Nuclear magnetic resonance spectroscopy and cyclic AMP studies of Candida albicans
by Pamela Jane Purus

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in MICROBIOLOGY
Montana State University
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Abstract:
Candida albicans is a polymorphic and opportunistic fungus. As a saprobe C. albicans exists in the yeast (Y) form. Upon tissue invasion, mycelial (M) forms are produced. Cyclic AMP is one of many factors that have been studied as possible initiation factors in morphogenesis. However the methods used have involved environmental parameters that do not reflect the in. vivo situation. Through the presence of an inhibitor of morphogenesis, morphogenic autoregulatory substance (MARS) produced by Y form cells the effects of cAMP were reevaluated by a system that more closely reflects the in vivo situation. In comparing levels of cAMP, no difference was found between the M and Y forms at the early time periods. There was an increase in the M form over the Y after morphogenesis was completed. These data indicate that cAMP is probably not important as an initiation factor in morphogenesis. Dimorphism assays are usually cell destructive techniques. Nuclear magnetic resonance (NMR) spectroscopy is an alternative method that allows live cells to be studied with out disrupting cellular integrity. The Y and M forms were found to be distinguishable from each other by NMR. Spectra obtained from 10 min incubated cells was different for the M and Y forms. However identification of specific peak differences was not possible because of reproducibility problems encountered. Therefore base line studies of the NMR spectra of yeasts were begun. C. albicans, C. tropicalis. C. krusei, C. guillermondii, C. pseudotropicalis and Torulopsis glabrata were used in an attempt to identify yeasts by their NMR spectra. Each of the species resulted in different NMR spectra that were reproducible. Twenty four C. albicans strains were grown under the same standard conditions. Three spectral types were noted, but no identification of strains was possible. Base line studies were done on C. albicans in order to determine what changes in growth conditions that affect the cells will change the NMR spectra. Changes in media, temperature of incubation, and growth phase of the organism change the NMR spectra. There were also effects on the cells and therefore the spectra due to continuous passage, mouse passage and mixed culture of the C. albicans cells. Variables involved in the growth of cells must be studied throughly before continuing with this complex problem.

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

AND CYCLIC AMP STUDIES

OF CANDIDA ALBICANS

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Pamela Jane Purus

This thesis has been read by each member of the
thesis committee and has been found to be satisfactory
regarding content, English usage, format, citations,
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ABSTRACT

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INTRODUCTION

Candida albicans is a normal inhabitant of the
mucocutaneous regions of the human oral cavity, upper
respiratory tract and intestinal tract (23, 59). This
endogenous species of yeast usually does not produce
disease in healthy individuals. However, through
alteration of host innate or acquired immune mechanisms,
an opportunistic infection may occur. Many factors can
compromise the host and possibly lead to disease. Among
these factors are: 1)endocrine dysfunction; 2)physiologic
changes such as pregnancy or early infancy; 3)prolonged
antimicrobic therapy; 4)immune deficiences; and
5)iatrogenic factors like indwelling catheters and
surgical procedures (37, 59, 78). The range of diseases
caused by Candida spp. is collectively called
candidiasis. Candidiasis can vary from acute to chronic,
superficial to disseminated disease (23, 59).

The most common etiologic agent of candidiasis is
Candida albicans. C. albicans is a polymorphic fungus. As
a saprobe, the organism exists as a budding yeast.
However, during tissue invasion, it undergoes morphologic
change to form hyphae, pseudohyphae and yeast cells
(34). Other dimorphic fungi such as Histoplasma
capsulatum, Blastomyces dermatitidis and Sporothrix
schenckii have been studied and morphogenesis has been

shown to be an important link to pathogenesis (59). In these latter cases the tissue form is the yeast form and the saprobic form is a mould.

