RESULTS AND DISCUSSION
Crude extract (pH 6.5) from GlcNAc-induced *C. albicans* SC5314

Add acetic acid to bring pH to 4.8

Supernatant  Pellet

Add PEG8000 to 5% (w/v)

Make supernatant to 25% PEG8000

Redissolve pellet in TSEM buffer pH 5.7

Bind to CM-cellulose column pH 5.7 and elute with 0-0.2 M KCl

Dialyze active fractions against TEM pH 8.8 and adjust pH=8.8

Bind to DEAE-Sepharose Column pH 8.8 and elute with 0-0.2M KCl and adjust active fractions to pH 5.6.

Concentrate by binding to 2 ml CM-Cellulose column and elute with 0.2 M KCl.

Pool fractions containing deaminase; dialyze and concentrate through Centricon-10

Fig. 2. Flow chart showing various steps involved in the purification of deaminase.
3.1 GLUCOSAMINE-6-PHOSPHATE DEAMINASE: PURIFICATION AND CHARACTERIZATION

3.1.1 Purification of Deaminase.

Deaminase was purified from GlcNAc-induced C. albicans SC5314 through various steps (Fig. 2). C. albicans grown up to mid log phase was induced with GlcNAc for 3 h. Cells were pelleted, homogenized and the crude extract prepared as described (Section 2.7.2). Yield of total protein was approximately 100 mg per gram wet weight of cells. Assay for deaminase activity was performed as described (Section 2.3). In the several batches of purification done, the specific activity of crude extract was between 0.04 and 0.08. pH of the crude extract (ca. 6.4) was reduced with acetic acid as described. Under these conditions, about 3-4 fold purification was obtained (data not shown). The supernatant obtained after acid precipitation was made up to 5% (w/v) PEG 8000; mode of PEG action is similar to that of organic solvents (Scopes, 1987). Precipitated proteins were removed and deaminase in the supernatant was precipitated by adding PEG to 25% (w/v). The precipitate containing deaminase was solubilized in the TSEM pH 5.7, CM-cellulose column buffer. The specific activity of the preparation after 5% and 25% PEG steps was lower than after acid precipitation step (data not shown); this is presumably due to partial inactivation of the enzyme. Though the PEG
steps apparently did not result in any improvement in purification, the steps were persisted with for two reasons: precipitation helps to equilibrate the sample with the column buffer prior to CM-cellulose chromatography and secondly the overall purification scheme resulted in a reasonable yield of the enzyme with almost consistent purity.

The sample was then chromatographed on CM-cellulose at pH 5.7 and eluted with a salt gradient (Fig. 3); deaminase was desorbed at about 75 mM KCl. This step resulted in about 20-fold purification (Table 2). The active fractions were pooled and dialyzed to remove the salt and pH readjusted to about 8.8. It was then bound to DEAE-Sepharose column at pH 8.8 as described. Deaminase was eluted with a salt gradient (Fig. 4). Under these conditions deaminase is weakly adsorbed and is eluted at about 30 mM KCl (data not shown). This step resulted in about 5-fold purification (Table 2). The overall yield was about 10% and the final specific activity obtained was between 15 and 22, with about 300-fold purification (Table 2). The fold purification depended on the specific activities of crude extract and that of the post-DEAE-Sepharose sample.

The DEAE-Sepharose step has been carefully optimized and any deviation in pH of the sample or buffer to the one described
Fig. 3. Elution profile of deaminase from CM-cellulose column. PEG-precipitated proteins were resolubilized in TSEM pH 5.7 and loaded to CM-cellulose column (2.6 x 25 cms) equilibrated with TSEM pH 5.7 and washed with the same buffer. Bound proteins were eluted with linear gradient of 0-0.2 M KCl (300 ml each) in TSEM pH 5.7. Five ml fractions were collected and assayed for deaminase activity. KCl concentration was determined in the fractions by conductivity measurements, and protein by absorbance at 280 nm. Deaminase eluted at 75 mM KCl. Large amounts of protein came unbound (not shown) and also preceeded the deaminase in the gradient elution.
TABLE 2. A typical result of a batch of purification of deaminase

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<tr>
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<tr>
<td>DEAE-Sepharose column</td>
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</table>

<sup>a</sup>The values shown correspond to the samples at the end of the indicated purification step.

<sup>b</sup>Specific activity is defined as μmoles of NADP converted to NADPH per minute per milligram protein.

<sup>c</sup>25% PEG step was preceded by 5% PEG and acid precipitation steps (data not shown; for a description see text).
Fig. 4. Elution profile of deaminase from DEAE-Sepharose column. Active fractions from CM-cellulose column were pooled, dialyzed against TEM pH 8.8, and loaded (50 ml) onto a DEAE-Sepharose column (2.6 x 20 cms) equilibrated with TEM pH 8.8. Column was washed until all unadsorbed proteins were removed. Bound proteins were eluted with 0-0.2 M KCl (250 ml each) linear gradient. Five ml fractions were collected, assayed for deaminase activity and for absorbance at 280 nm. From independent experiments, it was determined that deaminase eluted at about 30 mM KCl.
(Section 2.7.7), resulted in the enzyme coming unbound. The pH of the buffer and sample has to be constant, otherwise the enzyme will not bind to the column. Therefore, routinely a small-scale DEAE-Sepharose column was used to test the binding prior to loading the sample onto the preparative column. If this care is taken and other conditions are followed exactly, this procedure has been quite reproducible.

3.1.2 Purity and Molecular Weight of Deaminase

Purified deaminase was resolved in SDS-PAGE (Fig. 5). From the serial 2-fold dilutions of deaminase, it is apparent that at least 30 ng of protein can be detected by coomassie blue staining (Fig. 5). Deaminase band is just detectable at 256-fold dilution of purified deaminase; only a single band is seen in the lane with undiluted deaminase (8 ug), indicating that the deaminase preparation is at least 99% pure. In some batches of purification, a contaminating protein (35 kDa) is seen (Fig. 7B), which represented less than 1% of the total protein. From a plot of log molecular weight versus mobility, the molecular weight of deaminase was calculated to be about 28 kDa.

Native molecular weight was determined by gel permeation chromatography. Fig. 6 shows the elution profile of
Fig. 5. Analysis of purity of deaminase. Purified deaminase was serially diluted and resolved in 10% SDS-PAGE and proteins were visualized by staining with CBB R250. The top panel indicates amount of deaminase loaded. Molecular weight marker proteins (M) are from Sigma.
deaminase on a Superose 12 column (Pharmacia). About 0.4 units of purified deaminase was loaded onto the column. It can be seen that the enzyme activity peak correspond with the protein peak ($A_{280}$). Almost all the enzyme loaded was recovered from the column (data not shown). Molecular weight standards were run, their elution volumes were plotted and from the elution volume of deaminase, size was estimated to be about 44 kDa (Fig. 6, inset). 44 kDa is higher than the molecular weight determined by SDS-PAGE. Since only a single polypeptide is seen in SDS-PAGE, the idea was to see if native deaminase is a monomer or a multimer of identical subunits. E.coli deaminase has a molecular weight of 178 kDa, with six identical subunits (Calcagno et al., 1984). From the calibration plot (Fig. 6, inset) it can be seen that the standard proteins themselves have not run well and they do not fall in a line; log molecular weight versus elution volume is a linear relationship and a best fitting line would only give a rough estimate of size. On the other hand if size of the standard proteins is interpolated from the plot, then it can be seen that the estimated size is much different from that of the reported value; for example $\beta$-amylase (200 kDa) is about 240 kDa, BSA (67 kDa) is about 90 kDa, ovalbumin (43 kDa) is about 48 kDa, carbonic anhydrase (29 kDa) is 26 kDa, chymotrypsinogen A (25 kDa) is about 17 kDa. When a
Fig. 6. Determination of molecular weight by gel permeation chromatography. Purified deaminase (25 µg in 100 µl) was loaded onto a FPLC gel permeation column (Superose 12; 10 x 300 mm) and chromatographed in 0.05 M Na-phosphate, 0.15 M NaCl pH 6.8, at a flow rate of 0.5 ml/min. Proteins were detected at 280 nm using a UV monitor equipped with a 10 mm flow cell. 0.25 ml fractions were collected and assayed for deaminase activity. A big peak at the start (zero ml) represents 'injection peak,' probably due to pressure changes resulting from sample injection; another big peak around 18.5 ml that did not give deaminase activity probably results from absorbance of buffer components from sample. Under similar conditions standard proteins were run and their elution volumes determined and plotted against molecular weight (inset). Standard proteins used were (inset, 'o') Ferritin (440kDa), catalase (232kDa), β-amylase (200kDa), aldolase (158 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (67kDa), Ovalbumin (43kDa), carbonic anhydrase (29 kDa), chymotrypsinogen A (25 kDa) and cytochrome C (12.4 kDa). Deaminase had an elution volume of 14.1 ml and an estimated molecular weight of 44 kDa ('•', inset).
different gel permeation column, TSK G3000 SW was used, the elution volumes of standard proteins was not any better than the results presented here. In fact, the published calibration plot for Superose 12 from its supplier (Pharmacia, 1989 Biotechnology catalogue) was similar to the results obtained here. However, from this experiment, it can be inferred that deaminase exists as a monomer or at best a dimer of two identical subunits, otherwise the elution volume would have been significantly less. Among the other methods to determine native molecular weight, Ferguson plot can be used, which involves electrophoresis of the sample and standard proteins through various concentrations of polyacrylamide under native conditions as described (Chrambach, 1985).

