-phosphate deaminase. Furthermore, using RNA blot analysis we have shown that the *NAG1* transcript is GlcNAc-inducible and this pattern of expression of *NAG1* gene matches with that of the deaminase.

In *C. albicans*, GlcNAc has several functions. When cells are grown in a medium containing GlcNAc, the enzymes of the GlcNAc catabolic pathway are induced. These enzymes catabolize GlcNAc and provide energy for cell growth. GlcNAc, under certain conditions, also induces a change in the cellular morphology from the yeast form to the mycelial form (Simonetti *et al.*, 1974). GlcNAc is polymerized into chitin which is an integral component of cell wall. It is also present as part of carbohydrate moieties of certain glycoproteins. Besides, available evidence (Singh and Datta, 1979c) suggests that the capability to utilize GlcNAc as a carbon source is an attribute of pathogenic *Candida* species. However, more experiments are required to establish the relevance and role if any, of GlcNAc catabolic pathway for virulence of *Candida* species.

The *E. coli* deaminase is an oligomer of six subunits (Calcagno *et al.*, 1984), whereas the *C. albicans* enzyme is at best a dimer; the inability of GlcNAc-6-P to activate the *C. albicans* deaminase activity could be due to the non-oligomeric nature. This view is strengthened because the primary sequence of the *C. albicans* and the *E. coli* proteins are quite similar and so the allosteric activation site for GlcNAc-6-P in *E. coli* deaminase could arise when monomers associate to generate the quaternary structure. The glucosamine-6-phosphate de-

[2] K. Ganesan, A. Banerjee, and A. Datta, unpublished results.

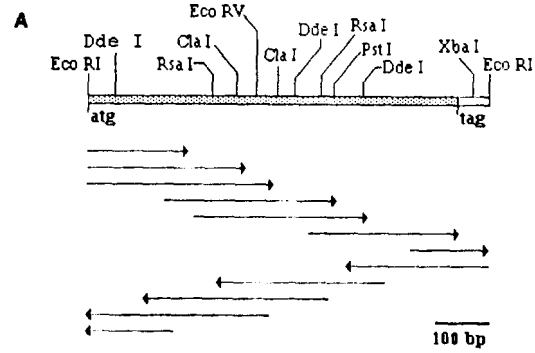

FIG. 5. **Primary structure analysis of *NAG1* cDNA.** *A*, restriction enzyme map and sequencing strategy. Restriction map of the D6 insert was constructed by single and double digestion of the plasmid subclone pD6 and the λ clone D6. The open reading frame is shown as a *stippled bar*; the positions of the start (*ATG*) and stop (*TAG*) codons are also shown. The *arrows* show the extent of sequence information obtained from each subclone in relation to the D6 clone; the direction of sequencing is also shown. *B*, sequence of *NAG1* cDNA and the deduced amino acid sequence. The 800-bp *Eco*RI insert of *NAG1* cDNA clone (D6) was sequenced by the dideoxy chain termination method using denatured double stranded DNA templates. The *Eco*RI ends originating from *Eco*RI linkers are shown in *lower case*. The deduced sequence of the Nag1 protein is shown below the DNA sequence. The NH_2-terminal 35 amino acids which are in complete agreement with the sequence obtained from purified deaminase are highlighted by an *underscore*.