The presence of hyphae and yeast cells in tissue of candidiasis patients has led to a controversy over which is the important form for pathogenesis. Investigators have shown that with natural and experimental infections the lesions may consist only of the yeast (Y) form (57, 79). However most investigators have implicated the mycelial (M) form in pathogenicity, perhaps due to it's ability to escape phagocytosis (19, 20, 43).

Studies on the polymorphic nature (or dimorphism) of Candida albicans were primarily directed towards surveying factors in vitro for their effect on morphogenesis. Some factors, for example proline (16, 17, 40) and zinc (4, 68, 81), when added directly to growing cultures promote an increase or decrease in Y-->M conversion. Intracellular levels of substances such as cyclic AMP (14, 53) and activities of enzymes like N-acetylglucosamine kinase (5, 62) and phosphofructokinase (12, 61) have been looked at in both the Y and M forms and differences in concentrations or activities have been noted.

The studies on dimorphism in C. albicans were
done by many researchers in various labs, using different
strains of organisms, types of media, methods of Y-->M
conversion, protocols for cell breakage, extraction of
the cellular products, and enzyme assay methods. This has
led to conflicting data and confusion over the question
of the role of specific factors in dimorphism. For
example N-acetyl-D-glucosamine is one of many factors
that have been implicated by different researchers to
both promote (36, 49, 66, 69) and inhibit (77) Y-->M
conversion. In an effort to dispel some of the confusion
surrounding dimorphism a standard system that perhaps
more closely reflects the in vivo situation was used to
reevaluate an extensively studied factor, cyclic AMP.

Cyclic adenosine 3',5'-monophosphate (cAMP) has
been studied as a possible factor in intracellular
regulation of fungi (54, 73, 74). cAMP has been shown to
be involved in the development of the cellular slime
mould, Dictyostelium discoideum (80) and is implicated as
important in the Y-->M form conversion in Histoplasma
capsulatum. In H. capsulatum, it was found that the level
of cAMP was approximately five times higher in the M form
than the Y form, and that addition of dibutryl cAMP
resulted in a Y-->M conversion (47, 51).

Work with the dimorphic fungus <u>Mucor</u> <u>racemosus</u>, by Sypherd et al (41, 55) indicated the reverse situation with <u>Mucor</u>. Intracellular levels of cAMP increased dramatically in the M-->Y conversion, before any visual signs of morphologic change. The level of cAMP reached a maximum at the time of conversion and dropped back to normal levels during exponential growth of the organism indicating that cAMP may be important in initation of morphogenesis.

cAMP levels in <u>C.</u> <u>albicans</u> have been studied by other groups of researchers. Both Niimi et al. (53) and Chattaway et al. (14) reported that the Y-->M conversion in <u>C.</u> <u>albicans</u> was accompanied by a rise in cAMP. Chattaway et al. (14) reported that the increase in cAMP only occurred when early stationary phase (18 h) $30^{\circ}C$ grown Y cells underwent a Y-->M conversion under the following conditions. The conditions were a temperature shift to $37^{\circ}C$, nutritional shift from Sabouraud broth with glucose to a starch, yeast extract, peptone broth (11) and the addition of partially purified serum protein (13). Niimi et al. (53) used early stationary phase (18 h) $37^{\circ}C$ grown cells shifted to $30^{\circ}C$ to induce the Y form cells and a shift to $40^{\circ}C$ to achieve Y-->M conversion or M form cells. Niimi's group (53) noted a higher level of cAMP in M vs Y form cells using this system of conversion

in either of 2 different media.

A major problem with the work of Niimi (53), Chattaway (14) and other researchers, is the use of temperature shifts to induce morphogenesis. Many investigators maintain Y form cells by growing them at 28-30°C. To acheive a Y-->M conversion, cells are shifted from 28 or 30°C to 37 or 40°C. This shift in temperature probably does not reflect what happens during a natural progression from commensalism to disease. C. albicans as a saprobe or member of the normal flora grows at 37°C as Y cells on the surface of mucosal membranes. Thus, a temperature shift does not occur upon invasion of tissue and formation of hyphal forms.