3.1.3 Confirmation of the Identity of Purified Deaminase

The identity of the purified protein is an important aspect; a single band seen in SDS-PAGE does not necessarily guarantee that it is the right protein. It is possible that the protein of interest is present in small amounts and is not detectable by coomassie blue staining of SDS-PAGE. By two additional fractionation steps - native PAGE and gel permeation chromatography - it was found that the 28 kDa band seen in SDS-PAGE copurifies with deaminase activity,
thus confirming the identity of deaminase as the 28 kDa band.

The enzyme preparation from DEAE-Sepharose column was resolved in its native form in polyacrylamide gel. Deaminase did not stack properly (data not shown) under non-denaturing conditions in polyacrylamide gel using discontinuous buffer system (Laemml, 1970). Thus, electrophoresis under non-denaturing conditions was performed at higher trailing phase pH values (Chrambach, 1985). When a buffer system giving a trailing phase pH of 10.5 at 0° and a trailing ion mobility of 0.064 was used, the proteins had stacked well. However, at least one protein did not unstack in the separating gel and thus co-migrated with the dye front because of low trailing ion mobility chosen here. A schematic diagram of coomassie blue staining pattern of such a gel is shown (Fig. 7A). Two bands, an upper band, and a lower band which migrated along with the dye front were seen after staining. Gel pieces corresponding to the bands were cut out and from an unstained gel eluted by diffusion into TSEM pH 5.7 (Section 2.6.2). Deaminase activity could be detected only in the band migrating with the dye and the top band did not give any activity. Part of the eluted samples were separated on SDS-PAGE (Fig. 7B). It can be seen that the gel slice that
Fig. 7. Confirmation of Deaminase Identity.

(A) Purified deaminase (20 μg), after DEAE-Sepharose chromatography step, was separated by native-PAGE. The figure shows a schematic representation of the coomassie blue G-250 staining pattern. In a parallel lane deaminase was resolved and slices corresponding to the positions shown were cut and protein eluted.

(B) SDS-PAGE Analysis: Equal volumes of samples eluted either from FPLC gel permeation column or native-PAGE was resolved in 15% SDS-PAGE and stained with CBB R-250. The samples loaded are indicated above the lanes. Deaminase activity (milli units) in the sample loaded on the gel are indicated below the lanes. 28 kDa protein co-purifies with deaminase activity and the 35 kDa protein is a contaminant. The sample applied to FPLC column and native-PAGE was applied to lane "load". Though the deaminase band in native-PAGE slice number 4 and loaded sample are nearly same, the activity values were very different; this is probably due to partial inactivation of deaminase during native PAGE (possibly because of high trailing phase pH).
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<td>Load</td>
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- 35 kDa
- 28 kDa

Deaminase (milli Units)

A: 

(-) Band

(+) Band + Dye

Slice No. 1 2 3 4
gave activity had an approximate molecular weight of 28 kDa and the top band seen in native gel had a molecular weight of 35 kDa. The sample before native gel electrophoresis had two bands 35 and 28.5 kDa. The 35 kDa protein is not a routine contaminant of the enzyme preparation, but when present, it constitutes less than 1% of the total protein. When this sample was passed through a gel permeation column (Fig. 6), a 28 kDa protein is seen (lanes 1-3 Fig. 7B) corresponding to the active peak in the gel permeation column. From these experiments, it is concluded that deaminase is a 28 kDa protein.

3.1.4 Determination of Isoelectric Point

Isoelectric point of deaminase was determined in a polyacrylamide isoelectric focussing gel containing ampholine 3.5-10 as described (Section 2.6.3). Proteins were visualized by coomassie blue staining. pH of the gel slices was measured after electrofocussing and from a plot of pH versus mobility, pI of deaminase was estimated to be approximately 7.4 (data not shown). This pI value is consistent with the behaviour of the protein during column chromatography steps; at pH 5.7 the protein has net positive charge and binds to CM-cellulose and at pH 8.8 the protein is negatively charged and is adsorbed to DEAE-Sepharose column (see Section 3.1.1).
3.1.5 N-Terminal Amino Acid Sequence

Deaminase purified as described (Section 3.1.1) was sequenced elsewhere (amino acid sequence was determined by Dr. R.K. Prusti, Seattle and Dr. M.A.Q. Siddiqui, New Jersey, U.S.A.). The N-terminal sequence shown in Fig. 8 was compared with that of the deduced N-terminal sequence of E.coli deaminase (Rogers et al., 1988). It can be seen that in the stretch compared, there is no obvious similarity between the two sequences. The N-terminal aminoacid sequence would be useful in the confirmation of the cloned gene (Section 3.3) and to assign the correct reading frame once the gene is sequenced.

3.1.6 Production of Anti-Deaminase Antibody

Antibodies against purified deaminase were raised in rabbits as described (Section 2.8). The titre of antibodies was monitored by double immunodiffusion technique (Garvey et al., 1977). Using about 5 μg of purified deaminase, a precipitin line could be seen at 8-fold dilution of the antiserum (data not shown).

In order to demonstrate that the antiserum can precipitate deaminase from solution, immunoprecipitation experiment was done as described (Section 2.12). Fig. 9 shows the coomassie stained pattern of SDS-PAGE; it can be seen that
Fig. 8. Comparision of N-terminal amino acid sequence of *C. albicans* deaminase and *E. coli* deaminase. N-terminal sequence of *C. albicans* deaminase was independently determined by Dr. R.K. Prusti and Dr. M.A.Q. Siddiqui, USA. The independent determinations gave identical results. Amino acid residues 1 to 23 matched completely in the two determinations and is indicated by underline. Apparently, there is no significant similarity between the amino acid sequences of *E. coli* and *C. albicans* deaminases. While *C. albicans* sequence was determined from the purified protein, the *E. coli* sequence was deduced from the deaminase gene sequence.
### A. N-terminal sequence of *C. albicans* deaminase

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### B. Deduced N-terminal sequence of *E. coli* deaminase.

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kDa

67 - 43 - 30 -

Deaminase

Pre-immune
Immune

kDa

94 - 67 - 43 - 30 - 20 - 14 -
Fig. 9. Immunoprecipitation of purified deaminase. Purified deaminase (5 μg) was reacted with IgG from preimmune or immune sera and bound protein was eluted by boiling in Laemmli buffer and resolved in 10% SDS-PAGE. Proteins were visualized by CBB R-250 staining. As a control, 5 μg deaminase was loaded in a parallel lane. Molecular weight markers from Pharmacia are shown on the left.
deaminase antibody can precipitate deaminase (28 kDa protein) which migrated to the same extent as the unprecipitated deaminase (Fig. 9). The intense protein band seen in all lanes is the heavy chain of rabbit IgG. Preimmune serum did not precipitate deaminase.

3.1.7 Affinity Purification of Deaminase-Specific Antibody

Deaminase (75 µg) was resolved in a preparative SDS-PAGE and the proteins were transferred to nitrocellulose. The protein band corresponding to deaminase was visualized by staining the blot with Ponceau S and was cut out and used for affinity purification of antibodies (Smith and Fisher, 1984; Elledge and Davis, 1987; Query et al., 1989) as described (Section 2.9). The titre of affinity-purified antibodies was determined by reacting antibodies at different dilutions to various amounts of immobilized deaminase (Fig. 10). It can be seen that at a dilution of 1:1000, the antibody can detect about 1 ng (in a two mm² slot) of deaminase. Thus for all immunoblot experiments, affinity-purified antibody was used at 1:1000 dilution.

3.1.8 Induction Kinetics of Deaminase

The affinity-purified antibody was used to study the GlcNAc-mediated induction of deaminase. C. albicans was grown in
Fig. 10. Titer of affinity-purified antibody. Indicated amounts of deaminase (five-fold dilutions) were bound to nitrocellulose by a slot blot device. Strips of nitrocellulose was individually reacted with different dilutions of antibody (1:50, 1:100 and 1:1000). Antigen-antibody complexes were detected with alkaline phosphatase-conjugated anti-rabbit IgG.
<table>
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glucose-containing medium until log phase and was washed and resuspended in growth medium containing GlcNAc instead of glucose. Fig. 11A shows the kinetics of induction of deaminase by GlcNAc. By 15 min of GlcNAc addition, deaminase activity was detected. Thereafter, the specific activity of the enzyme increased rapidly and the rate of synthesis of the enzyme was maintained until 90 min. But by about two hours of induction, the rate had reduced considerably. However, three to five hours the specific activity was constant. This indicates that the rate of synthesis and degradation have reached a steady-state. However, even if there is no further synthesis of the enzyme, a long half-life of the enzyme would maintain the level for the period till it is degraded.

