RNA synthesis in Candida albicans

by

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SUMMARY

The thesis describes the results from investigations into RNA synthesis in the dimorphic fungus Candida albicans. Methods were described, and evaluated, for the preparation of protoplasts and nuclei - two in vitro systems that were used to study RNA synthesis. It was found that most radiolabelled precursor was incorporated into low molecular weight RNA by nuclei. In contrast, the precursor was associated with higher molecular weight RNA species from protoplasts, from which the nuclei were prepared. Contamination by RNases was a serious problem in the preparation of this in vitro system from protoplasts.

The RNA polymerases were purified from yeast and part-purified from mycelial C. albicans. Three classes of RNA polymerase were resolved by ion exchange chromatography of cell-free extract from yeast, whilst only one was found in mycelia. Some characteristics of RNA polymerases from the yeast were described. All three isozymes had optimal activity in vitro when the media contained mono and divalent ion concentrations that were similar to those reported for Saccharomyces cerevisiae - a yeast often compared with C. albicans. In addition, the three isozymes from C. albicans had similar Km values for CTP to that of these enzymes from other eukaryotes. RNA polymerases I and III from yeast C. albicans showed similar sensitivities to α-amanitin as the corresponding isozymes from S. cerevisiae. RNA polymerase II was far more insensitive to the amatoxin than the corresponding enzyme from higher eukaryotes.

A variety of nucleoside analogues were suggested as potential antifungal agents warranting further investigations. These were capable of inhibiting RNA synthesis by C. albicans in vitro and/or in vivo assays.

Studies were also made on RNA synthesis during germ tube formation - the initial stage of the yeast-mycelial transformation. It was found that it was necessary to cultivate C. albicans yeast in nutritionally impoverished media and starved for 24 hr to achieve reproducible germ tube formation. The strain of C. albicans used in this research formed germ tubes when incubated in imidazole HCl buffer containing serum, N-acetyl glucosamine, glucose or glucose plus glutamine at temperatures above 35° C.. During germ tube formation there was an increase in the RNA content per unit yeast cell. Both the rate and maximum amount of radiolabelled precursor incorporated into RNA depended upon the conditions used to induce germ tube formation. Some inhibitors of RNA synthesis were capable of inhibiting germ tube formation. It was found that the ratio of high to low molecular weight RNA species changed over the period of germ tube formation.

The thesis concludes by evaluating the results of this research and suggests further directions for future research.

ABBREVIATIONS

adenosine diphosphate	ADP
adenosine monophosphate	AMP
adenosine triphosphate	ATP
10^{-3} Amperes	mA
base pairs	bp
Becquerel	Bq
bovine serum albumin	BSA
Centigrade	^{o} C.
counts per minute	c.p.m.
Curie	Ci
cytidine diphosphate	CDP
cytidine monophosphate	CMP
cytidine triphosphate	CTP
de-oxyribonucleic acid	DNA
dimethyl sulphoxide	DMSO
dithiothreitol	dTT
double stranded	ds
ethylenediaminetetra acetic acid	EDTA
glucose	glu
glucose/beef extract broth	G.B.E.
glutamine	gln
gramme	g
guanosine diphosphate	GDP
guanosine monophosphate	GMP
guanosine triphosphate	GTP
heterogenous nuclear ribonucleic acid	hnRNA
hour	hr
litres	L

messenger ribonucleic acid	mRNA
minimum inhibitory concentration	MIC
minute(s)	min
molecular weight	M.W.
N-acetyl glucosamine	NAG
National Collection of Pathogenic Fungi	NCPF
Newtons	N
nucleoside triphosphate	NTP
optical density	O.D.
Pearson product moment coefficient of correlation	r
phenylmethyl sulfonylfluoride	PMSF
polyacrylamide gel electrophoresis	PAGE
revolutions per minute (r.p.m.)	r.p.m.
ribonucleic acid	RNA
ribosomal ribonucleic acid	rRNA
Sabourauds dextrose agar	S.D.A.
Sabourauds dextrose broth	S.D.B.
seconds	sec
Shepherd and Sullivan	S. and S.
sodium dodecylsulphate	SDS
Svedberg units	S
trichloroacetic acid	TCA
Tetramethylethylenediamine	TEMED
Tris(hydroxymethyl)aminomethane	Tris
uridine diphosphate	UDP
uridine monophosphate	UMP
uridine triphosphate	UTP
ultra violet	UV
weight/volume	w/v
volume/volume	v/v
yeast extract/peptone broth	YEP

PREFACE

Many species of fungi are potentially pathogenic in man. The dimorphic fungus Candida albicans is possibly responsible for the greatest number of such infections in man. Although there are antifungal agents which are used against C. albicans, most have drawbacks such as showing toxicity to the host. Only the recently introduced antifungal agent ketoconazole has the advantage that it appears to have minimal side effects. RNA synthesis was studied as a possible target site for novel inhibitors of C. albicans that would not affect the mammalian host. This thesis will describe the results of such investigations.

The introductory chapter will examine some biochemical, epidemiological and physiological aspects of C. albicans and antifungal agents currently employed against various forms of candidosis. In addition, this chapter will review some aspects of eukaryotic RNA synthesis. Chapter 2 will describe the techniques required for research into any aspect of a pathogen, such as C. albicans, and the methods used to investigate RNA synthesis in this organism. The next four chapters will be concerned with evaluating the results obtained from investigations into RNA synthesis in C. albicans. The first of these chapters will describe and discuss the preparation of two *in vitro* systems that may be used to study RNA synthesis. The next chapter will describe the purification, and some characteristics, of RNA polymerases from yeast and the partial purification of this enzyme from mycelial C. albicans. The results obtained in these two chapters will be compared with other eukaryotes in general and Saccharomyces cerevisiae in particular. Chapter 5 will describe investigations into the effects of the inhibitor lomofungin on growth and RNA synthesis in C. albicans. (Lomofungin is a reputed inhibitor of these processes in other fungi). The effects of some nucleoside analogues on RNA synthesis in C. albicans were investigated using *in vitro* and *in vivo* systems prepared as described in the preceeding two chapters. The yeast-mycelial transformation is a distinguishing

characteristic of C. albicans, which may be important in the invasion of the host. Conditions will be described for the reproducible induction of germ tubes from the strain of C. albicans used for most of this research. RNA synthesis and the RNA species synthesised during the initial stages will be analysed in the hope of further understanding this morphological change. The final chapter will discuss the results of the thesis and suggest possible directions for future research.

CHAPTER 1 RNA SYNTHESIS IN *Candida albicans*

1.1 Introduction

The yeast *Candida albicans* is a pathogenic dimorphic fungus which is responsible for the largest number of infections of fungal aetiology in humans. Almost certainly, the most prevalent type of infection reported is vaginal candidosis in women of childbearing age. Recent (i.e. 1984) Department of Health statistics have shown an increase in the incidence of women showing symptoms of this disease over the past few years (89). Although treatment has improved recently, relapses are very common and a totally satisfactory systemic treatment is not yet available. There thus exists a huge market for suitable antifungal agents. Although there are pharmaceutical preparations available, it was not until the recent introduction of ketoconazole that a suitable treatment for candidosis could be taken orally. In order to assist in the development of other desirable antifungal agents that could be ingested orally, RNA synthesis was studied as a potential target site. RNA synthesis was chosen as it was hoped to find exploitable differences between this process in *C. albicans* and higher mammals.

In addition to existing as a pathogen, *C. albicans* may reside as a commensal in the human or animal host. Dimorphism may play an important role in the conversion from commensal to parasitic existence (see 6.1) . The involvement of RNA synthesis in this transformation was investigated to give an insight into this poorly understood phenomenon. This thesis will examine how successful these two aims were and also how apt RNA synthesis is as a site for novel inhibitors.

1.2.1 *C. albicans* - Characteristic morphology

C. albicans may exist in two distinct morphological forms. It can exist as a unicellular yeast (blastospore) or as an aggregate of multicellular hyphae (mycelia). *C. albicans* is taxonomically classified as an asporogenous yeast which may not be assigned to any of the more homogenous

yeast genera. In addition, it is unable to form a sexual stage in its life cycle. Therefore, its relationship to "classic" yeast genera, such as Saccharomyces, is obscure.

A wide variety of media have been described which promote the growth of either form of C. albicans. Most of these media favour growth of the yeast form in vitro. A yeast may be defined as "a fungus whose predominant morphological form is unicellular" (220). (It is this morphological characteristic that lead Lodder and Kreger Van Rij to classify the Candida genus as yeasts). Macroscopically, C. albicans yeast colonies have a white, creamy, moist and shiny appearance on solid media. Microscopically, the yeast blastospores are ovoid in shape, approximately 4 - 6 μm by 6 - 8 μm Blastospores arise through mitotic cell division (budding). The daughter cell grows, for a period of time, from a small, selected site on the apex of the parent. A septum (cross wall) is formed between the two cells just prior to separation (Fig. 1.1).