A better way to induce the Y-->M conversion may be the use of morphogenic autoregulatory substance (MARS) produced by the Y form cells of C. albicans (30, 31, 32). This substance promotes cell replication but suppresses germination (Y-->M conversion) under conditions that would normally promote germination. Through the use of MARS both M and Y forms can be studied at the temperature of 37°C, using the same medium with only the addition of MARS to suppress germination.

Another major problem in research on dimorphism in C. albicans is the 2 different approaches used in

studying dimorphism. Many investigators have studied the M and Y forms after completion of morphogenesis (52). These studies were done to determine differences in antigens (46, 71), cytoplasmic proteins (6, 8, 45) and cell wall compositions (11, 22, 70) of the 2 distinct M and Y forms. However, an alternative approach is to study the cells prior to a change in morphology and look at factors that may play a role in initiation of this morphogenic process. Problems arise when investigators, studying cells that have completed the morphologic change, try to make conclusions about initiation events. An example of this is the work of Niimi, et al. (53) and Chattaway, et al. (14) who report a rise in cAMP at the time of morphologic evidence and assume that cAMP was involved in initiation.

The studies described so far were done by traditional biochemical methods. For these investigations cells were broken open. The resulting cellular contents were studied through biochemical analyses for enzyme levels, RNA concentrations and concentrations of pathway intermediates. This process disrupts cellular integrity and compartmentalization. Membranes become broken and it should be expected that control substances of enzyme activity, such as allosteric effectors, are diluted away. This would lead to results that do not reflect the levels

within the cell. A technique that is not invasive and would therefore reflect the intracellular situation would be preferable.

Nuclear magnetic resonance (NMR) is an alternative method which allows subcellular analyses without disruption of cellular integrity. This form of spectroscopy relies on the existence of a magnetic moment in some isotopes of atomic nuclei. When placed in a stationary magnetic field these nuclei behave like tiny bar magnets, aligning with or against the field. When the nuclei in the magnetic field are pulsed with a range of radiofrequencies the nuclei absorb energy of a specific frequency that is dependent upon the chemical environment of the atoms of interest. Each chemically unique nucleus will therefore absorb energy of a certain resonant frequency. Once pulsed the nuclei are allowed to decay and the emission of the absorbed energy results in a free induction decay (FID) which is then Fourier transformed. The result is a spectrum of different energy shifts compared to a standard. This spectrum can be directly related to the atoms of the different compounds.

In living systems, the NMR nuclei most commonly examined are ^{31}P, ^1H and ^{13}C (60, 64, 65). There are 2 ways to use these nuclei to study living systems through

NMR. The first method is called natural abundance NMR because the NMR isotopes used are those that naturally occur within cells. There is a limited sensitivity however and only high concentrations (0.01 M), of low molecular weight compounds will be seen. ^{31}P accounts for nearly 100% of all phosphorus found in natural occurrence and therefore is readily detectable in living systems by natural abundance NMR. ^{31}P has been used to look at phosphorus pools in both cell extracts (26, 38, 39) and intact organisms (2, 7, 25). ^{13}C has also been used in natural abundance work (39, 48, 72) although not to the full potential because of the low natural abundance of ^{13}C nuclei.

The second method involves the addition of labelled compounds to metabolizing cells. ^{13}C is ideal for this work because it has a low natural abundance of only 1.1 %. This allows the cell sample to be enriched with a ^{13}C labelled compound and there will be very low natural background of ^{13}C. The metabolism of the labelled compound can be followed by following the changing peak heights of intermediary metabolites in a spectrum over time (3, 18, 21, 26).