The extracts were also analyzed by Western blot; equal amounts of protein were reacted with affinity-purified deaminase antibody (Fig. 11B). Deaminase band could be seen by 15 min of induction (lane 2) and after three hours there was no further increase (lane 6 and 7). From the enzyme assay data (Fig. 11A) and Western blot data (Fig. 11B), it can be concluded that deaminase is induced rapidly by GlcNAc.
Fig. 11. Kinetics of deaminase induction. C. albicans SC5314 grown to log phase in YNB-glucose was washed and resuspended in YNB-GlcNAc. At different intervals cells were collected and homogenized.

[A] Cell extracts from 0 min, 15 min, 30 min, 1h, 2h, 3h and 5h of induction were assayed for deaminase activity.

[B] Crude extracts containing equal amounts of protein (10 ug) from cells induced for 0 min (lane 1), 15 min (2), 30 min (3), 1 h (4), 2h (5), 3h (6) and 5 (7) were resolved on 12% SDS-PAGE, transferred to nitrocellulose and immuno-detected with affinity-purified deaminase antibody. Positions of molecular weight standard proteins (Sigma) are shown on the right.
A

INDUCTION PERIOD (hours)

ENZYME ACTIVITY (units per mg protein)

B

1234567 kDa

66

45 36 29 24 20
3.1.9 Deaminase is Induced Over 200-Fold by GlcNAc

In GlcNAc-grown cell extracts (Fig. 12 lanes 1-9) the 28 kDa deaminase can be detected even when the extract is diluted 256-fold (lane 8; here the band can barely be seen, but it could be easily seen in the blot). However, even in undiluted glucose-grown extract (lane 10) deaminase could not be detected. This indicates that deaminase is induced over 200-fold in GlcNAc-grown extracts. The intensity of purified deaminase (lane 11, 10 ng) is comparable to the deaminase from about 3 μg GlcNAc crude extract (lane 5); this indicates that when deaminase is induced about 200-fold, it constitutes about 0.3-0.5% of total protein.

The affinity-purified deaminase antibody (Section 3.1.7) reacts with two proteins from GlcNAc-grown cell extracts; one is the 28 kDa deaminase that is absent in the extract of glucose-grown cells and another is a 43-45 kDa protein, which is present in glucose-grown cells also. Since an affinity-purified antibody was used in this experiment, the reactivity to the 45 kDa protein indicates that it is antigenically related to deaminase. Though the identity of this protein is not known, it is possible an antigenic relationship is likely between deaminase and C. albicans glucosamine synthetase, which catalyses the conversion of fructose-6-phosphate to glucosamine-6-phosphate (Milewski et al., 1985).
Fig. 12. Quantification of Deaminase by Western blot. *C. albicans* SC5314 was grown to log phase in peptone-KH$_2$PO$_4$ medium in the presence of either glucose or GlcNAc. Crude extracts from these cells and purified deaminase were resolved on 13% SDS-PAGE, transferred to NC and immunodetected with affinity-purified deaminase antibody. Lanes: GlcNAc-grown extracts 50 μg (1), 25 μg (2), 12.5 μg (3), 6 μg (4), 3 μg (5), 1.5 μg (6), 0.8 μg (7), 0.4 μg (8), 0.2 μg (9); glucose-grown extract 50 μg (10); purified deaminase (—he) 10 ng (11), 50 ng (12). Positions of molecular weight marker proteins are shown on the right. A protein (43-45 kDa)(—he) that is present in both glucose- and GlcNAc-grown extracts is antigenically related to deaminase.
Glc-grown *C. albicans*  

\[ \text{Isolate poly(A)}^+\text{RNA through oligo(dT) Cellulose.} \]

\[ \text{Synthesize single-stranded } ^{32}\text{P-labeled cDNA Probes} \]

\[ \text{Screen replica nitrocellulose filters of } C. albicans \text{ genomic library in YEp13} \]

(1) Select colonies giving signals with only GlcNAc cDNA

(2) Select colonies giving weak signal with Glc cDNA but strong signals with GlcNAc cDNA and

(3) as a control select colonies giving signals with only Glc cDNA.

\[ \text{Rescreen and purify colonies} \]

\[ \text{Isolate plasmid DNA, slot blot and hybridize with Glc and GlcNAc cDNA} \]

\[ \text{Hybrid-selected mRNA translation.} \]

**Fig. 13. Strategy to isolate GlcNAc-inducible genes by differential screening**
3.2 ISOLATION OF GlcNAc-INDUCIBLE GENES FROM C. albicans

3.2.1. Overview

GlcNAc induces the synthesis of its catabolic enzymes permease, kinase, deacetylase and deaminase; experiments with inhibitors of RNA and protein synthesis had indicated that for the appearance of enzyme activities, transcription and translation are necessary. These studies gave us the preliminary idea that genes are regulated at the level of transcription and not by other means such as post-translational events. Since the assays used to measure enzyme levels are not very sensitive, it could not be clearly known if the basal enzyme activities seen in glucose-grown cells is real. So we started with the premise that the mRNA for the enzymes are either absent or present at much lower levels in glucose-grown cells. This differential gene expression, brought about by simple changes in the laboratory growth conditions, such as change in carbon source, is an ideal system to study regulation of gene expression. Cloned genes are prerequisites for these studies and this section focusses on the approach to clone (fig. 1), the differentially expressed genes. The next section describes the cloning of the gene for glucosamine-6-phosphate deaminase, one of the enzymes of the GlcNAc catabolic pathway.
3.2.2 Differential Screening of \textit{C. albicans} Genomic Library

If two populations of mRNA share sequences that are present at different concentrations or if some mRNA species are present in one and absent in other populations, then the genes coding for these mRNAs can be isolated by differential hybridization technique. This approach was first successfully applied in \textit{S. cerevisiae} for isolation of galactose-inducible genes (St. John and Davis, 1979); thereafter success have been reported from other systems (for references, see Sambrook et al., 1989).

3.2.2.1 YEp13 Library of \textit{C. albicans} Genomic DNA

\textit{C. albicans} B792 genomic DNA library (Rosenbluh et al., 1985) was obtained from Y. Koltin. Briefly, genomic DNA was isolated (Riggsby et al., 1982) and was partially digested with Sau3A I. It was then size fractionated and fragments in the 10 kb size range were ligated to BamH I-cut and dephosphorylated YEp13 (Broach et al., 1979). About 16,000 independent transformants were obtained; out of 100 independent colonies, all were tetracycline-sensitive (cloning into the unique BamHI site would render YEp13 tetracycline sensitive) indicating very low frequency of non-recombinants.
3.2.2.2 Poly(A)+ RNA isolation from *C. albicans* SC5314

*C. albicans* SC5314 was grown in GPK broth (see Media and Solutions) until log phase (OD$_{595}$=1.2). Then to one set of flasks glucose was added to 0.1% (w/v) final concentration and to the other set of GlcNAc was added to 0.1% (w/v) final concentration. Growth was continued for another 8h, during which OD$_{595}$ of culture increased from 1.2 to 3.4. To monitor the induction of enzymes, deaminase was assayed from both cell populations; the specific activity in glucose medium was 8.4x10$^{-3}$ units mg$^{-1}$ protein and in GlcNAc medium the specific activity was 0.084 U mg$^{-1}$ protein. So addition of GlcNAc to growing culture brought about ten-fold induction in the level of deaminase; in a different experiment using immunoblot technique, it has been shown that GlcNAc induces the deaminase level by 200-fold (Fig. 12). Total RNA and poly(A)$^+$RNA were isolated as described (Sections 2.13,2.14).

3.2.2.3 Single stranded cDNA Probe Synthesis and Colony hybridization

Single-stranded cDNA probes from glucose- and GlcNAc-poly(A)$^+$ RNA were synthesized as described (see Methods). The specific activity of both probes were about 19 x 10$^6$ cpm/µg cDNA. From the amplified library, about 60,000 colonies plated on LB amp plates were transferred to
duplicate nitrocellulose filters (15,000 colonies per 132 mm filter) as described (see Methods). One set of filters were hybridized to glucose cDNA probe and the other set with GlcNAc cDNA probe at $10^6$ cpm/ml hybridization solution, washed under high stringency (0.1xSSC, 65°, 20 min) and autoradiographed as described (see Methods). A representative result of the first screening is shown in Fig. 14. It can be seen that signals of different intensities were obtained (Table 3). A total of 89 differential GlcNAc-specific signals were obtained; of which 19% belong to category A, 20% to category B, 34% to category C, and 27% to category D. All 89 GlcNAc-specific colonies, 5 colonies giving glucose-specific signals and a control E.coli containing only YEp13 were picked from master plates, subcloned and single colonies were patched onto gridded filter on LB amp. Duplicate filters were again screened with glucose- and GlcNAc cDNA probes. The patches giving GlcNAc-specific signals were again colony-purified, patched onto duplicate nitrocellulose filters and rescreened. Twenty nine GlcNAc-specific clones were obtained.