B

```
gaattccCATGAGACAAGCTATATTTTCCAACCCTAACGATGCTGCTGAGTATTTGGCAA      60
        M  R  Q  A  I  F  S  N  P  N  D  A  A  E  Y  L  A  N  18

ACTATATCATTGCCAAAATCAACTCCACCCCCAGAACATTTGTTCTTGGCCTTCCAACCG     120
  Y  I  I  A  K  I  N  S  T  P  R  T  F  V  L  G  L  P  T  G  38

GGTCATCCCCTGAAGGCATTTATGCCAAATTGATCGAAGCCAACAAGCAAGGCCGTGTTA     180
  S  S  P  E  G  I  Y  A  K  L  I  E  A  N  K  Q  G  R  V  S  58

GTTTCAAAAACGTCGTGACCTTCAACATGGACGAGTATTTGGGATTTGCCCCATCTGACT     240
  F  K  N  V  V  T  F  N  M  D  E  Y  L  G  F  A  P  S  D  L  78

TGCAGTCGTACCATTATTTCATGTACGACAAGTTTTTCAACCATATCGATATCCCGCGTG     300
  Q  S  Y  H  Y  F  M  Y  D  K  F  F  N  H  I  D  I  P  R  E  98

AAAATATCCACATCTTGAACGGATTGGCCGCAAACATCGACGAGGAGTGTGCCAACTACG     360
  N  I  H  I  L  N  G  L  A  A  N  I  D  E  E  C  A  N  Y  E  118

AAAAGAAAATCAAGCAATACGGAAGAATCGATTTGTTCTTAGGCGGGTTAGGCCCAGAAG     420
  K  K  I  K  Q  Y  G  R  I  D  L  F  L  G  G  L  G  P  E  G  138

GTCATTTGGCATTCAACGAAGCGGGATCATCAAGAAACTCAAAAACAAGAAAGGTCGAGT     480
  H  L  A  F  N  E  A  G  S  S  R  N  S  K  T  R  K  V  E  L  158

TGGTCGAAAGTACCATCAAGGCAAACTGCAGGTTTTTCGGGAACGACGAGAGCAAGGTCC     540
  V  E  S  T  I  K  A  N  C  R  F  F  G  N  D  E  S  K  V  P  178

CTAAATATGCATTGAGTGTTGGTATTTCCACCATCTTGGACAACTCAGACGAAATTGCCA     600
  K  Y  A  L  S  V  G  I  S  T  I  L  D  N  S  D  E  I  A  I  198

TTATCGTGTTGGGCAAAAGTAAACAATTTGCATTGGACAAAACTGTAAACGGGAAACCAA     660
  I  V  L  G  K  S  K  Q  F  A  L  D  K  T  V  N  G  K  P  N  218

ACGACCCAAAATACCCATCAAGCTATTTACAAGACCACGCAAATGTCTTGATTGTTTGCG     720
  D  P  K  Y  P  S  S  Y  L  Q  D  H  A  N  V  L  I  V  C  D  238

ATAACGCTGCCGCTGGATTAAAGTCAAAGTTGTAGAGATGTATAGAGTCTAGAATATACA     780
  N  A  A  A  G  L  K  S  K  L  *                              248

GGTAAATCATGACggaattc                                            800
```

aminase from *C. albicans* (this work) and from *Proteus vulgaris* (Nakada, 1966) are the only two known cases that are not activated by GlcNAc-6-P.

It has been reported that of the 4 cysteine residues in the *E. coli* deaminase, two of them at positions 118 and 239 of the primary sequence (Fig. 6), form a thiol pair and are important for catalytic function (Altamirano *et al.*, 1992). In the *NAG1* sequence too, 2 cysteine residues occupy comparable positions, at 114 and 237 (Fig. 6), suggesting that these cysteinyl residues could be important for the activity of *C. albicans* deaminase as well. In both *E. coli* (Plumbridge, 1990) and *C. albicans* (Shepherd and Sullivan, 1983), uptake of GlcNAc is apparently necessary for induction of the catabolic enzymes. Furthermore, unlike in *E. coli*, glucose does not repress the synthesis of the *C. albicans* GlcNAc catabolic pathway enzymes (Singh and Datta, 1978).