^{31}P NMR has been used to study dimorphism in C. albicans by Cassone et al. (9). Cassone et al. (9) looked at the Y-->M form conversion in C. albicans by using many

of the same methods used by Niimi et al. (53), Chattaway et al. (14) and others. The Y form cells were grown in a nondefined (yeast-extract, glucose) medium at 28°C and incubated for 8, 40 or 48 hours before NMR analysis. The Y-->M conversion was acheived by inoculating 24 h Y form cells into a second medium at the higher temperature of 37°C. M form cells were harvested at 90 and 240 min. for NMR analysis. Although the use of temperature and nutritional shifts are standard procedure in most laboratories for Y-->M form conversion, these procedures may lead to uncertain results when using the NMR approach. The differences in spectra obtained due to changes in either temperature, media or growth phase of organism can be drastic. Also the use of chemically defined media is important for reproducibility of spectra and attempts at identifying intracellular compounds, because of the known and consistant ingredients in defined media.

A Y-->M conversion system that does not depend upon a temperature shift (ie., all growth at 37°C) and utilizes a defined medium, would reduce the variables encountered by Cassone, et al. (9). In addition, the use of MARS as a germination suppressor would control for the effect of the nutritional shifts. However, in working out

a system of Y-->M conversion that reflected the in vivo situation and was compatable with NMR, another group of variables was encountered. Dimorphism is not a simple biological event in which the Y form converts into 100% true hyphae. A number of intermediate forms, apparently each with their own biochemistry, form during a Y-->M conversion. This results in a multitude of spectra due to the varying phenotypes.

Along with this problem is the strain variation of C. albicans. In the early 1960's, Hasenclever, et al. (27, 28, 29) described 2 serotypes A and B of C. albicans. Since we found that the spectra between strains vary the different spectra may relate to these antigenic groups of C. albicans. Also specific strains of C. albicans appear to change with time in their ability to achieve Y-->M conversion and also in NMR spectra.

Biological applications of NMR are just beginning to be investigated. This study examined the reproducibility of NMR spectra of C. albicans under varied environmental conditions. The study adressed the stability of the organism and the resulting NMR spectra, under prolonged culture passage, animal passage and mixed culture conditions. Experiments that involved different environmental conditions such as media, temperature and growth phase of the organism were

perfomed. This study establishes base line NMR spectra for further study of the complex events in fungal dimorphism.

MATERIALS AND METHODS

Organisms

Candida albicans strain 394. Strain 394, used in
all experiments on morphogenesis, was a gift from R.P.
Morrison (University of Oklahoma, Norman, OK). For use in
experiments, the yeast was grown in glucose (2%), yeast
extract (0.3%), peptone (1%) broth (GYEP) at $37^{\circ}C$ under
constant aeration by rotation of flasks at 160 rpm
(incubator shaker, New Brunswick Scientific, Edison, NJ)
and transferred every 48 h. Under these conditions over
99% of the cells were yeast (Y) form. The organism was
stored on potato flakes agar (PFA) slants (58) at $4-6^{\circ}C$
and transferred every 6 months.

Other strains,species and genera used for NMR
analysis. Candida albicans strains 16-149 were obtained
from the Clinical Microbiology Laboratories, University
Hospital, University of Washington, Seattle, WA.
C. albicans strains 158-164 were a gift from Dr. T.G.
Mitchell (Duke University, North Carolina). All strains
were clinical isolates passed in culture no more than two
times before being received. C. albicans strains 172-175
were obtained from the American Type Culture Collection
(Rockville, Maryland). C. tropicalis, C. krusei, C.
psuedotropicalis, C. guillermondi and Torulopsis glabrata
were from the Montana State University mycologic culture

collection. All organisms were stored on PFA slants.
Biochemical confirmation of species was performed on all
strains, species and genera by using API strips from
Analytab Products, Ayerst Labs (Plainview, N.Y.).

Media

Germination medium (GM-2). GM-2 was prepared from a
10x stock solution (Table 1) by adding 1 part stock to 7
parts sterile deionized water and 2 parts sterile, 0.05M
pH 7.4 (at 37°C) tris(hydroxymethyl)aminomethane
hydrochloride buffer (pH 7.4 TRIS buffer).