### 3.2.3 Slot Blot Differential Hybridization

Plasmid DNA was isolated from all GlcNAc-specific clones, four glucose-specific clones and a YEp13 clone. To see
Fig. 14. Differential screening of *C. albicans* genomic library. *C. albicans* genomic library in YEpl3 was plated out to give about 20,000 colonies per plate; colonies were transferred in duplicate to nitrocellulose membranes and DNA was immobilized. ($^{32}$P) dCTP labeled single strand cDNA probes synthesized from Glc-poly(A)$^+$ RNA or GlcNAc poly(A)$^+$ RNA were hybridized to replica filters at 10$^5$ CPM/ml in 50% formamide, 6xSSC at 42$^{\circ}$. Filters were washed at high stringency of 0.1xSSC at 68$^{\circ}$. A typical result containing Glc-specific (G) and GlcNAc-specific signals (arrows) are shown. Many genes are apparently induced by GlcNAc, whereas some are repressed.
Table 3. Differential signals obtained with Glc and GlcNAc cDNA probes hybridized to replica of genomic library

<table>
<thead>
<tr>
<th>Category</th>
<th>Signal Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glc Probe</td>
</tr>
<tr>
<td>A</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>+/-</td>
</tr>
<tr>
<td>C</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>+/-</td>
</tr>
<tr>
<td>E</td>
<td>+++</td>
</tr>
</tbody>
</table>
whether the differential signals obtained during colony hybridization is real, the plasmid DNA (equal volumes of minipreps) was immobilized onto nitrocellulose using a slot-blot manifold in duplicate and probed with glucose- and GlcNAc-specific cDNA probes (Fig. 15). Out of 29 clones obtained by colony hybridization only 17 clones gave GlcNAc-specific signals in slot-blot hybridization. From the differential hybridization it can be inferred that expression of seventeen clones was induced when GlcNAc was added to the culture. Interestingly, some clones were repressed under the same conditions (glucose-specific clones).

3.2.4 Transformation of S. cerevisiae With GlcNAc-Inducible Genes

The deaminase gene was also expected to be present among the GlcNAc-specific clones. To assay for the presence of the gene there are two approaches: (i) if a mutant is available, then complementation of the defect can be attempted. Since such a mutant was not available C. albicans could not be used. (ii) The other alternative is to transform a suitable heterologous host and monitor the expression by either assaying the enzyme activity or detecting the protein by immunoblot analysis.
Fig. 15. Slot blot differential hybridization. Plasmid DNA, isolated from Glc- and GlcNAC-specific clones obtained by colony screening, were bound to nitrocellulose in duplicate and hybridized with Glc-specific (A) or GlcNAC-specific (B) cDNA probes. Specific activity of probes were $2 \times 10^6$ CPM/µg cDNA. The filters were washed in 0.1xSSC at 68°, and exposed to X-Omat film for about 12 h at -70°. A representative result shows both GlcNAC-specific and Glc-specific hybridization. Control YEpl3 did not bind to any probe sequences.
Plasmid DNA from each GlcNAc-specific clone was used for transforming *S. cerevisiae* using lithium acetate procedure (Section 2.22). The transformants were grown in selective medium and cell extracts from all transformants were assayed for deaminase activity. Deaminase activity could not be seen in any of the clones. It is possible that either a full length gene is not present or the protein if expressed is not enzymatically active in a heterologous host. In order to detect if at all any deaminase-related protein is expressed in *S. cerevisiae* transformants, the cell extracts were subjected to SDS-PAGE, protein, transferred to nitrocellulose and probed with deaminase antiserum. Though some non-specific reactivity of the crude antiserum to *S. cerevisiae* proteins were seen, no transformant-specific protein was detected.

Atleast two possibilities existed at this stage: (1) The failure of expression of candidate deaminase gene could be due to the following reasons: (a) Unusual codon usage of *C. albicans* gene would result in poor expression; this may not be true because several *C. albicans* genes have been isolated by complementation of *S. cerevisiae* mutants e.g., URA3 gene (Gillum et al., 1984), HIS3, TRP1 (Rosenbluh et al., 1985), ADE1, ADE2, LYS2 (Magee et al., 1988); (b) *S. cerevisiae* can not utilize GlcNAc as carbon source. If
some activator gene or its product regulates expression of deaminase gene in *C. albicans*, such control might be lacking in *S. cerevisiae*. At present, sufficient results are not there to comment on this possibility. (2) The other possibility is that for some reasons deaminase gene is not represented among the GlcNAc-specific clones. In order to investigate this, hybrid-selected translation was done.

3.2.5 Hybrid-Selected mRNA Translation

To study what kind of polypeptides are coded by the GlcNAc-specific clones, hybrid-selected translation (Ricciardi, et al., 1979) was done as described (Maniatis et al., 1982). Plasmid DNA from all 17 GlcNAc-inducible clones, glucose-specific clones and YEp13 clone were bound to nitrocellulose, and hybridized to total RNA from GlcNAc-induced *C. albicans* (Section 2.2). mRNA hybridized to the bound plasmid DNA were individually eluted and translated in a rabbit reticulocyte translation system (Section 2.2†). The polypeptide sizes encoded by the clones are shown in Table (data kindly provided K. Ganesan and A. Banerjee).

It can be seen that of the 17 GlcNAc-specific clones obtained from slot blot hybridization, four clones (3, 11, 13 and 16) did not give any detectable protein. The other thirteen clones can be grouped into six sets depending on
the kind of polypeptides they code for. Some clones code for more than one polypeptide (Table 4); one possibility is that the insert might contain more than one coding region and this could give multiple bands. Another possibility is that complementary DNA strands could code for different proteins (anti-sense transcription).

Clones 2, 5 and 9 encode a protein of approx. 29.5 kDa and the size of the purified deaminase is approx. 28 kDa (Fig. 5). Since the two sizes are quite close it was expected that these three clones could code for deaminase; the size difference could be due to an electrophoretic artefact. In order to confirm the size of the polypeptide encoded by clones 2, 5 and 9, the experiment was repeated. About 8 µg each of purified plasmids 2, 5, 9 and YEp13 were bound to nitrocellulose and specific mRNA was hybrid-selected and translated in vitro. The translation mix was analyzed on 12% SDS-PAGE. In a parallel lane, purified deaminase (3 µg) was also electrophoresed. The gel was stained and destained to see the positions of marker proteins and that of deaminase (Fig. 16). The translated proteins were visualized after fluorography.

If clones 2, 5 and 9 are true deaminase clones, then the size of polypeptide they encode should correspond to that of
Table 4. Proteins synthesized in vitro from hybrid-selected mRNA

<table>
<thead>
<tr>
<th>Group No.</th>
<th>Clone Number</th>
<th>Polypeptide size (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>1, 8, 10, 12</td>
<td>51, 30</td>
</tr>
<tr>
<td>2.</td>
<td>2, 5, 9</td>
<td>29.5</td>
</tr>
<tr>
<td>3.</td>
<td>4, 14</td>
<td>56, 48, 31.5</td>
</tr>
<tr>
<td>4.</td>
<td>6, 7</td>
<td>40, 32, 28.5, 26</td>
</tr>
<tr>
<td>5.</td>
<td>15</td>
<td>58</td>
</tr>
<tr>
<td>6.</td>
<td>17</td>
<td>39.5</td>
</tr>
<tr>
<td>7.</td>
<td>3, 11, 13, 16</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>YEp13 and Glc-specific clones</td>
<td></td>
</tr>
</tbody>
</table>