Although the deaminase has been studied from a variety of sources (Noltmann, 1972), the *E. coli* enzyme is the one that has been most thoroughly studied; besides, the organization and regulation of expression of the *E. coli* GlcNAc catabolic pathway is also being studied in detail. However, very little is known at the molecular level about the pathway from *C. albicans*. Incidentally this is the first report on the molecular characterization of a constituent gene of the GlcNAc catabolic pathway from a eukaryotic organism. Not all regulatory mechanisms known for the *E. coli* pathway, such as the catabolite activator protein-cAMP system (which is nonexistent in eukaryotes), are expected to be operative in *C. albicans*. This implies that the *C. albicans* GlcNAc catabolic pathway might be controlled by other forms of regulation. To gain an insight into the regulation of the *NAG1* gene, we have isolated genomic clones and are characterizing them. Furthermore, to

```
                  10         20         30         40         50
NAG1    MRQAIFSNPNDAAEYLANYIIAKIN----STPRTFVLGLPTGSSPEGIYAKLIEANKQGR
        ::   .......... :..:...::    .. :.:::::::::.: ..:  :.: .:.:.
NAGB    MRLIPLTTAEQVGKWAARHIVNRINAFKPTADRPFVLGLPTGGTPMTTYKALVEMHKAGQ
                10         20         30         40         50         60

          60         70         80         90         100        110
NAG1    VSFKNVVTFNMDEYLGFAPSDLQSYHYFMYDKFFNHIDIPRENIHILNGLAANIDEECAN
        ::::.:::::::::.:.. .. .::. ::. .::.:.::: :::..::: :..::.:: .
NAGB    VSFKHVVTFNMDEYVGLPKEHPESYYSFMHRNFFDHVDIPAENINLLNGNAPDIDAECRQ
                70         80         90         100        110        120

          120        130        140        150        160        170
NAG1    YEKKIKQYGRIDLFLGGLGPEGHLAFNEAGSSRNSKTRKVELVESTIKANCRFFGNDESK
        ::.::. ::.:.::.::.: .::.::::..:: .:.:: .:...: ::.:::.:: ..
NAGB    YEEKIRSYGKIHLFMGGVGNDGHIAFNEPASSLASRTRIKTLTHDTRVANSRFFDNDVNQ
                130        140        150        160        170        180

          180        190        200        210        220        230
NAG1    VPKYALSVGISTILDNSDEIAIIVLGKSKQFALDKTVNGKPNDPKYPSSYLQDHANVLIV
        ::::::.::..:.:: ..:. :.::::. :..::. .:.:   ..... :.:: :.....:
NAGB    VPKYALTVGVGTLLD-AEEVMILVLGSQKALALQAAVEG-CVNHMWTISCLQLHPKAIMV
                190        200        210        220        230

          240
NAG1    CDNAAA-GLKSKL
        ::.... .:: :
NAGB    CDEPSTMELKVKTLRYFNELEAENIKGL

          240        250        260
```

FIG. 6. **Alignment of the NAG1 amino acid sequence with the *E. coli* nagB sequence.** The deduced amino acid sequence of *C. albicans NAG1* (*NAG1*) was used to search the SWISS-PROT protein sequence data base (release 21) using Mail FASTA program, and the *E. coli nagB* (*NAGB*) sequence was obtained. Identical amino acids between the two sequences are shown by a *colon* and similar amino acids representing conservative changes are shown by a *period*.

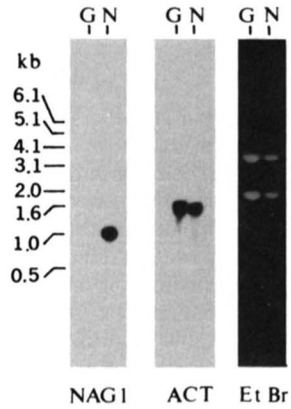

FIG. 7. **RNA blot analysis to study pattern of expression of the *NAG1* gene.** Ten micrograms of Glc-grown (*lane G*) and GlcNAc-grown (*lane N*) total RNA was denatured using glyoxal/dimethyl sulfoxide, resolved in an agarose gel, and blotted to GeneScreen Plus membrane. DNA fragments (1-kb ladder; Bethesda Research Laboratories) denatured exactly like RNA samples, were used as markers. *NAG1* cDNA probe was hybridized to the RNA blot and exposed to preflashed X-Omat AR film at room temperature for 10 h. For control hybridization, *C. albicans* actin gene, *ACT1*, was hybridized to the same blot and exposed to preflashed X-Omat AR film for 1.5 h at room temperature. An 1.6-kb RNA species is seen in both *G* and *N lanes*. A part of the RNA gel was stained with ethidium bromide (*right panel*) to see the rRNA levels; the small rRNA (1.8 kb) and the larger rRNA (3.5 kb) can be clearly seen in both lanes.

study if the GlcNAc utilization is necessary for *C. albicans* virulence, the *NAG1* cDNA clone would be used to construct mutant strains by the gene disruption technique (Kelly *et al.*, 1987); such strains would be tested in experimental infections.

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