Aside from the inability to form a sexual stage in the life cycle, the principle characteristic of the Candida genus is the ability to form chains of elongated yeast cells (pseudo-hyphae). Pseudo-hyphae are formed by a process similar to blastospore development. However, the daughter cells remain attached to the parent. In addition, subsequent daughter cells are more narrow and elongated than the original parent cell (Fig. 1.2).

Many workers have confused hyphae with pseudo-hyphae even though their mode of formation is different. Hyphal formation is characterised by cellular material growing from the blastospore in narrow cylindrical tubes. This process is termed germ tube formation. In certain media (e.g. some peptone broths) blastospores may be produced along the hyphal shoot. However, in media such as serum more hyphal branches may be formed as the hypha extends (Fig. 1.3). All hyphae usually aggregate as mycelia and can be seen macroscopically as large "clumps" of cells in suitable media.

C. albicans is able to form chlamydospores in certain nutritionally impoverished media containing surfactants. These characteristic out-

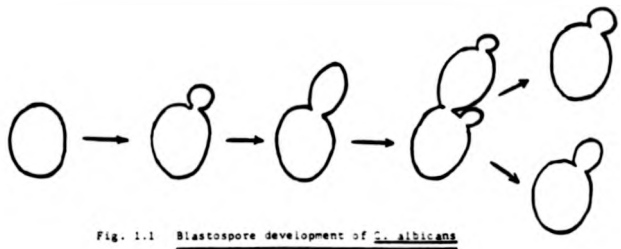


Fig. 1.1 Blastospore development of C. albicans

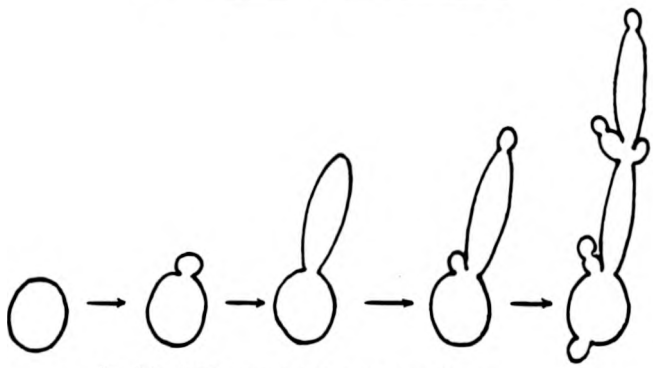


Fig. 1.2 Pseudo-mycelial development of C. albicans

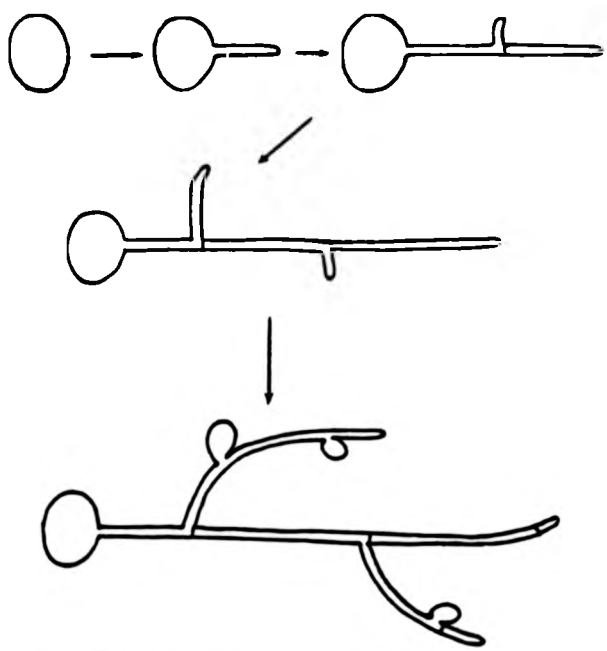


Fig. 1.3 Mycelial development of C. albicans

growths of cellular material are attached to the main body of yeast or hyphal cells by a suspensor cell. Chlamydospores have a thick double-layered cell wall: the outer layer containing $\beta(1 - 3)$ glucans and other polysaccharides, whilst the inner is mostly protein (176). This cell wall surrounds a cell membrane, within which are large amounts of storage lipids, polysaccharides and proteins (263). It has been suggested that chlamydospores are a dormant phase of the fungus (17).

The appearance of chlamydospores and germ tubes are generally accepted as positive presumptive evidence for the identification of C. albicans. However, other Candida species, such as C. tropicalis, have been reported as having the ability to form chlamydospores (154). In addition, there are some strains of C. albicans that do not readily form germ tubes. As a result, many medical mycologists use such physiological and biochemical properties as the ability to ferment and assimilate suitable compounds as tools for the identification of C. albicans.

1.2.2 C. albicans - Biochemical and Physiological properties

In recent years there has been an increase in the number of papers dealing with the biochemistry and physiology of C. albicans. However, in comparison to the extensive literature concerned with these aspects in another yeast, Saccharomyces cerevisiae, such studies on C. albicans are far from complete. A great deal of the new research is concerned with studies on the mode of action of inhibitors (see 1.2.5) and the dimorphic transformation (see chapter 6).

C. albicans is able to grow using a variety of compounds as sole carbon or nitrogen sources. In the laboratory more complex media are used to cultivate the organism. These conditions usually result in cultivation of the yeast form of C. albicans. Growth is possible on media that have a pH in the range 3 - 8 and an incubation temperature of 20 - 40° C.. Growth of C. albicans is usually much slower in synthetic media compared to that in ill-defined, complex media. As a consequence, cultivation of large quantities of the organism rapidly for biochemical

investigations usually involves growth in peptone-glucose media. Fairly high growth rates have been reported in these media. Indeed, doubling times, in the exponential phase of growth, of 70 min are not unusual (109).

Studies of C. albicans have so far indicated that the biochemistry of this organism is typical of other lower eukoryotes such as S. cerevisiae. Much research on the metabolism of C. albicans has investigated oxidative phosphorylation and carbohydrate utilisation. As an aerobic eukoryote possessing mitrochondria, the oxidative phosphorylation mechanism of C. albicans is reportedly similar to that of S. cerevisiae (410). However, despite possessing all the enzymes necessary for anaerobic glycolysis (299), C. albicans is unable to grow anaerobically (266). In addition, it has also been found that C. albicans possesses all the enzymes of the hexose monophosphate pathway and most of those required for the tricarboxylic acid cycle (299). Hexoses may be diverted from glycolysis to form polysaccharides (64). The enzymes responsible for the synthesis of glucans and mannans have been extensively studied. These polysaccharides are important cell wall constituents of C. albicans and other fungi (85, 240).

The cell wall and membrane have both been fairly well studied - the latter as it is believed to be a site of action for a variety of inhibitors (see 1.2.5). The cell wall maintains the distinctive ovoid shape of C. albicans and also acts as a boundary between the cell and environment. Early research showed that the cell wall possessed large quantities of glucans and mannans with small amounts of protein, lipid and chitin (65). The glucans and mannans consist of backbones of hexose (glucose in the former, mannose in the latter) residues which contain extensive branches and cross links. The glucans have a $\beta(1 - 6)$ linked glucose backbone with $\beta(1 - 3)$ linked branch points. Mannans contain $\alpha(1 - 2)$ branch points of mannose units attached to an $\alpha(1 - 6)$ linked mannose backbone (287). There may be as many as eight layers of polysaccharide in the cell wall (294).

The polysaccharides have often been found associated with proteins as glucoproteins, mannoproteins or glucomannoproteins (189) Recent studies have shown that there are differences between strains in the length of side chains of mannose residues from extracted mannoproteins (304) These mannoproteins have been

implicated in affecting the ability of C. albicans to bind to buccal epithelial cells (96, 320). They may thus have an important role to play in the invasion of mucous epithelia. The cell wall glucoproteins and mannoproteins are the antigenic determinants by which strains of C. albicans can be differentiated into one of two types. Hasenclever's group first demonstrated this phenomenon and termed the two antigenic types group A and group B (153, 155). The former type (group A) possessed the same complement of antigens as the other type (group B), plus at least two further antigens (370).

Enclosed within the cell wall is the cell membrane. This contains both free sterols and sterol esters, triglycerides and carboxylic acids of a chain length C_{18} (239). Phosphatidylcholine and phosphatidylethanolamine have been found to be the major phospholipids (102). The quantitive levels of the various membrane lipids have been shown to vary from strain to strain (149) and can also be changed by the chemical composition of the media (194). The cell membrane is also the site of a variety of lipid soluble enzymes. These include such enzymes as β(1 - 3) glucan synthetase (125) and chitin synthetase (73) - enzymes implicated in the dimorphic transformation (see chapter 6).

The limited research into the molecular biology and macromolecular synthesis of C. albicans has shown similarities between these and appropriate processes in S. cerevisiae. Such similarities have tempted several workers to use this non-pathogen as a model for drug and inhibitor studies (212). However, as mentioned in 1.2.1, C. albicans differs from S. cerevisiae in two important respects: the inability of the former to produce a sexual stage and the ability to form hyphae and pseudo-hyphae under suitable conditions.