Fig. 16. Hybrid-selected Translation. Total RNA isolated from GlcNAc-induced *C. albicans* was hybridized with plasmid DNA bound to nitrocellulose membrane. Hybridization was in 65% formamide, at 50°C for 3h. Filters were washed and specific mRNA was eluted and translated in rabbit reticulocyte lysate. 35S-labeled translation products from a reaction without any added RNA (lane E), or programmed with RNA recovered after hybridization (lane H), or with total RNA before hybridization (lane T), or with mRNA eluted from immobilized YEpl3 (lane C), or from GlcNAc-specific clones 2, 5 and 9 (lanes 2, 5 and 9) were resolved on 12% SDS-PAGE and detected by fluorography. Purified deaminase (5 μg) resolved in an adjoining lane was detected by coomassie blue staining and its position in the gel is indicated ( ). Molecular weight of bands seen in the fluorograph is shown on the right.
deaminase. However, they code for a polypeptide slightly larger (1.5 kDa) than deaminase. That the size of purified deaminase is not an artefact, due to degradation or some modifications during purification step, is ruled out from immunoblot experiments (see Fig. 12); mobility of purified deaminase and the deaminase from GlcNAc-grown cell extracts is identical. This showed that deaminase in the cytosol of *C. albicans* is of the same size as that of the purified enzyme. Is it possible that there is a precursor for deaminase that is not processed in vitro? In vitro translation of total RNA from glucose- and GlcNAc-grown cells and immunoprecipitation revealed a polypeptide from GlcNAc RNA that migrates to a similar position in 12% SDS-PAGE, as the purified deaminase (Fig. 17). A precursor protein for a cytosolic protein is uncommon and together with the results of the immunoblot analysis and immunoprecipitation experiment, it was clear that clones 2, 5 and 9 are not related to deaminase and are some other GlcNAc-inducible clones, whose identity is presently not known. The other GlcNAc-inducible clones could represent genes coding for other enzymes of the GlcNAc catabolic pathway and certain other inducible genes hitherto unknown.
Fig. 17. SDS-PAGE of in vitro translated proteins Total RNA isolated from C. albicans grown in peptone-KH$_2$PO$_4$ medium with either glucose or GlcNAc, was translated in vitro in a rabbit reticulocyte lysate system. Equal volumes of translated products were either directly analyzed (Total: glucose, G (35,000 dpm); GlcNAc, N (20,000 dpm)) or immunoprecipitated (Immunoppt: glucose, G (840,000 dpm); GlcNAc, N (535,000 dpm)) using immobilized deaminase-antibodies and analysed on 12% SDS-PAGE, with purified deaminase (6 µg) in an adjoining lane. Gel was stained with coomassie blue R 250 to visualize molecular weight standards and purified deaminase, fluorographed at -70$^\circ$ for 2 days (Total) or 15 days (immunoppt). Position of purified deaminase (28 kDa) is indicated with an arrow head (►). Positions of molecular weight standards (Sigma) are shown on the right. In immunoprecipitated samples, a 40 kDa band is seen in both lanes, and 37, 35 and 28 kDa bands are seen only in GlcNAc lane.
3.3 CLONING AND CHARACTERIZATION OF GLUCOSAMINE-6-PHOSPHATE DEAMINASE GENE

3.3.1 Strategy for Isolation of Glucosamine-6-Phosphate Deaminase Gene

Several options are available for isolation of genes from a library (Kimmel, 1987). They are nucleic acid hybridization (using either heterologous gene probe or synthetic oligonucleotide probes derived from the amino acid sequence of the proteins), differential hybridization, complementation of mutants and screening of an expression library with antibody probes. Genetic complementation of mutants has been successful in E. coli, and several fungi including Saccharomyces cerevisiae. As discussed elsewhere (Section 1.2.4), C. albicans is naturally diploid and therefore stable mutants are difficult to obtain. Efforts from this laboratory to isolate mutants defective in GlcNAc utilization was not successful (K. Ganesan and A. Banerjee, personal communication). So, cloning of gene by complementation was not possible. Differential hybridization, though gave several GlcNAc-inducible clones (Section 3.2.3) hybrid selected translation revealed that none of the clones encoded proteins of the size of deaminase (Fig.16).
3.3.1.1 *E. coli* Deaminase Gene Does Not Hybridize To *C. albicans* Genomic DNA

Recently, glucosamine 6-phosphate deaminase gene, nag B, has been isolated from *E. coli* (Rogers et al., 1988). A comparison of the N-terminal amino acid sequence of the proteins from *E. coli* and *C. albicans* (Fig. 8) indicate that they do not share a common region in the stretch compared. However, it is possible that some conserved domains could be present at other regions of the protein, for example at the catalytic site. *C. albicans* genomic DNA (2 μg each) was digested with EcoRI or ClaI (EcoRI has three sites and ClaI has no site within *E. coli* deaminase gene), resolved in 1% agarose gel and DNA fragments were transferred to Gene Screen Plus membrane as described. *E. coli* deaminase gene (nag B) was $^{32}$P-labeled by random primer method as described, and hybridized to the blot in 1M NaCl, 50% formamide at 37°C for 26 h. The blot was then washed in 2xSSC at 47°C for 60 min with three changes. On autoradiography, even after 3 days, no hybridization was seen. Under the same conditions, the probe hybridized strongly to the SspI-ClaI double digest of the plasmid pB35-25, from which the probe DNA was derived (Rogers et al., 1988), indicating that the hybridization experiment had worked. Lack of hybridization of the nag B probe to *C. albicans* genomic DNA, under the stringency used,
indicated that there is no appreciable stretch of homology. So, use of a heterologous probe to isolate glucosamine-6-phosphate deaminase gene was not a feasible approach.

3.3.1.2 Immunoscreening Of Expression Library, The Right Approach

Since deaminase antibody was available (Section 2.8), a more direct approach was to screen an expression library. Two types of expression libraries can be used for C. albicans: cDNA and genomic DNA library. Since deaminase is expressed at about 0.4-0.5% of the total protein when C. albicans is induced with GlcNAc, use of cDNA library was preferred. Assuming a transcriptional control of deaminase expression, deaminase mRNA should be present at high abundance; even if present at an abundance of 0.1% of the total mRNA, the minimum number of recombinants to be screened is only about $6 \times 10^3$. In a genomic expression library, on the other hand, a 1 kb gene from C. albicans can be expected to express as a fusion protein only once in every $10^5$ recombinants. So, for a gene expressed between high and a medium level, a cDNA expression library seems to be the best approach particularly if the number of recombinants is not large.

$\lambda gt11$ was constructed by Young and Davis (1983). It has a unique EcoRI site near the 3' end of the lacZ gene, 53 base
Isolate poly(A)+ RNA from GlcNAc-induced *C. albicans*

Synthesize Oligo (dT)-primed first strand cDNA

Convert to ds cDNA by RNase H - DNA Pol I method.

Treat with EcoR I Methylation to block EcoR I sites

Treat with T4 DNA Pol and Klenow to create blunt-ended ds cDNA

Add EcoRI linkers

Treat with EcoR I to generate ds cDNA with EcoR I termini

Purify EcoR I-ended ds cDNA from linkers

Ligate

Package Recombinant λgt11 DNA

Infect E. coli Y1090

Induce fusion proteins with IPTG, transfer to NC and immunodetect with deaminase antiserum.

Fig. 18. Strategy for construction and screening of λgt11 cDNA expression library.
pairs upstream from the \( \beta \)-galactosidase translation termination codon. Foreign DNA inserted into the unique EcoRI site have the potential to be expressed as a \( \beta \)-galactosidase fusion protein which confers enhanced stability to the foreign protein. Also, foreign genes under \textit{lac} Z control have a better chance of being expressed in \textit{E. coli}.

Advantages of expression vectors are: a) in vitro packaging reaction and infection of \textit{E. coli} are highly efficient, and would aid cloning of rare or low abundance sequences; this should help in the construction of high complexity libraries. b) antibody screening of plaques give much better signal/noise ratio than immunoscreening the colony blot (Snyder et al., 1987). c) \textit{Agt11} fusion proteins constitute between 0.1\% and 0.4\% of the total \textit{E. coli} proteins. The various steps in the construction of \textit{Agt11} cDNA expression library are shown in Fig. 18.

3.3.2 Construction of \textit{Agt11} cDNA Expression Library

3.3.2.1 Isolation of poly(A)+RNA.

\textit{C. albicans} SC5314 was grown in yeast nitrogen base broth (without amino acids) containing GlcNAc 0.5\% w/v) as the carbon source. Under these conditions, deaminase is present at an abundance of approx. 0.5\% in the total
protein extract, as determined by Western blot (Fig. 12). Cells were rapidly harvested when OD595 of culture reached 1.3 (ca. 9.4 x 10^7 cells/ml), washed with sterile water and total RNA was isolated by guanidine thiocyanate method (see Methods). A typical result of Glyoxal-denatured total RNA is shown (Fig. 19). Two major RNA bands around 3.5 kb and 1.8 kb are large and small rRNA respectively. 5.5 S rRNA and tRNA have a size around 0.3-0.4 kb. Two other bands close to the two rRNA possibly could be mitochondrial rRNAs. Poly(A)^+ RNA was purified from total RNA by two cycles of oligo-(dT) cellulose chromatography as described by Okayama et al. (1987). Starting from 2 mg total RNA, the yield of poly(A)^+ RNA was about 100 µg, at an abundance of about 3-5%. A260/A280 ratio was about 2.0, indicating very low protein contamination. Integrity of poly (A)^+ RNA preparation was checked by translation in a rabbit reticulocyte cell free system (see Methods).