GM-2 with morphogenic autoregulatory substance
(MARS). Cell filtrates containing MARS were prepared as
previously described from our laboratory (31, 32).
Briefly, medium made of 2 parts sterile 0.05M, pH 7.4 (at
37°C) Tris buffer and 8 parts sterile deionized water was
inoculated with a 48 h GYEP grown C. albicans 394 (3x
washed with sterile 0.15N NaCl) to acheive $2x10^8$ Y
cells/ml of medium. The cultures were incubated for 15-18
h at 37°C in sterile, glass 1 liter graduated cylinders
without shaking. After incubation, suspensions were
centrifuged at 1500xg for 2 h and the supernatant fluid
was collected and filtered through a 0.45 um porosity
membrane filter. The filtrates were lyophilized and

Table 1. Formulation for 10x concentrated GM-2

Ingredients[a]	Amount(mg/l)
folic acid[b]	10
L-arginine HCl	1266
L-methionine	150
L-proline	692
L-histidine	420
glucose	5000
d-biotin	10
$(NH_4)_2SO_4$	250
$NaH_2PO_4 \cdot H_2O$	413
$Na_2HPO_4 \cdot 12H_2O$	717

[a] All chemicals are products of Sigma Chemical Company (St. Louis, MO) except glucose and $(NH_4)_2SO_4$ (J.T. Baker Chemical Company, Philipsburg, NJ)

[b] component was heated into solution, the other components were added, and the final solution was sterilized by filtration through 0.45 um porosity membranes (HAWP, Millipore Corp., Bedford, MA)

reconstituted with 1x GM-2 to achieve a 2-4x concentration of MARS.

Synthetic amino acid medium for fungi (SAAMF). SAAMF at a concentration of 2x was prepared by combining 19.5g of glucose and one packet of SAAM base powder (Table 2) in 500 ml sterile deionized water. The mix was heated to 40-50°C for 1-2 h while stirring, then sterilized by membrane filtration (0.45 um porosity). SAAMF broth (SAAMFB) was prepared by adding 500 ml of sterile deionized water to the 2x SAAMF, resulting in 1 liter of 1x SAAMFB. SAAMF agar (SAAMFA) was prepared by adding 500 ml of 2x SAAMF to 500 ml 2% agarose (catalog # 27392-444, VWR, San Francisco, CA) in deionized water for a final agarose concentration of 1%.

Germination Assessment

The percent of germination, in experiments involving dimorphism in Candida albicans, was calculated by counting the number of germ tubes in 100 groups of cells. The morphological forms of C. albicans were categorized as germ tubes, pseudotubes, or yeast forms according to previous descriptions (10, 33). A germ tube was defined as a cell with a length at least twice that of the mother cell and with parallel walls throughout

Table 2. Formulation for Synthetic Amino Acid Medium Base

Ingredients[a]	Amount(mg/l)
L-arginine	1050
L-lysine	580
L-histidine	310
L-tyrosine	360
L-tryptophane	100
L-phenylalanine	320
L-cystine	240
L-methionine	150
L-threonine	480
L-leucine	520
L-isoleucine	520
L-valine	460
L-proline	1000
glycine	500
L-glutamic acid	2520
L-asparagine	1000
d-glucose	1000
fumaric acid	1500
sodium pyruvate	1000
ammonium acetate	500
$K_2HPO_4 \cdot 3H_2O$	500

Table 2 (cont'd)

Ingredients	Amount(mg/l)
MOPS[b]	16450
Tris[c]	10450
biotin	0.5
folic acid	5
choline chloride	25
nicotinamide	5
d-calcium pentothenate	25
pyridoxal HCl	5
thiamine HCl	5
riboflavin	5
i-inositol	25
$MgCl_2$ anhydrous	95.35
$FeCl_3.6H_2O$	2.7
$ZnSO_4.7H_2O$	0.8
$MnSO_4.4H_2O$	0.36
$CaCl_2$ anhydrous	5.57
phenol red	2

[a] GIBCO catalog # M00898, all components come premixed from GIBCO (Grand Island, New York)

[b] MOPS= 2-(N-morpholino)propane sulfonic acid

[c] Tris= 2-amino-2-(hydroxymethyl)-1,3-propanediol

the length. Blastoconidia with no more than 3 attached buds were designated as yeast forms. All other morphologies not fitting into either category were termed pseudotubes.