1.2.3 C. albicans - Ecology and Epidemiology

C. albicans is distributed worldwide and found in terrestrial and aquatic environments. As the causal agent responsible for candidosis, the fungus has been found in man and the primates and other warm blooded mammals (122). C. albicans may be recovered from a variety of superficial sites on individual organisms showing no symptoms of candidosis. As such, a number

of reviews of yeast epidemiology have drawn the conclusion that C. albicans, and a few other yeast species, are common and harmless commensals (98, 122 238). The fungi have been recovered from samples taken from mucous membranes and the digestive tracts of "normal" individuals.

Studies into the frequency of recovery of C. albicans from the mouths of "normal" individuals have reported the presence of the fungus in between 2 and 23% of test subjects (238,278). However, in hospitalised individuals the presence of the yeast, from oral sources, may be as high as 70% (24). The oral populations of yeasts are not static and have been found to fluctuate day by day and with the age of the individuals (122). C. albicans has also been recovered from faecal samples with recovery frequencies of around 15% (94). Most publications concerning the frequency of recovery of C. albicans from human sources use the vagina as the source. The frequency of recovery of C. albicans is higher in women suffering symptoms of vaginal thrush than hospitalised or "normal" individuals. C. albicans is most often recovered from samples taken from women of childbearing age (278). Samples from other sites, such as skin, oesophagus and stomach, have been found to contain C. albicans. These are usually taken from hospital patients showing signs of other debilitating illnesses (278). A high proportion (60 - 70%) of C. albicans isolates, from all sites, have been found to be of the A serotype (156).

1.2.4 C. albicans - Candidosis and pre-disposing factors of the host

Section 1.2.3 indicated that many people harbour within them commensal yeasts, such as C. albicans, which have a potential to become pathogenic. Indeed, it has been suggested that "C. albicans is a better clinician and can discover abnormalities in persons much earlier in the course of the development of such abnormalities than we can with our chemical tests" (400). There are esentially four types of candidal infection. Mucosal infections are those of the mucus membranes, such as mouth and vagina. Cutaneous infections affect skin and nails. Muco-cutaneous candidal infections are

those affecting both mucosal and cutaneous sites. This form of infection, and systemic candidoses, affecting the internal organs, are by far the most serious clinically.

A whole variety of pre-disposing factors can account for susceptibility of individuals to candidosis. These can be classified as "natural", dietary, mechnical and iatrogenic medical factors (278). "Natural" factors include diabetes mellitus (and other debilitating diseases and disorders) and digressions from normal physiological status (e.g. pregnancy). The excess or deficiency of foodstuffs may alter the composition of endogenous flora permitting alterations in the levels of C. albicans. Mechanical factors such as trauma (e.g. burns) or local tissue disruption (e.g. wearing dentures) may permit invasion of the affected tissue area by C. albicans.

Possibly the most important factor for serious infection by C. albicans at present is that arising as a consequence of iatrogenic medical factors. The incidence of such serious infections is increasing as new techniques in medicine have been introduced. Treatment with antibiotics or corticosteroids alters the gut floral population of treated individuals. This has been found to result in an increase in the incidence of individuals showing symptoms of candidosis (278). Surgical procedures - such as the introduction of a catheter into the blood vessels or urinary tract - may also be accompanied by serious candidosis.

The range of situations that can dispose an individual to either local or widespread candidal infections is extensive. The possibility of serious systemic candidosis in severly ill hospital patients is considerable. Thus, where systemic candidosis is diagnosed, the primary consideration for treatment should be to attempt to minimise the many factors that can potentiate the disease.

In present day medicinal practice vaginal candidosis is commonly seen. There is thus a good deal of interest shown in this disease. This may be illustrated by the large number of publications concerned with the carriage of fungi in the vagina and also trials with antifungal agents (see 1.2.5).

The classical lesions of the disease are a white discharge from the vagina coupled with soreness, inflammation and itching of the affected area. However, accurate diagnosis may only be confirmed by detection of C. albicans in swabs.

Many authors believe that vaginal candidosis has become more common in the post-antibiotic and post-oral contraceptive age. However, definitive proof is not easy to find (278). The fact that the number of diagnoses of vaginal candidosis has risen annually (89), may be an indication of increasing numbers of women contracting the disease or physicians are increasingly correctly interpreting the symptoms. Vaginal thrush is commonly seen during pregnancy. In addition, the prevalence of vaginal yeasts is higher with women taking oral contraceptives compared to those who are not (278). Some authorities believe that the increased wearing of occlusive nylon tights may increase the likelihood of contracting the symptoms.

A consequence of the increasing numbers of women contracting the disease has been that many pharmaceutical companies have made strenuous efforts to produce suitable antifungal agents. There are probably more formulations of pharmaceuticals currently available for the treatment of vaginal candidosis than for any other type of candidal infection. These drugs - the mode of action of which will be discussed in 1.2.5 - may be supplied as creams, lotions or pessaries. The literature available on treatment of vaginal candidosis suggests that most cases will respond to topical treatment alone. However, the extent of re-infection, or possible relapse of the disease, means that an aggressive schedule of treatment must be attempted for some patients at least (278).

Oral thrush is another mucosal infection which, although less common than vaginal candidosis, may be observed in elderly, debilitated patients. New born infants may also be susceptible to C. albicans infections because of their immature antimicrobial defences. This disease may be treated using similar topical regimes as that for vaginal candidosis. Extensive topical regimes are also necessary for cutaneous candidoses of the skin and nails.

In contrast to candidal infections of mucous membranes, not fatal and commonly seen in medical practice, systemic candidoses affecting the central nervous system, and heart, used to be fatal until the introduction of suitable antifungal agents. Candidal infections of bone and joint, respiratory and genito-urinary tracks and kidney have all been reported. The severity of these systemic candidal infections is in proportion to the extent which the host's immune response system is suppressed. In addition, chronic muco-cutaneous candidosis may arise as a consequence of a deficient immune system. Thus, fatal widespread lesions arising due to infection by C. albicans, are only now usually seen in patients with extreme debilitation (278).

1.2.5 C. albicans - Antifungal agents currently available for the treatment of Candidosis

The only suitable treatment for superficial candidosis up to the 1950's was provided by formulations containing Gentian violet and similar non-specific antiseptics (400). Nystatin, the first specific antifungal agent, was discovered in 1950 (158). Since then, the literature concerned with compounds that show inhibitory activity against fungi has increased enormously.

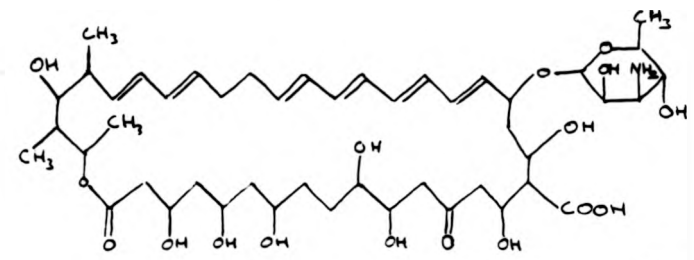
The route from in vitro efficacy of a compound, to commercial availability for treatment, is both time consuming and expensive. Firstly, a compound must be identified that shows in vitro inhibitory activity against C. albicans - and hopefully other fungi. This must then be followed by investigations into the mechanism of action of this potential drug. A successful candidate should show differential sensitivity between mammalian cells (the host) and the fungus (the pathogen). This must be followed by detailed pharmacokinetic studies on in vivo models - infected laboratory animals. This should indicate whether the candidate drug is safe for clinical trials on human volunteers - the penultimate step before the compound is licensed for general medical use.

The cost of research and development of a potentially successful antifungal agent has been estimated at £25 million (318). Only the large multinational drug companies of the western world - financed by profits from pharmaceutical sales - can afford such costs. As a consequence, research can only be directed towards illnesses and infections of sufficient magnitude that guarantee financial reward. Mucosal candidal infections are widespread (see 1.2.4) and thus represent a large market for potential novel inhibitors. Any drug that shows good inhibitory activity against less common fungi, or has high production costs, is unlikely to be developed. It is therefore perhaps not surprising that there are few pharmaceutical agents that are widely used in antifungal chemotherapy. These are the polyene antibiotics, the substituted imidazoles and the nucleoside analogues.

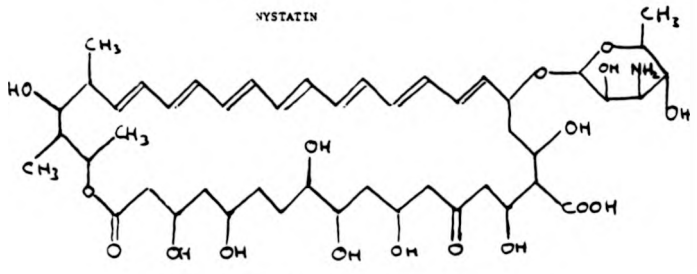
The two polyene antibiotics most commonly employed as antifungal agents are nystatin and amphotericin B (see Fig. 1.4). Other polyene antibiotics, which have been used against candidal infections, include candicidin, pimaricin trichomycin and hamycin. These polyenes are isolated from a variety of Streptomyces species (148). Minimum inhibitory concentrations (MIC) of 0.4 μg/ml (0.4 μM) to 50 μg/ml (53 μM) for nystatin and less than 1 μg/ml (1 μM) are usually reported for these two common antibiotics in vitro against C. albicans (318). These MIC values may be affected by the media composition. The MIC is not affected by the inoculum size or incubation time (118,165).