3.3.2.2 Double Stranded cDNA Synthesis and Cloning into λgt11

Starting from 5µg poly (A)^+ RNA, approx. 4µg of ds cDNA was obtained. ds cDNA that was treated with methylase, ligated to EcoRI linkers and cut with EcoRI (ready to be cloned) was analyzed on non-denaturing 2% agarose gel (Fig. 20). It covers a size range of 0.2-4.5 kb with an average size
Fig. 19. Agarose Gel Electrophoresis of Glyoxal-Denatured RNA. Total RNA (20 μg each) from C. albicans grown on glucose or GlcNAc, were denatured with glyoxal. DNA standards, (HindIII fragments of DNA, BRL) and the samples were resolved in 1.5% agarose gel and were visualized by ethidium bromide staining.
Fig. 20. Size distribution of double stranded cDNA. Double stranded cDNA was synthesized from poly(A)$^+$RNA isolated from GlcNAc-grown *C. albicans*. ds cDNA was methylated, EcoRI linkers added and digested with EcoRI. EcoRI-ended ds cDNA was resolved in non-denaturing 2% agarose gel and the size estimated from the mobility of HindIII-digested $\lambda$ DNA standards.
around 0.8 kb. The efficiency of cDNA cloning was about $10^6$ plaque forming units/µg cDNA, using packaging extracts (Amersham) with an efficiency of $6 \times 10^7$ plaque forming units/µg control vector DNA.

3.3.2.3 Immunoscreening of λgt11 Plaques With Deaminase Antiserum

110,000 plaques were plated out at a density of 100 plaques/cm² and fusion proteins were induced by overlaying plaques with IPTG-saturated nitrocellulose filter circles. Proteins that were transferred from plaques were then reacted with deaminase antiserum at a dilution of 1:5000. The antigen-antibody complexes were then detected using anti-rabbit IgG- alkaline phosphatase conjugate. A total of seven positives were obtained, with plaque color intensity ranging from deep purple to very light purple and this can be seen from the picture of the blots from final screening (Fig. 22).

Fig. 21 is a representative result of immunoscreening of the λgt11 primary library. A plaque showing positive signal is shown by an arrow. The plaques lighting up in the background are due to cross reactivity of rabbit antiserum to E. coli proteins (Huynh et al., 1985; Snyder et al., 1987). In a pilot experiment, it was seen that deaminase
Fig. 21. Immunoscreening of λgt11 cDNA expression library. 20,000 primary plaques were induced with IPTG, proteins transferred to nitrocellulose and immunodetected with deaminase crude antiserum. Note the overall reactivity of the crude antiserum to plaques. Arrow shows a positive signal on the plaque lift.
antiserum has considerable reactivity to \textit{E. coli} proteins. This can be circumvented by adding excess \textit{E. coli} lysate to diluted antiserum prior to screening (Super Screen Immunoscreening Protocol, Amersham). But treatment of deaminase antiserum with \textit{E. coli} lysate did not remove the reactivity even after addition of excess amount of lysate (0.5 mg/ml). In a separate experiment it was observed that affinity-purified deaminase-specific antibody reacted with a protein of 50 kD from \textit{E. coli}; the background reactivity can be attributed to the binding of antibody to this protein from \textit{E. coli}.

The positive signals were then replated in the range of 80-350 plaques per 90 mm plate. On screening these plaques as described above, positives were obtained from all the clones; the frequency of positive plaques ranged from 8-35\% of the plaques screened. The plaques giving positive signals during the second screening were replated, which was about 10-90 plaques per 90 mm plate. These were then rescreened as described. All the positive plaques from first screening consistently gave signals and the signal intensity was also consistent. A representative area from the final plaque screening from all plates are shown (Fig.22). All the plaques were clonally pure at this stage. Intensity of signals from different clones on a
Fig. 22. Final screening of deaminase-antibody reactive clones. About 30-100 plaques were induced with IPTG for fusion protein expression and proteins were transferred to nitrocellulose and immunodetected with deaminase antiserum at 1:5000 dilution. 1 to 7 are clone numbers and 'C' is control (λgt11). Different signal intensities were obtained and all plaques appeared to be clonally pure.
relative scale with control (λgt11) as reference (1+). 
are: clone 1 (5+), clone 2 (6+), clone 3 (3+), clone 4 (5+), clone 5 (2+), clone 6 (4+), clone 7 (1+). Phages from all these pure plaques were used for further work.

3.3.3 Analysis of Fusion Proteins from Recombinant Clones. 
3.3.3.1 Preparation of lysogens: A crude lysate containing a particular recombinant antigen can be prepared by expressing a λgt11 recombinant as a lysogen in E. coli Y1089. The features of this strain are: a mutation hflA150, which enhances the frequency of phage lysogeny; a deletion in the lon protease gene which enhances the stability of foreign proteins; presence of lacI gene coding for lac repressor which prevents lacZ-directed gene expression until derepressed by the addition of IPTG (Huynh et al., 1985).

Phages from the purified plaques were used to infect Y1089 at a multiplicity of infection of five as described (Huyuh et al. 1985); cells were then plated and allowed to grow at 32°C. Then single colonies from all clones were individually patched onto a grid plate in duplicate. One plate was incubated at 42°C and the other at 32°C. About 2-8% of the colonies were lysogens, as judged by their inability to grow at 42°C.
3.3.3.2 Western Blot Analysis of Fusion Proteins from Lysogens

Fusion proteins were prepared from lysogens as described. Equal volumes of crude lysate (10 μl each) were resolved in SDS-PAGE (5%) transferred to nitrocellulose and immunodetected as described, with crude deaminase antiserum at a dilution of 1:5000 (results not shown). The size of fusion proteins are shown in Table 5. Clones 6 and 7 did not show specific immunoreactive proteins.

In a similar experiment, but using affinity-purified deaminase-specific antibody (Fig. 23), no cross-reacting proteins could be seen with clones 3 and 5, indicating that these two clones are unrelated to deaminase. So, from a total of 7 positive signals, only three clones gave fusion proteins that reacted with specific antibody. Of the others that did not give any bands on the Western blot, clones 3 and 5 are false positives, clones 6 and 7 (and λgt11) did not give any immunoreactive fusion proteins even with crude antiserum.

3.3.4 Analysis of Insert DNA from Deaminase Antibody-Reactive Clones

3.3.4.1 Determination of Size of the Insert.

The usual practice for determination of insert size involved digestion of the clone DNA with appropriate restriction
TABLE 5. Specificity of the fusion protein immunoreactivity

<table>
<thead>
<tr>
<th>Clone No.</th>
<th>Fusion protein size (kDa)</th>
<th>Immunoreactivity</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>with deaminase</td>
<td>with</td>
<td>affinity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>antiserum purified</td>
<td>antibody</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>125</td>
<td>+</td>
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<td>+</td>
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<tr>
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<td>+</td>
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<td>+</td>
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<tr>
<td>5</td>
<td>138</td>
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<tr>
<td>6</td>
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<td>7</td>
<td>-</td>
<td>_1</td>
<td>_1</td>
<td>-</td>
</tr>
<tr>
<td>λgt11</td>
<td>-</td>
<td>_1</td>
<td>_1</td>
<td>-</td>
</tr>
</tbody>
</table>

*Only an overall reactivity to E. coli proteins was seen using deaminase crude antiserum.*
Fig. 23. Fusion protein analysis by Western blot. *E. coli* Y1089 lysogenized with recombinant phages (lanes 1-7 represent clone nos.), and λgt11 (C, control) were induced with IPTG. Equal volume of crude extracts of the lysogens were separated on 5% SDS-PAGE, blotted to nitrocellulose and immunodetected with affinity-purified deaminase antibody. Fusion proteins are seen only in clones 1, 2 and 4. Size of fusion proteins is shown on the left.
enzymes, resolving the vector from the insert on agarose gel and visualizing the insert by ethidium bromide fluorescence. This method is alright if the ratio of insert size to vector size is high.

In \( \lambda \)gt11 cDNA library the average size of the insert (if the cDNA has not been size fractionated as in this case) would be 0.5 kb; this is approximately 1% of the vector size. Consequently much more DNA has to be loaded to see the insert. To circumvent this problem, it has been suggested to end-label the EcoRI-termini specifically using \((\alpha^{32}P)dATP\) and Klenow polymerase (Amersham cDNA cloning kit \( \lambda \)gt11 manual). Since the vector and insert would be radiolabeled at the termini, the intensity of the vector and insert bands would be comparable (Section 2.28).

DNA was isolated from \( \lambda \)gt11 cDNA clones as described (see Methods) and about 50 ng each were digested with EcoRI and end labeled as described (Amersham cDNA Cloning System Manual). The fragments were then resolved on 2% agarose gel, TCA fixed, dried and autoradiographed at -70° with screen. Fig. 24 shows the labeled insert DNA from the clones. Clones 1, 2 and 4 that gave fusion proteins (Fig.23) had insert sizes of 0.23, 0.29 and 1.3 kb \((0.67 + 0.62)\) respectively. Clone 4 when digested with EcoRI, gave
only a single insert fragment in the end laneled analysis (Fig. 24). However two bands were seen when a Southern blot containing EcoRI-cut DNA from clones, was self-hybridized with clone 4 probe derived from its gel purified insert (Fig.25). It appears that the two fragments (0.67 and 0.62 kb) were co-migrating in the gel used for end-labeled insert analysis (Fig. 24). Clone 6 had an insert of 0.78 kb (Fig.24). The position of vector EcoRI cut and labeled vector arms is indicated on the side. Here the difference in intensity of vector and insert fragments can be explained if there was a partial EcoRI digestion of the clones. The other partial digestion products have possibly not been resolved in 2% gel. No insert is seen for clone 7; this could be because either EcoRI has not cut at all or this clone might well be a non-recombinant phage (cf. plaque signal intensity between Fig.22(7) and22(C)). All the positive clones have been designated by a prefix λ followed by d, for deaminase and the respective clone numbers; for example clones 1,2,4 and 6 would be λd1, λd2, λd4 and λd6.