Cyclic AMP Assay

Preparation of cells. The cells from 100 ml of a 24h C. albicans 394 culture grown in GYEP were washed 3x with sterile 0.15 N NaCl or deionized water. Forty milliliters of GM-2 or GM-2 + MARS in 50 ml Erlenmeyer flasks were inoculated with the washed cell suspension to a final density of 5×10^5 cells/ml of medium. The cultures were incubated at 37°C, 180 rpm in a shaking water bath (Gyrotory Water Bath Shaker, New Brunswick Scientific, Edison, NJ) for between 0.5 and 4 h. [Some difficulty was encountered in optimizing the final protocols. For more information see Appendix I].

cAMP extraction. After incubation, a sample of cells was removed from each culture flask and the percent of germination determined. The cells were collected by membrane filtration (25 mm, 0.45 um porosity) and the filter was placed in 2.5 ml of 6% ice-cold trichloroacetic acid (TCA) within 30 sec of removal of the flask from the water bath. The samples were subjected

to 5 cycles of freezing (dry ice: ethanol bath, $-70^{\circ}C$) and thawing (water bath: $37^{\circ}C$), filters were removed and the TCA mix was extracted 3x at $23-27^{\circ}C$ with an equal volume of water-saturated diethyl ether. The remaining ether was evaporated off under a stream of nitrogen gas, and the sample was centrifuged at 1500xg for 15 min. The supernatant fluid was removed, and 2M Tris base was added to achieve a pH of 7. The neutralized supernatant fluid was then lyophilized. The pellets were frozen ($-70^{\circ}C$) for use in protein determination.

Protein determination. Each frozen pellet was resuspended in 0.5 ml of 1N NaOH, boiled for 20 min and centrifuged at 1500xg for 15 min. The supernatant fluid was removed and assayed for protein concentration by the method of Lowry (44).

cAMP assay. The lyophilized samples were reconstituted in 0.5 ml of acetate buffer. An [125]I cAMP RIA kit [New England Nuclear(NEN), Boston, MA] was used to determine cAMP content of the samples by following the NEN procedure for acetylated samples. (For further enzyme studies see Appendix III.)

Cell Preparation For NMR

Dimorphism studies on C. albicans 394. The cells from 500 ml of a 48h C. albicans 394 culture grown in GYEP

were washed 3x with sterile 0.15 N NaCl. Eight hundred milliliters of GM-2 or GM-2 + MARS in each of 30 1-liter Erlenmeyer flasks were inoculated at 5×10^5 cells/ml of medium and incubated at 37°C under constant aeration by rotating at 160 rpm. After incubation flasks were placed in an ice-water bath and samples were taken for percent germination assessment. The cells from the 30 1-liter flasks were pelleted by continuous flow centrifugation at 5000 x g in an RC2-B Sorvall centrifuge. The pelleted cells were washed 3x with 0.15 N NaCl and $0.5-2 \times 10^{10}$ cells were transferred to a 10 mm NMR tube, which was held on ice until NMR analysis.