The mode of action of these drugs is believed to be that they bind with sterol components of the cell membrane, which is ergosterol in susceptible fungi. This binding alters the permeability of the membrane to allow leakage of cytoplasmic materials (318). Resistance to nystatin and amphotericin B has only been induced in vitro by growth on media containing the polyene antibiotics. These drug resistant strains show reduced virulence when infected in laboratory animals (278).

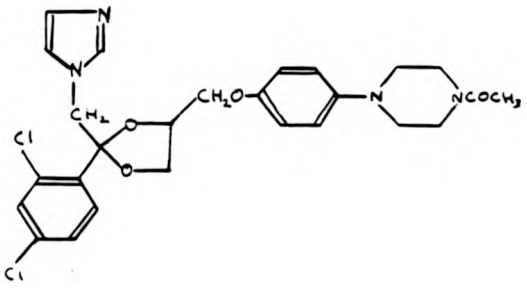
Clinically, amphotericin B is the most important polyene antifungal agent. Although nystatin is used fairly extensively for treatment of superficial



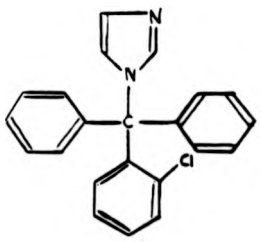
NYSTATIN



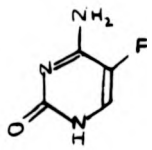
AMPHOTERICIN B



KETOCONAZOLE



CLOTRIMAZOLE



5-FLUOROCYTOSINE

Fig. 1.4 Some antifungal agents currently used against C. albicans

candidoses, the toxicity of this compound to the mammalian host system reduces its usefulness. Amphotericin B has been applied to virtually every form of candidosis. However, this compound also shows toxicity towards humans, although the side effects are less severe than other polyene antifungal agents. The main side effects are kidney damage, uraemia and hypokalemia (278).

One possible method of reducing the effective cidal concentration of a compound, when treating candidosis, is to use the drug in synergystic combination with amphotericin B. It has been known for some time that antibacterial therapy can be improved by the use of two antibiotics acting synergistically against a particular organism (177). A synergistic *in vitro* antifungal response has been found using sub-lethal levels of amphotericin B with any of the following:- ascorbic acid and cysteine (26), tetracycline and actinomycin D (206), 5-fluorocytosine (253) and rifampicin (27). It is believed that the levels of amphotericin B used enable a sufficient number of "holes" to develop in the membrane, allowing access of the antibiotics to their sites of action (254).

The polyene antibiotics have now been superseded by the mono-N-substituted imidazoles for the treatment of superficial fungal infections. High cure rates have been reported for clotrimazole - the first such compound used against vaginal candidosis (278). Other substituted imidazoles now used against superficial infections include miconazole, econazole and, recently, ketoconazole (see Fig. 1.4). The minimum inhibitory concentrations of the substituted imidazoles *in vitro* against *C. albicans* vary from 0.02 μg/ml (0.06 μM) to 4 μg/ml (11.6 μM) for clotrimazole (278,318). The other substituted imidazoles show minimum inhibitory concentrations of similar magnitudes (343). The wide variation of such values may be accounted for by inoculum density (151), media composition (407), pH (417) and morphological form (183), all of which can alter the sensitivity shown by *C. albicans* to the substituted imidazoles. In addition, the minimimum inhibitory concentration may be affected by the incubation period and also

the method used to determine the value (145).

The mode of action of the substituted imidazoles is believed to be inhibition of ergosterol biosynthesis. Van den Bosche et al found that, at concentrations of 9.05 µg/ml (0.1 µM) miconazole inhibited ergosterol biosynthesis by blocking demethylation of the precursor sterol at C_{14} (386). This same group found higher concentrations of substituted imidazoles were required for inhibition of rat liver cholesterol biosynthesis (385). Thus, the substituted imidazoles indirectly affect the membrane permeability of susceptible organisms and cause visible damage to the structure and function of intracellular organelles (318). However, other groups (80, 101) feel that the substituted imidazoles have a direct effect with the membrane causing rapid leakage of K^+ ions and high and low M.W. cytoplasmic molecules.

Clotrimazole and miconazole are poorly absorbed from the digestive tract and consequently low serum levels are found. This may be compounded by liver microsomal enzymes that rapidly breakdown the active drugs (99). However, much higher levels in serum of water soluble ketoconazole have been obtained (318), which suggest a more promising role for this substituted imidazole against systemic candidosis.

The nucleoside analogue most widely used against C. albicans is undoubtedly 5-fluorocytosine (5FC) (see Fig. 1.4). Since its commercial introduction in the late 1960's, this fluorinated pyrimidine has been used successfully, primarily against systemic candidoses (278). Concentrations of 0.1 µg/ul (0.8 µM) to 15 µg/ul (116 µM) are usually inhibitory to C. albicans in vitro (278). However, not all yeast isolates have been found to be sensitive to the drug. Estimates of primary resistance of between 10% (278) - 20% (13) of clinically isolated strains tested have been reported. The B serotype has been found to show a higher incidence of resistance to 5FC than the A (13). Resistance to 5FC may be easily induced in vitro by growth of C. albicans on media containing low levels of the drug (87). Medical practitioners should bear this in mind for clinical treatment of candidosis.

The mechanism by which 5FC inhibits C. albicans growth is believed to be by disruption of translation. 5FC is taken up into C. albicans by a cytosine permease. The pyrimidine is then de-aminated, phosphorylated to 5-fluorouridine triphosphate and incorporated into RNA. The presence of the fluorinated pyrimidine base in the RNA disrupts the normal protein synthetic activity of the cell and normal development is therefore grossly impaired. As much as 50% of the uracil may be replaced by 5-fluorouracil. Incorporation of 5FC into the host mammalian cell is limited due to lack of the specifically required permease (291).

As a water-soluble pyrimidine, 5FC may easily be absorbed from the gut, hence the preferred formulation is in tablet form. Peak serum levels of 5FC follow after 5 hr ingestion (278). Unfortunately, high levels of 5FC have been found to cause liver damage (302) and show bone marrow toxicity (290). This problem, coupled with the ease some strains of C. albicans show resistance to 5FC, indicates that there is a requirement for anti-fungal agents that do not have these drawbacks.

Overall, with the possible exception of ketoconazole, present anti-fungal agents show much to be desired. As indicated above, problems such as drug toxicity, limited absorbtion from the intestinal tract, and the ease of development of resistance by the fungi abound. As a consequence, any new drug should show a wide spectrum of activity, the possibility of oral administration with freedom from toxicity and low production costs. It was to achieve such an end that RNA synthesis in clinically the most important species, C. albicans was studied.

1.3 RNA synthesis

These following sections of this introductory chapter will discuss some aspects of DNA directed RNA synthesis (i.e. transcription), with particular reference to eukaryotes. Included in these sections will be discussions on the general RNA species that are transcribed from the DNA, the organisation of the DNA template in the eukaryote and the general mechanisms involved in

the process of RNA synthesis. It must be stressed that this is in no way a comprehensive study of this ever-expanding field - it should be considered an overview of current trends and ideas in the area.

1.3.1 Transcription of DNA coded RNA species

The "central dogma" of molecular genetics is now a firmly established scientific principle (i.e. DNA → RNA → protein) (394). These processes apply to all living cells. In eukaryotes, the cell's complement of DNA may be found in the nucleus, which is delineated by a double-membraned nuclear envelope. The possession of a nucleus distinguishes eukaryotic cells from prokaryotes. Eukaryotic transcription and processing of the newly synthesised RNA (i.e. post-transcriptional modification) is performed within the confines of the nucleus. Eukaryotic protein synthesis (translation) occurs on the ribosomes (highly complex ribonucleic acid - protein assembly), which are located on the outer membrane of the endoplasmic reticulum in the cytoplasm. In contrast, prokaryotic protein synthesis occurs concurrently with one end of the transcribed RNA translated whilst the other end may still be being transcribed (394).

The DNA may be divided into 2 sorts - that which codes for RNA and that which is non-coding. The functions of the non-coding regions are not yet fully understood. However, some workers (e.g. 119) believe that these sequences are important in determining the patterns of DNA folding in the nucleus (see 1.3.2). The DNA that is transcribed may be used as a template for translation (i.e. messenger RNA) or to form the ribosome (ribosomal RNA) or that which carries the amino acid in the translation step (transfer RNA). Although the general principle of eukaryotic DNA transcription is the same as in prokaryotes, the machinery necessary to control gene activation and repression and "unpack" the DNA is considerably more complex. In the eukaryote transcription of all 3 RNA species is usually followed by post transcriptional modifications (285).