3.3.4.2 Southern Blot Analysis: Atleast Two Sets Of Clones are Present

To establish the relatedness of the clones, insert DNA from clones λd1, λd2, λd4 and λd6 were labeled and separately used for Southern hybridization. DNA from all the clones
Fig. 24 Determination of insert size. About 200 ng of phage DNA from different clones were digested with EcoRI. The EcoRI ends were specifically labeled with ($\alpha^{32}$P)dATP and Klenow and were resolved in 2% agarose gel. Fragments in the gel were fixed, dried and autoradiographed at -70° with intensifying screen. The background smear migrating ahead of the 0.23 kb fragment is probably degraded chromosomal DNA which are also $^{32}$P-labeled after EcoRI digestion. The intense band near the top, seen in all lanes, is due to end-labeled vector arms.
were EcoRI digested, electrophoresed and immobilized to Gene Screen Plus membrane. \( \lambda d_1 \) and \( \lambda d_2 \) probe hybridization pattern (Fig. 25 A,B) indicates that \( \lambda d_1 \) and \( \lambda d_2 \) are related to \( \lambda d_6 \). \( \lambda d_4 \) probe does not hybridize to any other clones (Fig. 25C) indicating that this is a unique clone. However, \( \lambda d_6 \) probe hybridises to \( \lambda d_1 \) and \( \lambda d_2 \) (Fig. 25C), indicating that they are all related. \( \lambda d_4, \lambda 3 \) and \( \lambda 7 \) are unique and are not related to the other group of clones.

Though \( \lambda d_1 \) and \( \lambda d_2 \) are related to \( \lambda d_6 \), they do not show any appreciable homology to each other. Since first strand cDNA was synthesized using oligo (dT) primers, related clones are expected to share a common 3'-end of the mRNA. But it appears that one of the two clones \( \lambda d_1 \) and \( \lambda d_2 \) was initiated at an internal site during reverse transcription. Yeast mRNA in general is reported to have between 40-60 adenosine residues as part of poly (A) tail (McLaughlin et al., 1973; Reed et al., 1973). Only a minimal number of adenosine residues may be required at the reaction conditions used (42°, approximately 0.1 M KCl), for the formation of stable hybrids and serve as primer for reverse transcription; it may not be very improbable to expect about 3-4 lysine codons (AAA) in the coding region. Poly(A)\(^+\) tail may not be the only site for oligo(dT) primer hybridization and situations such as the one described could
Fig. 25. Southern blot analysis of relatedness of λgt11 cDNA clones. Recombinant phage DNA were treated with EcoRI and separated on a 2% agarose gel. Fragments were blotted to Gene Screen Plus and probed with 32P-labeled insert from clones λd1 (A), λd2 (B), λd6 (C) and λd4 (D). Size of the hybridizing inserts are shown in the centre. λgt11 vector arms (V) also hybridize to the probe due to small amounts of vector contamination in the insert used as probe. The blot was hybridized first with λd4, stripped off the probe and the same blot was rehybridized sequentially with λd2, λd1 and λd6. Hybridization was in 1M NaCl, 10% Dextran sulfate, 50% formamide, with random-primer labeled probes at 0.4x10⁵ CPM/ml, at a specific activity of 5x10⁹ CPM/µg. Blots were exposed for about 3-10 h.
potentially serve as sites for internal initiation of reverse transcription. The hybridization pattern of \( \lambda d_1 \), \( \lambda d_2 \) and \( \lambda d_6 \) reveal that \( \lambda d_1 \) and \( \lambda d_2 \) are subsets of the larger clone \( \lambda d_6 \); they are homologous to different regions of \( \lambda d_6 \), and one of the two being internally initiated.

3.3.5 \( \lambda d_6 \) Does Not Express Fusion Protein

From the Southern hybridization experiments it is clear that \( \lambda d_6 \), \( \lambda d_1 \) and \( \lambda d_2 \) are related clones, but it is intriguing as to why no fusion protein was seen with \( \lambda d_6 \), using either deaminase crude antiserum or the specific antibody.

One of the reasons could be poor or no induction of phages from lysogens. Even when the entire experiment was repeated, no fusion protein was detected. When lysogens were induced for fusion protein analysis (Fig. 23), phages were taken, lysed with phage cracking buffer (25mm EDTA, 0.5% (w/v) SDS, 15% (v/v) glycerol and 0.003% (w/v) bromophenol blue) and analysed on agarose gel (Data not shown). Phage DNA could be seen, indicating that phages were induced from lysogens. Though \( \lambda d_6 \) phage was produced, fusion protein was apparently not synthesized. Under the same conditions, fusion proteins have been expressed from other lysogens. \( \beta \)-galactosidase might still be expressed
well but if a translation stop codon is encountered in the 5'- untranslated region (Haymerle et al. 1986) of the insert cDNA, translation would cease after β-galactosidase. Since, λd6 clone has been obtained after repeated selection by antibody screening of plaques, the following explanation is offered: Plaque screening was done using E. coli Y1090 as host. Y1090 has a mutation, supF, due to which amber (UAG)-suppressor tRNA is expressed; whereas lysogens were made in E. coli Y1089 which lacks the suppressor tRNA. If a translation stop codon (UAG) is present in the deaminase mRNA (in clone λd6), then the amber-suppressor tRNA (in supF host) would help to alleviate the defect in Y1090 and not in Y1089. If anti-β-galactosidase antibody is used to probe a blot containing extracts from lysogens, then lacZ gene expression can be monitored. Another approach is to use a supF host to make lysogens and analyze the fusion proteins on Western blot; also plaques can be used as a source of fusion proteins and analyzed by Western blot (Saul and Yeganeh, 1986).

3.3.6 Northern Blot Analysis Reveals Pattern of Expression

In order to identify the deaminase clone, pattern of expression of the clones were studied using Northern blot analysis. Deaminase is synthesized only in presence of GlcNAc as a carbon source and the protein level is induced
over 200-fold. *In vitro* translation and immunoprecipitation experiments reveal that deaminase mRNA is absent in cells grown with glucose, whereas it is seen in cells grown with GlcNAc as carbon source (Fig.17). This indicates that deaminase gene is regulated at the transcriptional level.

So, RNA levels were analysed by Northern blot, using the cloned DNA as a probe. Fig.26 shows the pattern of hybridization of the probes from $\lambda d_1$, $\lambda d_2$, $\lambda d_4$ and $\lambda d_6$ in separate experiments. $\lambda d_4$ probe picks up a mRNA of 2 kb that is constantly expressed in glucose-and GlcNAc-grown cells. The other three clones detected a single mRNA of 0.9 Kb that is expressed only in presence of GlcNAc as carbon source. The amount of RNA loaded in each lane was seen by ethidium bromide staining of the gel (Fig.26). 20 $\mu$g glucose and 15 $\mu$g GlcNAc total RNA was used for blot experiments and for ethidium bromide staining. It can be seen from ethidium bromide staining pattern, almost equal amount of RNA was loaded. So the signal intensity in the two lanes should actually reflect the level of expression of the particular mRNA. Thus it is clear that $\lambda d_1$, $\lambda d_2$ and $\lambda d_6$ all code for a mRNA of about 0.9 Kb, whose transcription is induced by GlcNAc; whereas $\lambda d_4$ code for a mRNA of about 2 kb that is transcribed in both glucose- and GlcNAc-grown cells.
Fig. 26. Pattern of expression of clones analyzed by Northern blot. Total RNA was isolated from *C. albicans* SC5314 grown in peptone, KH$_2$PO$_4$ medium with either GlcNAc or Glc as carbon source. 15 µg GlcNAc RNA and 20 µg Glc RNA were resolved in 1.5% agarose gel, blotted to Gene Screen Plus and hybridized with random-primer labeled probe. For hybridization with Ad1, Ad2 and Ad4 probes, and for ethidium bromide staining, RNA samples and DNA standards were glyoxalated and resolved in agarose gel. For hybridization with Ad6 probe, RNA samples were denatured with 2.2 M HCHO and resolved in HCHO-containing gel. Size of hybridizing RNA species was estimated using glyoxal-treated DNA fragments as standards. In an independent experiment, the size of Ad6 hybridizing RNA was estimated to be 0.9 kb using Glyoxal system (not shown).
<table>
<thead>
<tr>
<th>PROBE</th>
<th>RNA SIZE</th>
</tr>
</thead>
<tbody>
<tr>
<td>λd1</td>
<td>0.9 kb</td>
</tr>
<tr>
<td>λd2</td>
<td>0.9 kb</td>
</tr>
<tr>
<td>λd6</td>
<td>0.9 kb</td>
</tr>
<tr>
<td>λd4</td>
<td>2.0 kb</td>
</tr>
<tr>
<td>Et Br stain</td>
<td>3.2 kb (Large rRNA)</td>
</tr>
<tr>
<td></td>
<td>1.8 kb (Small rRNA)</td>
</tr>
</tbody>
</table>
3.3.7 Genomic DNA Blot Hybridization