Strain and genera variation. From cultures maintained at $4-6^\circ$C on PFA slants, 2 ml of SAAMFB in 5 ml tubes were inoculated and incubated at 37°C overnight under constant aeration by rotating at 160 rpm. One hundred and fifty milliliters of SAAMFB in 250 ml Erlenmeyer flasks were inoculated with 2 drops of the overnight culture and incubated at 37°C, 160 rpm for 24h. For growth curve experiments the 150 ml of SAAMFB was inoculated to a total cell density of 10^8 cells from 24h cultures grown in 10 ml of SAAMFB. Cells obtained for 28°C temperature experiments were grown in a shaking water bath at 160 rpm. Cells were collected, washed 3x

with 0.15 N NaCl and $0.5-2 \times 10^{10}$ cells were packed into a 10 mm (OD) NMR tube, on ice.

NMR of mixtures of C. albicans. One hundred and fifty milliliters of SAAMFB in 250 ml Erlenmeyer flasks were inoculated with C. albicans 16 and C. albicans 113, 109 or 122 and incubated at 37°C, 160 rpm for 24h. Strain 16 was used because it does not agglutinate with an agglutinating monoclonal antibody (H-9, # 35 antibody isolated in Dr. Cutler's lab by Diane Brawner). The other strains (113, 109, and 122) used always gave a 3 or 4+ agglutination with the antibody. After incubation, the mixed cultures were plated onto SAAMFA and incubated at 37°C for 24 h. Isolated colonies were tested with the antibody and specific colonies were chosen on the basis of agglutination. Cells from these colonies were used to inoculate SAAMFB. The cultures were grown for NMR analysis following the strain and genera procedures.

NMR of C. albicans passed through mice. The cells from 10 ml of a 24 h SAAMFB culture of different C. albicans strains were washed 3x with sterile 0.15 N NaCl, and suspensions of 1×10^7 cells/ml were prepared. BALB/cByJ mice, obtained from Jackson Laboratories (Bar Harbor, ME), were injected intravenously with 0.1 ml of the washed cell suspensions. The mice were sacrificed 3 days after injection. The kidneys of the mice were

removed, homogenized and plated on SAAMFA following the procedure in our lab (15). The plates were incubated at 37°C for 24 h and cells from isolated colonies were used to inoculate 150 ml SAAMFB in 250 ml Erlenmeyer flasks for incubation following strain and genera procedure.

NMR Parameters

^{13}C NMR spectra of the cells were recorded at 2°C by means of a Bruker WM-250 spectrometer working at 62.83 MHz, interfaced to an Aspec-2000 computer, under conditions of broad band decoupling. The field/frequency ratio of the spectrometer was locked onto a coaxial D_2O sample and referenced with an internal tetramethylsilane (TMS) capillary. For the dimorphism studies, each sample was recorded as an 8K data file of 5000 accumulated free induction decays (FIDS) obtained by 55° pulses with an acquisition time of 0.2 sec, relaxation delay of 0.2 sec and sweep width of 20000 Hz. Strain and genera variation experiments were stored as 8K data files obtained by 600 scans of 85° pulses with an acquisition time of 0.54 sec, relaxation delay of 3 sec and sweep width of 15151.51 Hz. The 8k FIDS were truncated to 4k, Fourier transformed and then the power spectra were obtained and used in evaluation.

RESULTS

Cyclic AMP and Dimorphism

To determine if cyclic AMP (cAMP) plays an important role in morphogenesis using the system of MARS suppression of germination (Y-->M conversion), intracellular levels of cAMP were evaluated. Candida albicans strain 394 ($5x10^5$ cells/ml) in duplicate flasks of GM-2 or GM-2 + MARS were incubated at 37°C for 0.5, 1 and 3 h time periods. After incubation, the cells were collected, lysed, extracted and centrifuged. The supernatant fluid was analyzed with an ^{125}I-cAMP radioimmune assay kit (New England Nuclear) to determine intracellular levels of cAMP. There was no significant difference in cAMP levels (Figure 1) between germinating (GM-2) and germination-suppressed (MARS-treated) cells noted at the early time periods (0, 0.5 and 1 h). Multiple repetitions of this experiment, as well as experiments looking at other intermediate time periods, all revealed no increase in cAMP. Only when the Y-->M conversion was morphologically complete at 3 h did the cAMP in the M form cells increase.