Ribosomal RNA synthesis occurs in the nucleolus of the eukaryotic nuclei.

The genes coding for ribosomal RNA (rRNA) are highly re-iterated. There have been reports of anything between 50 and 2×10^4 copies of the gene present in each cell (140). These multiple gene copies are linked in tandem array along the genome of the organism. The transcription unit is relatively constant in size, with a molecular weight (M.W.) of 4×10^6 to 5×10^6. However, the non-transcribed spacer region varies in length from organism to organism (140). In eukaryotes the rRNA unit is transcribed as a 37 S to 45 S precursor, from which the mature rRNA species are processed. Multiple copies of DNA sequences coding for transfer RNA (tRNA) and another rRNA - 5 S rRNA - are present in the eukaryotic genome in tandem arrays. These RNA species may also be processed to produce the mature products.

The transcribed genes which are translated during protein synthesis are often termed structural genes. The structural genes for eukaryote proteins are large complex DNA molecules. In many cases, these contain a number of intervening sequences not found in the mature messenger RNA (mRNA) (264). These intervening sequences are not found in prokaryotes. Transcription by the eukaryotic cell of the structural gene coded DNA produces long mRNA precursor molecules. These precursor molecules are commonly referred to as heterogenous nuclear RNA (hnRNA). The hnRNA molecule is "capped" by the addition of a 7-methyl guanosine residue almost immediately. On completion of the transcript, a poly-A polymerase enzyme adds 100 to 200 residues of adenylic acid to the 3' end of the RNA chain. After splicing - i.e. removal of the intervening sequences - the mRNA migrates as a ribonucleo-protein assembly to the ribosome. It is thought the 5' cap allows binding to the ribosome. The function of the poly-A tail is not known for certain, but it seems to help mediate subsequent RNA processing and the migration from the nucleus (285). Table 1.1 shows the number of nucleotides, molecular weights and sedimentation coefficients of some RNA species transcribed from the DNA template.

RNA SPECIES	RNA POLYMERASE RESPONSIBLE FOR SYNTHESIS	SEDIMENTATION COEFFICIENT	MOLECULAR WEIGHT ($\times 10^{-6}$)	NUMBER OF NUCLEOTIDES
Mammalian Ribosomal RNA	RNA Polymerase I	28 S 18 S 5.8 S	1.9 0.71 0.06	5,000 2,000 130 - 160
S. cerevisiae Ribosomal RNA		25 S 17 S 5.8 S	1.3 0.7 0.06	4,000 2,000 158
Typical Mammalian hnRNA	RNA Polymerase II	Smaller than 4 S to greater than 45 S	Around 2.2 (Anything up to 18)	6,000 (Anything up to 50,000)
Typical mRNA		Usually 8 S - 15 S	Around 0.5 (Between 0.2 to 1.1)	1,500 (Anything in range 500 - 3,000)
Small Ribosomal RNA	RNA Polymerase III	5 S	0.04	120
Transfer RNA		4 S	0.03	76
Prokaryotic Ribosomal RNA	Prokaryotic RNA Polymerase	23 S 16 S 5 S	1.05 0.55 0.04	3,000 1,500 120

Table 1.1 The sedimentation coefficients, molecular weight and number of nucleotides in some RNA species and the RNA polymerase isozyme responsible for their synthesis. (Taken from Stewart and Latham (163)).

S. cerevisiae			Cauliflower*			Mouse plasmacytoma**		
I	II	III	I	II	III	I	II	III
185[a]	205		190	180		195	240	
		160			150			155
137	145	128	125	140	130	117	140	138
		82						89
					70			70
						61		53
48					50	49		49
44	46							
41		41		40			41	41
36								
	33.5	34						33
28	28	28				29	29	29
			25	25	25		27	
24	24	24			24			
					23			
20		20	22	22	22		22	
	18						19.5	
				19	17.8	19	19	19
			17.5	17.5	17.5			
				17				
				17				
				16.2			16.5	
				16				
14.5	14.5	14.5		14				
13.3	12.5	11						

Table 1.2 Subunit composition of several eukaryotic RNA polymerases

The table shows the subunits that make up the RNA polymerase isozyme from three different eukaryotes

a - numbers refer to M.W. x 10^{-3}

▭ - shown to be identical polypeptides by at least two criterion

—— - these polypeptides have the same M.W.

(Taken from * Paule (281) and ** Roeder (310).)

1.3.2 Organisation of the eukaryotic DNA into chromosomes

As noted in 1.3.1, the major difference between prokaryotes and eukaryotes is the latter has a nucleus where most of its DNA is located. This section will examine how the DNA is packaged in a eukaryote such as C. albicans. Other differences are found between prokaryotes and eukaryotes, e.g. the DNA content of the former is usually much smaller, whilst that of the latter is usually tightly coupled with specialised proteins (histones). However, these differences may be regarded as secondary as the largest DNA complement of bacteria may be as large as that of the smallest DNA complement of some eukaryotes (119). Also one class of eukaryotes - the dinoflagellates - does not possess histones, although it does possess the ubiquitous nuclear membrane (309).

The DNA from a eukaryote is usually in the form of a linear polymer containing anything from 1×10^7 to 1×10^{11} base pairs (bp). In a lower eukaryote, such as C. albicans, there are approximately 1.2×10^7 bp in the total genome (142). The folding of the DNA is an important consideration for eukaryotic cells as it is essential to pack the very long polymer in an ordered manner. In C. albicans the DNA has to fit into the nucleus with a radius of 1 μm (36) and hence with a volume of $4 \times 10^{-18} m^3$ - assuming the nucleus forms a perfect sphere in the organism. The conformation the DNA double helix may adopt depends upon a variety of external parameters. Under "normal" physiological conditions the most stable conformation of the DNA double helix is the right hand B form (93). In such conditions a polymer of DNA (with a diameter of 2.4 nm and 0.34 nm between each bp) of 1.2×10^7 bp would occupy a volume of $2 \times 10^{-20} m^3$. However, it is necessary to fold the DNA in such a manner that there is easy access, when required, by the transcription enzyme(s) and factors. The histones pay a central role in storage and packing of the DNA.

Histones are small proteins (molecular weights of 10,000 to 15,000) that contain a high proportion of positively charged amino acids. These allow tight binding to the DNA. Four of the five histones are present in an

octamer in duplicate amounts. These histones - termed histone H_{2A}, H_{2B}, H_3 and H_4 - comprise the central core of the nucleosome - the fundamental packing unit of eukaryotic DNA. These histones must rank amongst the most highly evolutionary conserved proteins known. For example there are only 2 differences in amino acid sequence of histone H_4 from cows and peas (173).

X-ray diffraction patterns of crystals of the nucleosomes reveal that 146 bp of DNA are wrapped around the disc shaped histone octamer core. Each core is connected by a "linker" sequence of approximately 60 bp in length (307). The standard nucleosome consists of about 200 bp of DNA. In a lower eukaryote such as C. albicans one would expect a total of 5×10^4 nucleosomes. The strand of DNA containing the histone octomers may be visualised by electron microscopy giving a "beads on a string" appearance (199). The DNA in this order of assembly may be termed chromatin.

The nucleosome is folded into the next level of organisation (the so called 30 nm chromatin fibre) (350) and held by co-operative binding of the 5th histone - H_1. Two models have been proposed for the packing of nucleosomes into the 30 nm chromatin fibre. Both would allow a 1 cm strand of DNA in the B conformation (i.e. approximately 2.9×10^7 bp) to be packed into a shorter strand of 0.24 mm in length (250). Thus, assuming the packaged chromatin of C. albicans adopts the form of a cylinder, the volume of the 30 nm chromatin fibre in this lower eukaryote may be estimated at $7 \times 10^{-20} m^3$. The DNA must be further folded into "domains" using specialised scaffolding proteins. Such a general folding pattern for the genome has been adopted in both prokaryotic (e.g. Escherichia coli) and eukaryotic (e.g. Man, Drosophila melanogaster) organisms (29). In eukaryotes the loops have an average length of 400 nm of 30 nm chromatin fibre. These fibres are attached to the inner nuclear membrane by a specialised laminar protein assembly.