The hybridization pattern of different clones to *C. albicans* genomic DNA blots is presented in Fig. 27. The results show that all the clones are complementary to sequences in the genomic DNA. \( \lambda d4 \), and \( \lambda d6 \) and its subsets are apparently single copy genes because with five different enzymes they gave a unique fragment; if more than one gene is present then the size of the hybridizing fragment would not be constant due to variation in the sequence of the DNA in its neighbourhood. The hybridization pattern of \( \lambda d4 \) is different from that of \( \lambda d6 \) and its subsets, confirming the observation that these two sets of clones are unrelated. \( \lambda d4 \) hybridization reveals an internal site each for EcoRI and HaeIII. The two EcoRI fragments migrated very closely; with a lesser agarose concentration, the two bands could be resolved better. Two fragments of approximately 0.67 and 0.62 kb can be seen in the blot hybridization of clones (Fig. 25). The presence of internal EcoRI site shows that EcoRI methylase treatment had completely protected the site during library construction. The two fragments of nearly equal size are not unrelated inserts, because when the same probe \( \lambda d4 \) is used for genomic blot hybridization, it gave a single band with BamHI, HindIII, PstI, SspI and XbaI-digested genomic DNA (Fig. 27).
\( \lambda d_1, \lambda d_2 \) and \( \lambda d_6 \) hybridizations gave interesting results (Fig. 27). A single fragment was seen in each lane corresponding to genomic DNA digested with BamHI, EcoRI, HaeIII, HindIII, SspI, and XbaI. When PstI-treated genomic DNA blot is hybridized with \( \lambda d_1 \), a 8.6 kb fragment is seen; with \( \lambda d_2 \) probe a 3.3 kb fragment is seen; but when \( \lambda d_6 \) probe is used, it picks up two fragments of 8.6 kb and 3.3 kb (Table 6).

These results confirm the observation that \( \lambda d_1 \) and \( \lambda d_2 \) are subsets of \( \lambda d_6 \); there is a PstI site that apparently separates the two smaller clones. Either the PstI site is not there in the smaller clones or is present at one of the ends; the latter possibility could result in a small region of homology and it might be missing under the stringency used. The difference in signal intensity of the 8.6 and 3.3 kb fragments with \( \lambda d_6 \) probe could be due to non-uniform transfer of larger and smaller fragments from gel during blotting. The other possibility of them being partial digestion products is not true because \( \lambda d_1 \) and \( \lambda d_2 \) hybridization picks up only one of the two fragments.

These possibilities can be verified by restriction enzyme mapping of all the clones. Hae III-digested genomic DNA blot hybridized with the probe derived from \( \lambda d_6 \) reveals a
Fig. 27. Genomic DNA blot hybridization. *C. albicans* SC5314 DNA (1 µg each) treated with BamHI, EcoRI, HaeIII, HindIII, PstI, SspI or XbaI was resolved on a 1.2% agarose gel. DNA fragments were blotted to Gene Screen Plus membrane and probed sequentially with $^{32}$P-labeled insert from clones λd1, λd2, λd6 and λd4, stripping the probes off the membrane each time before rehybridization. Positions of DNA size markers (λHindIII and 1 kb ladder) are shown in the centre. Blots were exposed for 6-12 h to X-Omat film at -70° with intensifying screen.
TABLE 6. Size of DNA fragments obtained during genomic DNA blot hybridization with probes from various clones

<table>
<thead>
<tr>
<th>Enzymes used</th>
<th>Fragment size (kb) picked up by probe derived from clone</th>
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<tbody>
<tr>
<td></td>
<td>λd1</td>
</tr>
<tr>
<td>BamHI</td>
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</tr>
<tr>
<td>EcoRI</td>
<td>13.3</td>
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<tr>
<td>HaeIII</td>
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<tr>
<td>HindIII</td>
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<tr>
<td>Pst I</td>
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<tr>
<td>Ssp I</td>
<td>2.95</td>
</tr>
<tr>
<td>Xba I</td>
<td>4.5</td>
</tr>
</tbody>
</table>
0.64 kb fragment; since this size is smaller than that of the insert DNA from this clone, there is at least one internal Hae III site (Fig. 24 and Table 6). Conclusions from the genomic blot hybridization are: \( \lambda d_1 \) and \( \lambda d_2 \) are subsets of \( \lambda d_6 \); there is an internal Hae III and PstI site in \( \lambda d_6 \) and an EcoRI and HaeIII site in \( \lambda d_4 \); \( \lambda d_4 \) and \( \lambda d_6 \) are single copy genes; and the EcoRI ends of all these inserts are from linkers as can be seen from the size of EcoRI fragments in the genomic DNA.

### 3.3.8 \( \lambda d_6, \lambda d_2 \) and \( \lambda d_1 \) are the Candidate Deaminase cDNA Clones

A near full length deaminase cDNA clone has been isolated. Evidences in favour of this conclusion are:

1. The affinity-purified deaminase antibody reacts with only two proteins from \( C. albicans \) extracts: 29 kDa deaminase, which is induced over 200-fold by GlcNAc and a 45 kDa protein, whose expression is constant in presence of glucose and GlcNAc (Fig. 12).

2. The fusion proteins react with affinity purified deaminase antibody.

3. \( \lambda d_1 \), \( \lambda d_2 \) and \( \lambda d_6 \) are related clones and \( \lambda d_6 \) is the largest.
(4) All three clones hybridize to \textit{C. albicans} genomic DNA indicating their common origin.

(5) Expression of $\lambda d_1$, $\lambda d_2$ and $\lambda d_6$ are tightly regulated; GlcNAC is essential for the transcription of deaminase mRNA.

(6) Expression of $\lambda d_4$ is not affected by GlcNAC (Fig. 26).

(7) GlcNAC-induced RNA of 0.9 Kb can code for a protein of 29 kDa.

(8) The largest clone $\lambda d_6$ has an insert of 0.78 kb indicating that it is a near full length cDNA of the 0.9 Kb deaminase mRNA.

3.3.9 Confirmation of the Validity of Clones

Two independent approaches that have been taken to identify the deaminase clone are: (1) Use of affinity purified antibody against fusion proteins and (2) study of the levels of RNA under induced and uninduced conditions, assuming a transcriptional regulation of gene expression. Kimmel (1987) has suggested "keeping your plasmids circular and your arguments linear", wherein best confirmation of clones come from evidences utilizing diverse properties of the gene or its gene products. Accordingly, further work needed to confirm the deaminase clone are: (1) DNA
sequencing and comparison of the deduced amino acid sequence with the N-terminal sequence of deaminase (Fig. 8). (2) Hybrid-selected translation of specific mRNA using the clone DNA and immunoprecipitation. (3) Expression of the 0.78 Kb clone in *E. coli* or in yeast with the help of suitable expression vectors and assay for enzyme activity.

### 3.3.10 Conclusions

Addition of GlcNAc to *C. albicans* growth medium has been shown to result in a rapid induction of deaminase, the terminal enzyme of the GlcNAc metabolic pathway. The expression of deaminase (and other enzymes of the pathway) is tightly regulated; addition of GlcNAc to *C. albicans* culture results in about 200-fold increase in deaminase level as compared to glucose-containing culture. In glucose-grown cells, there is no detectable deaminase mRNA and its synthesis is initiated only on addition of GlcNAc to the culture. Though the kinetics of transcription is yet to be studied, results from the protein blot analysis and the RNA blot analysis indicate that mRNA should also be rapidly transcribed on addition of GlcNAc.

In *C. albicans* the control of expression of the catabolic enzymes is brought about by GlcNAc. Uptake of GlcNAc into the cells is necessary for the induction of the enzymes.
(Sullivan and Shepherd, 1982). Since GlcNAc induces the expression of all catabolic genes, it is likely that a master control gene (MCG) is involved. The rapid kinetics of expression of the catabolic genes suggest that the MCG product might be constitutively present but inactive in cells, and GlcNAc would directly or indirectly activate the MCG product; this then would turn on the expression of the target genes. If this is true then the regulatory MCG product is expected to be a DNA-binding protein. As future plan, upstream sequences from deaminase genomic clone can be used to detect the DNA-binding protein by gel shift assays and by DNase foot printing technique.