NMR and Dimorphism

To determine if different morphological forms of Candida albicans have different NMR spectra, $5x10^5$
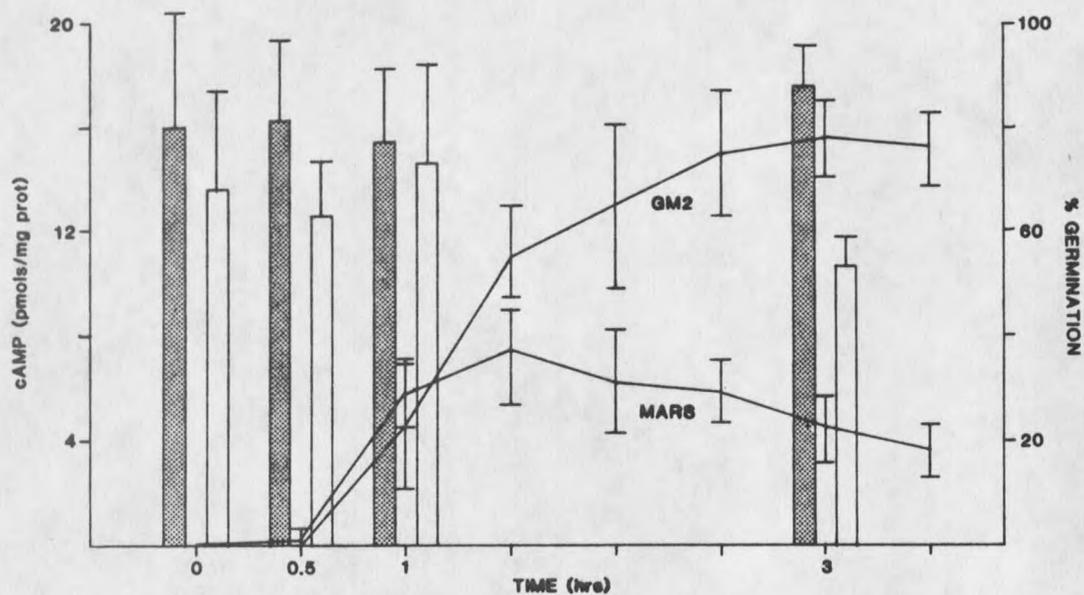
Figure 1. Germination and intracellular cyclic AMP levels
of <u>Candida</u> <u>albicans</u>. The bars indicate levels
of cAMP assayed at 4 different time periods.
Shaded bars are cAMP levels of cells under
germinating conditions (GM-2). Open bars are
levels under conditions of suppressed
germination (GM-2 + MARS). Curves represent
percent germination measured every 0.5 h of the
experiment.

cells/ml in flasks of GM-2 or GM-2 + MARS were incubated at 37°C for 3 h. The cells were collected, washed and packed into a 10 mm (OD) NMR tube. The spectra obtained (Figure 2) were reproducible over multiple repetitions when percent germination and morphologies between runs were similar, and the temperature in the NMR sample was 2°C. The Y control spectrum was obtained on Y cells that were not released into GM-2 or MARS + GM-2. Since the Y control cells were grown in GYEP but not released into GM-2, these cells served as a control of the media difference from maintenance in GYEP to experimentation in GM-2 by noting peak differences in the spectra. The control cells also showed any changes in the strain that had occurred over continuous passage in GYEP. The base line spectra obtained serve as a source of comparison between runs and as a control see that the spectrometer is running properly.

For ease of discussion of figures, all spectra were divided into 3 sections. Section I (10-35 ppm) was referred to as the aliphatic region. This region contains most of the peaks for aliphatic carbons, for example methyl side chains on amino acids and methyl and methylene carbons on fatty acids (1, 42, 48). The region of peaks due to the hydroxyl, ether, sulfhydryl and amine