1.3.3 DNA directed RNA polymerases

The DNA directed synthesis of RNA is catalysed using a DNA dependent

RNA polymerase (nucleoside triphosphate : RNA nucleotidyl transferase, E.C. number 2.7.7.6). Over the past 10 - 15 years the subcellular localisation, subunit composition and reaction properties of these complex enzymes - from both eukaryote and prokaryotic sources - have been elucidated. Initial work in the early 1960's (62) resulted in the isolation of a protein that was capable of DNA directed RNA synthesis in E. coli. Since then, it has been found that prokaryotes possess a single class of enzyme responsible for the synthesis of all DNA coded RNA species. The more complex transcription machinery of eukaryotes is reflected in most eukaryotes possessing three classes of RNA polymerase. This is not a definitive difference between prokaryotes and eukaryotes. It has been found that in at least one lower eukaryote - Trypanosoma cruzi - there is only one RNA polymerase isozyme (192)

The enzymes from both prokaryotes and eukaryotes have several factors in common for synthesising RNA. Firstly, both are DNA directed (i.e. the enzyme requires a DNA template to show activity). RNA synthesis occurs in a 5'⟶3' direction. The enzyme is able to accurately add one of the four ribonucleotide bases, that is complementary to the DNA strand, to the nascent RNA chain. Also, the enzymes require a divalent metal ion - almost always Mg^{++} or Mn^{++} - to show activity. All RNA polymerases examined so far are Zn-containing metalloenzymes. The Zn is tightly bound to at least one of many subunits that are a further characteristic of RNA polymerase. The four stages of DNA directed RNA synthesis will be discussed in the next section of this chapter (1.3.4). Also, 4.4.3 will further discuss the requirements for divalent metal cations of RNA polymerases in general and those of C. albicans in particular.

The 3 classes of RNA polymerase usually isolated from eukaryotic sources are termed RNA polymerases I, II and III. This terminology is based on that of Roeder and Rutter (311). Each class of enzyme was distinguishable on the basis of its elution from DEAE-Sephadex when the salt concentration of the eluting buffer was increased. Chambon and co-workers proposed a nomenclature for the RNA polymerases based on their sensitivity

to the fungal toxin α-amanitin (63). In this scheme, enzymes that are insensitive, extremely or moderately sensitive to α-amanitin are termed classes A, B and C enzymes respectively. The general pattern of α- amanitin sensitivity for RNA polymerases from higher eukaryotes, such as mammals, has led some workers to equate RNA polymerases I, II and III with A, B and C. However, as will be discussed in 4.4.3, these may not necessarily correspond to RNA polymerases I, II and III. RNA polymerases I, II and III are responsible for the synthesis of the large 37 S - 45 S ribosomal RNA precursor heterogenous nuclear and small 5 S ribosomal and transfer RNA species. respectively.

Valenzuela et al (383) first reported inhibition of RNA polymerases I and II from rat liver and sea urchin by the zinc chelator 1, 10 - ortho phenanthroline. This suggested the necessary involvement of Zn for the RNA polymerases to show activity. Recent work has suggested that, under certain circumstances, this compound may inhibit enzyme activity by degrading the DNA template (97). However, other studies, using techniques such as atomic absorption spectroscopy (e.g. 353), have suggested all nucleotidyl transferases are Zn metalloenzymes. Inhibition with other Zn chelating agents, such as 8 - hydroxyquinoline, and the lack of inhibition using 3, 7 - phenanthrolene (which does not chelate with Zn), are further evidence to suggest Zn has an important role (112). RNA polymerases from a number of viral (59, 77), prokaryotic (146,334) and eukaryotic (180,209) sources have all been shown to contain zinc. Indeed, it has been found that all nucleotidyl transferases contain Zn . As removal of this Zn results in complete loss of RNA polymerase activity, it is probable that the Zn moiety is a necessary component for activity (286). Although very tightly bound, the zinc can, in certain circumstances (i.e. growth in Zn limiting media), be substituted by cobalt in RNA polymerase from prokaryotes and some lower eukaryotes (358,111). This has enabled studies to be made of the role of zinc in transcription.

The zinc content of RNA polymerases, from various sources, have been

found to differ widely. This may be illustrated by considering the Zn content of S. cerevisiae RNA polymerase II (1 g atom Zn/M.W. enzyme (209)) and wheatgerm RNA polymerase II (7 g atom Zn/M.W. enzyme (286)). As a further comparison the Zn content of RNA polymerase II from calf thymus has been measured at 5 g atom Zn/M.W. enzyme (37). A similar spread of estimations of Zn content for RNA polymerase I are apparent: this isozyme from calf thymus has 7 g atom Zn/M.W. enzyme (37) compared to 2 g atom Zn/ M.W. enzyme from S. cerevisiae (14) and Euglena gracilis (113). The Zn content of RNA polymerase III has been reported to vary from 2 g Zn atom/ M.W. enzyme in S. cerevisiae (391) to 4 g atom Zn/M.W. enzyme from calf thymus (37).

The function of the zinc in RNA polymerase enzyme, and the reason why there is such a variation in the Zn content of the different isozymes from different species, is not yet fully understood. Almost certainly, at least 1 Zn is involved in the catalytic mechanism of the enzyme (see 1.3.4). It is possible that the other Zn atom(s) are involved in maintaining structural stability. The Zn is so tightly bound that removal of this cofactor results in denaturation of the enzyme (391). Thus, other methods (e.g. n.m.r. studies) must be used to elucidate its role. Studies on the role of Zn in eukaryotic RNA polymerases should result in assigning the Zn atom to one or more of the many subunits that are typical of these enzymes. The two Zn atoms present in E. coli RNA polymerase have been assigned to particular subunits. This enzyme from this prokaryote has been extensively studied. It consists of five subunits, four of which form the active core of the enzyme. These are termed the α subunits (2 per enzyme core M.W. 40,000), β (M.W. 155,000) and β' (M.W. 160,000). Although this core enzyme can direct DNA dependent RNA synthesis using naked DNA, the fifth subunit, termed σ factor, is necessary for accurate transcription initiation of specific DNA sequences (50). One of the Zn atoms has been reportedly associated with the β' subunit, whilst the other may be found at the junction of the β and β' subunit (403). However, other reports assign the second

Zn atom to the β' subunit (260). Unfortunately the unambiguous location of the Zn atom in the eukaryotic enzyme has not yet been realised.

The typical RNA polymerase, from both eukaryotic and prokaryotic sources, is a large molecule (with a molecular weight of 400,000 to 700,000) consisting of multiple subunits. By no means all DNA dependent RNA polymerases are such large molecules - those associated with mitochondrial or chloroplast RNA synthesis and some viral induced enzymes (e.g. phage N_4 (59)) have molecular weights of 150,000 to 200,000 (138).

In contrast to the five subunits possessed by E. coli, most of each class of RNA polymerase from a eukaryotic source consists of some seven to fifteen polypeptides. The complexity of these enzymes has not always been fully realised. Some studies have failed to analyse the RNA polymerase under conditions which allow the subunit composition to be fully elucidated. It is necessary to employ gel electrophoresis conditions which allow resolution over the range of molecular weights of 10,000 to greater than 200,000 in order to determine the subunit composition. Table 1.2 shows the subunit composition of RNA polymerases from three eukaryotic sources.

The subunits of eukaryotic RNA polymerases may be conveniently divided into two groups: large (i.e. those having a M.W. greater than 100,000) and small (i.e. those having a M.W. smaller than 60,000). RNA polymerase III does possess a subunit intermediate in size, at least according to the literature cited for this isozyme shown in Table 1.2. The subunit composition of the RNA polymerases cited in the table illustrates the complexity of these large molecules. The table shows the M.W. of the component subunits, of the RNA polymerases for the organisms indicated. Other groups have reported different values for the M.W. of these subunits. This is presumably because M.W. estimation of large polypeptides by PAGE is difficult as there are few high M.W. markers available.

RNA polymerases are composed of polypeptides, most of which are coded for at different genetic loci - the smaller polypeptides do not usually appear to arise as a consequence of proteolytic cleavage. This may be con-

firmed by different cleavage patterns (using two dimensional gel electophoresis) and the lack of antigenic cross-reactivity between most subunits. However, some subunits of RNA polymerases, from the same organism, do appear to be identical - at least as far as these parameters are concerned (138). These are indicated in the table.

Unambiguous assignation of the roles the subunits play in transcription may not be fully achieved until techniques have been developed to reassemble the RNA polymerase. E. coli, and other prokaryotic RNA polymerases, may be reassembled from their individual subunits to form active enzyme (50). Unfortunately, there are no reports of this having been achieved in a eukaryotic enzyme. Thus, the elucidation of the subunit functions has necessitated other approaches. Antigenic cross-reactivity and antibody studies, in addition to the use of chemical probes and genetic mutations, have helped in unravelling the role of the RNA polymerase subunits.

As a lower eukaryote in possession of all three RNA polymerases, S. cerevisiae has lent itself to extensive studies on these enzymes. RNA polymerases constitute only a small proportion of the cells total protein, thus large numbers of cells are required to obtain sufficient quantities of the enzyme for studies. As an example only about 8 - 15 mg, 20 mg and 3 - 10 mg of RNA polymerases I, II and III may be purified from 1 Kg of S. cerevisiae (141 ,142 ,384). Such large quantities of S. cerevisiae yeast may easily be cultivated. Also, strains of this yeast have been isolated that show mutant RNA polymerase activity. One mutant shows defective RNA polymerase II activity, the rpo B mutant, has been isolated and characterised Although the RNA polymerase from this mutant does not possess the subunits with molecular weight 32,000 or 16,500 it is believed that it is in fact the large 220,000 molecular weight subunit that is altered. Evidence for this is based on different peptide maps of this large subunit from rpo B, mutants and wild type strains of S. cerevisiae. Removal of the 32,000 and 16,500 molecular weight subunits by electophoresis, or mild urea treatment, from the wild type enzyme did not result in the differences in RNA polymerase

activity found (i.e. alteration of salt and temperature optima and a relative deficiency in RNA chain initiation and elongation) (317).

The immunological relationship between RNA polymerases, and their subunits, has allowed comparisons to be made both between the components of each class of enzyme and between RNA polymerases from different species. It was first reported by Hildebrandt et al (164) that there were similar antigenic determinants of RNA polymerases I and II from S. cerevisiae. Antibodies raised against S. cerevisiae RNA polymerases I could inhibit in vitro activity of RNA polymerase I and II and formed immuno-precipitates with both isozymes. Reciprocal experiments, using antibodies raised against RNA polymerase II showed similar results (164). Studies by Buhler et al (45) indicated that the cross-reactivity was not due to antigenic similarities of the largest subunit of RNA polymerase I with other subunits from the same enzyme or any from RNA polymerase II. This group has prepared a detailed immunological study of S. cerevisiae RNA polymerases, and found that antibodies against native RNA polymerase I and II and all subunits of RNA polymerase I showed cross-reactivity with those common to all isozymes. In addition, the large subunits of RNA polymerase I did show some cross-reactivity with those of Candida tropicalis and Endomycopsis fibuligeria, two distantly related fungal species. However, no cross-reactivity was observed with Schizosaccharomyces pombe, a third species of yeast (44).

Recent work on silkworm (Bombyx mori) RNA polymerase I has shown that monoclonal antibodies against the second largest subunit cross-react with RNA polymerases I and II from the same species (129). This finding indicates that these two RNA polymerases share commonly exposed antigenic determinants. This is a similar result to that found for S. cerevisiae, reported in the preceding paragraph. Monoclonal antibodies have also been raised against RNA polymerases from higher (e.g. calf thymus (56) and rat hepatoma (312)) and lower (e.g. the fungi Podospora comata (388) and S. cerevisiae (169)) eukaryotes. Results from these investigations have implicated several subunits in roles in the mechanism of RNA synthesis (see 1.3.4).

RNA polymerases might have part of their sequences conserved over evolution. Evidence for this is provided by antibodies against RNA polymerase II from insects (D. melanogaster and Chironomus tentons) showing cross-reactivity with the same isozyme from sheep (201). Also, it has recently been reported that the gene coding for part of RNA polymerase II, from D. melanogaster, hybridises with RNA species from mammalian systems (172). This finding implies some degree of conservation over evolution for RNA polymerase II. Antibodies against S. cerevisiae snowed some cross-reactivity with the largest, and one of the smallest, subunits of wheatgerm RNA polymerase II (44). However, this group reported little, or no, binding between these antibodies and either calf thymus RNA polymerase II or E. coli RNA polymerase. This indicates that over long periods of time the sequence and structure responsible for antigenic cross-reactivity may change.

The topographic arrangement of the subunits of the eukaryotic RNA polymerase is still being elucidated. Investigations into the three dimensional structure of E. coli RNA polymerase have been more successful. The review by Meisenberger et al (256), on the structure of the holoenzyme, indicated that the enzyme had a maximum dimension of 24 nm. This was determined by small angle X-ray studies of holoenzyme, core and subunits in solution. The volume of the holoenzyme was determined as 790 nm^3. This group suggested a triangular arrangement for the subunits, with a "Y" shaped σ subunit occupying the centre of the arrangement. Another group (364), has postulated a similar arrangement for the core structure. This model, based on small angle neutron scattering, is three elongated elipsoids with axial ratios of between 7 : 1 and 9 : 1. Recent work by Tichelaar et al (375) suggested that the RNA polymerase may even adopt a cylindrical shape when studied using scanning transmission electron microscopy. It should be borne in mind that the method used for measurement of the dimensions of the enzyme may affect the results obtained. The conditions used to investigate the RNA polymerase may be different from those found in vivo and could hence alter the structure of the enzyme

The RNA polymerase from E. coli has a sedimentation coefficient of 15 S (50). As a comparison this value for RNA polymerase I from S. cerevisiae has been measured as 16 S (384). Recent work by Bull and Garrido (46) has shown that this isozyme, and RNA polymerases II and III, are roughly spherical in shape. The dimensions of RNA polymerases I, II and III using scanning electron microscopy are 12.7 nm by 11 nm, 12.7 nm by 12.2 nm and 13.6 nm by 11.5 nm respectively. Assuming RNA polymerase I forms a sphere of approximate radius 6.25 nm, the volume may be estimated as 1,020 nm^3. This value was 1.3 times that estimated by Meisenberger et al. for E. coli RNA polymerase of 790 nm^3 (256). Such a ratio is similar to that reported by Bull and Garrido, who estimated the volume of the eukaryotic RNA polymerases to be 1.33 times as large as that reported by Slayter and Hall (355). This group also used electron microscopy techniques to measure the dimensions of E. coli RNA polymerase. Unfortunately the localisation of specific subunits has not yet been realised for eukaryotic RNA polymerases.

The requirement of eukaryotes for RNA polymerases with multiple subunits is still not yet fully understood. Recently, Grossman postulated that the large number of subunits may reflect a requirement, by eukaryotes, for RNA polymerases possessing a large number of nucleotide binding sites. This author believed that the nucleotide and divalent cation - as the direct substrate for RNA polymerase - were bound to several subunits to ensure high fidelity and high rates of transcription (133). It might also be possible that the high number of subunits possessed by eukaryotic enzymes is a reflection of the complex nature of eukaryotic transcription. Section 1.3.1 discussed the highly complex nature of the stored genetic information, in the nucleus, as a DNA-protein assembly. The "extra" subunits may be required for interaction between RNA polymerase and the DNA-protein complex. In addition, other factors - as will be discussed in Section 1.3.4 - are required for accurate transcription. It is possible that the multiple subunits may be required to interact with these factors.

1.3.4 Some aspects of the transcription mechanism.

The complex process of RNA synthesis by the cell may be understood best by dividing this process into its component stages. Transcription of a DNA sequence has four steps. The binding of RNA polymerase to a specific site (step 1) is then followed by initiation of the RNA chain (step 2). The RNA chain is elongated (step 3) until, finally, the process is terminated (step 4). Although the general principle of prokaryotic transcription appears to be the same as that of eukaryotes, the former is better understood. This is presumably because faithful prokaryotic RNA synthesis has been characterised for at least the last ten years. Accurate prokaryotic transcription requires only a suitable section of DNA, the RNA polymerase, the presence of a divalent cation and the four ribonucleoside triphosphates (61). In contrast, eukaryotic RNA polymerases require additional factors for faithful transcription of DNA sequences. As a consequence, it was not until 1979, when systems became available that could accurately transcribe eukaryotic genes in vitro (396), that advances were made.

RNA polymerases, and other DNA binding proteins, recognise specific DNA sequences by hydrogen bonding to base pairs and by "sensing" helix geometry. The B form DNA double helix - the most stable form the DNA adopts under physiological conditions - has two separate grooves running between the two strands. Portions of each bp are exposed into these two separate channels. The arrangement of protruding atoms in a sequence can be uniquely recognised by interaction with Hydrogen bonding amino acid residues of a protein. Thus, a sequence of amino acid residues in a DNA binding protein can be arranged to maximise the H-bonding between it and the recognised sequence. Another criterion for recognition of a specific DNA sequence can be the composition of bases in that sequence. Both the exact tilt of the bases and the helical twist angle between the bp have been found to depend upon which nucleotides are next to each other in a sequence. The small variations in the helix will cause atoms to be displaced from their ideal positions - distorting the phosphate-sugar backbone of the DNA by

± 0.1 nm. It is believed by Trifonov (378) that any DNA binding protein should recognise this displacement.

The first stage of transcription involves the binding of the RNA polymerase to a specific DNA sequence, i.e. promoter recognition. It has been known for some time that prokaryotic RNA polymerases require two regions of conserved DNA sequence, located about 10 (the "Pribrow box") and 35 bp upstream of the transcription startsite (295,328,337,371). These two highly conserved sequences, of TTGACA , around 35 and TATAAT, around 10 bp upstream from the startsite, have a sequence of 15 - 21 bp between these regions (314). Promoters may be expected to share common structural features, reflecting a similar interaction with RNA polymerase. However, a comparison of prokaryotic promoter sequences has revealed considerable sequence diversity outside the two conserved regions (348). A recent compilation and analysis of E. coli promoter sequences has shown that mutations in the highly conserved sequences affect the strength of E. coli RNA polymerase-promoter binding (157). The σ subunit of the RNA polymerase is responsible for binding to the DNA sequence containing the promoter region (272). Different σ factors appear to be responsible for different promoter selection in prokaryotes (95). It is thought that σ subunit-DNA binding results in changes in the secondary structure of the DNA (144).

Eukaryotic RNA polymerases require other factors for correct promoter recognition in addition to DNA sequences. However, there does appear to be some requirement for a nucleotide sequence. Genes transcribed by RNA polymerase II were amongst the earliest characterised, having several control regions upstream of the site at which transcription is initiated. The development of cell-free systems that allowed selective and accurate initiation of transcription has enabled studies to be made on the control of eukaryotic gene expression (396). Two methods have been described that support faithful RNA polymerase II directed transcription. These are derived from either ammonium sulphate precipitates of cell lysates or supernatants from high speed centrifugations. The latter method usually

requires the addition of exogenous RNA polymerase II.

Promoter DNA sequences for RNA polymerase II have been found to contain an A-T rich region. There is typically a TATA sequence (the "Goldberg-Hogness box" (41)), which seems to be present at about 30 bp from the nucleotide coding for the first base of the mature mRNA (the cap site). This is the point at which transcription begins (416). Two other DNA sequence elements have been implicated in the control of initiation of transcription. The first is located around the cap site region and is typically a PyCATTCPu sequence (132), whilst sequences far upstream (70 - 80 bp) from the TATA box constitute the second sequence - typically CCATT (28).

Deletion of the capsite and CCATT sequences may not affect efficient transcription of some genes in vitro e.g. rabbit β globin (131), but the CCATT sequence is important in promoting high efficiency transcription of this gene in vivo (130). The cap sequence apparently directs the selection of the exact starting point of transcription (314). The TATA box appears to be important in directing the transcription of several genes in vitro. Evidence for this is provided by deletions, or single base changes, in the TATA sequence has been found to abolish accurate transcription of some cloned genes (e.g. ovalbumin (121) and Simian virus 40 (244) genes). In addition, deletions of a given size downstream from the TATA sequence have been found to result in shifting the initiation site downstream a similar distance (131). When cloned into a plasmid DNA, the TATA sequence can direct initiation of transcription 25 bp from the introduced sequence (325). These findings indicate that the DNA sequences in a promoter region are important in directing the RNA polymerase II to correct initiation site.

The ease of analysis of the RNA polymerase III transcription products, and the ability to initiate in vitro RNA synthesis, has permitted the study of transcription directed by this isozyme in nuclear preparations and reconstituted systems. Initial work by Wu demonstrated accurate transcription of a defined template - containing 5.5 S rDNA - using a supernatant from a high speed centrifugation prepared from KB tumour cells (404). Extracts from

Xenopus laevis oocytes and KB cells have been fractionated to reveal at least three proteins required for accurate 5 S rRNA and tRNA transcription. One of these proteins is believed to bind to internal regions of the gene. The protein factor binds to 5 S rDNA at positions +45 to +96 (319). Similarly accurate transcription of the tRNA gene requires a protein factor binding to internal regions. There appears to be two intragenic control regions for transcription of the tRNA gene: the first lies near the 5' end (at positions +5 to +25) whilst the second lies at positions +50 to +72 (313). The DNA sequences of 4 $tRNA^{lys}_{2lys}$ genes have revealed a highly conserved sequence located around position -20 (166). However, the exact significance of this conserved region is unknown, as its deletion does not affect in vitro transcription in this system (88). It is probable that it is involved in in vivo transcription, as several groups (e.g. 344 ,360) have noted that sequences upstream of initiation of the tRNA gene can affect transcription. The promoter regions of tRNA and 5 S rRNA genes appear to be conserved in diverse eukaryotes (75). This indicates that the control of transcription of these genes may be conserved over evolution.

It is believed that transcription of the large 37 S - 45 S rRNA precursor by RNA polymerase I is mediated by DNA sequences both downstream and upstream of the start site (196,261). Expression of the rDNA gene from Drosophila required a promoter sequence lying in the region -43 to -27 (197). In addition to RNA polymerase I, a supernatant from a high speed centrifugation was required for accurate transcription initiation. The rDNA genes from other organisms have been investigated to determine sequences that influence the activity of RNA polymerase I. These investigations have found a region of approximately 40 bp upstream for the start of rDNA from mice (136) and a sequence lying between -145 and +16 of Xenopus rDNA (267). required for initation by RNA polymerase I. There does not seem to be any great level of sequence homology between the RNA polymerase I promoter regions This may be illustrated by the finding that sequence homology among three Xenopus species is restricted to the portion -11 to +4. This suggests that

this sequence has a major role in RNA polymerase I recognition (15). However, other species, such as S. cerevisiae (193), Drosophila (223), mice and humans(115), have rDNA that does not show conservation of such sequences. In all the species noted rDNA transcription begins with an A residue and the sequence AGGTA occurs within ten nucleotides of the initiation site.

An RNA polymerase enzyme may bind to DNA at a recognised promoter sequence or arbitrarily as non-specific binding (often termed non-promoter binding by some workers (e.g. 365)). As bacterial polymerase holoenzyme require only a promoter sequence for specific binding, it is not surprising that this process is better understood in prokaryotes. The sequence of events for promoter selection and RNA chain initiation by E. coli RNA polymerase on T7 DNA and the Lac UVS promoter of E. coli DNA have been described in some detail (120). Firstly, the bacterial RNA polymerase locates and binds selectively and stably to the DNA promoter sequence - forming an "I" or "closed" complex. This binary complex may be formed at low temperatures (0° C.) and is stable in the absence of nucleoside triphosphates. This complex has a half life for dissociation of approximately 40 min (61).

The holoenzyme is thought to facilitate the unwinding and separating of the DNA strands to form an "open" or "RS" (rapid start) complex. In contrast to the "I" complex, this binary "RS" complex does not form at low temperatures, but on forming is stable for hours in the absence of nucleotides. The RNA polymerase is believed to cover approximately 30 bp on binding (303). Methods such as UV hyperchromaticity (168), and DNA sequencing of the holoenzyme opened sequence (348) have suggested that between 10 and 12 bp are melted on formation of the RS complex. Other groups have studied the extent of DNA unwinding, based on sensitivity of DNA to dimethyl sulphate methylation (257) and the migration of unwound DNA by agarose gel electrophoresis (120). These studies have indicated that 15 - 17 bp may be unwound on "RS" formation. The "RS" complex allows the rapid formation of the first phosphodiester bond to be catalysed in the presence of nucleotides. This process can be termed initiation of RNA

synthesis as the binary complex becomes a ternary complex of RNA polymerase - DNA-nascent RNA chain.

Eukaryotic RNA polymerases, like prokaryotic enzymes, are able to transcribe both double (ds) and single stranded (ss) DNA templates. Single stranded templates generally promote higher rates of transcription by eukaryotic RNA polymerases. For example, Dezelee et al (92) have reported that S. cerevisiae RNA polymerases I and II transcribe un-nicked ds DNA very inefficiently. However, the introduction of unpaired gaps into the duplex DNA, by treatment with nucleases that produce ss regions, stimulate transcription by these enzymes. The same group also reported that a gap of 25 - 50 nucleotides in length was required for S. cerevisiae RNA polymerase to initiate RNA synthesis. Just as RNA polymerases from S. cerevisiae require nicks for efficient transcription of ds DNA, these enzymes from higher plants and animals are similarly stimulated (63, 103). The eukaryotic RNA polymerase has been found to protect 34 - 38 bp from nuclease digestion on binding to DNA (103).

Selective, sequence specific binding has been reported for wheatgerm RNA polymerase II on de-proteinised Adenovirus DNA (402), Simian virus 40 DNA (323) and plasmid DNA containing a cauliflower mosaic virus sequence (129). The binding sites on Adenovirus DNA corresponded to similar binding sites for human placental RNA polymerase II (402). It is thought that wheatgerm RNA polymerase II is able to form two types of binary complex analogous to the "I" and "RS" complexes described for bacterial RNA polymerases. The stability of the binary complex of wheatgerm RNA polymerase II and SV 40 DNA is greater on the superhelical form of the DNA compared to either relaxed circular or linear forms (324). This may be due to unwinding of the supercoiled DNA providing energy for denaturation of the DNA strands. It should be noted that although the reports cited above do imply some degree of selective binding and initiation of transcription, in no case has the selectivity been definitively correlated with an in vivo promoter. As noted earlier in this section, a number of other factors are required

to obtain faithful in vitro transcription of eukaryotic genes besides eukaryotic RNA polymerases.

The formation of the first phosphodiester bond characterises transcription initiation. It has been postulated that the Zn metal cofactor plays an important role in initiation and elongation in the formation of this bond. It was suggested in the early 1970's, the function of Zn in a nucleotidyl transferase was to activate the 3'OH group of the ribose (354). Thi would facilitate a nucleophillic attack of the primer - or growing chain - towards the α phosphate group of the nucleotide entering the active site (Fig. 1.5). It is thought (10, 49) that a divalent metal ion-nucleotide complex serves as a substrate for this reaction. The divalent metal ion co-ordinates with the oxygen atoms of the β and γ phosphate and is held at a specific site - the nucleotide binding site - possibly through a hydrogen bond. The charge on the α phosphate group is neutralised by a positive moiety on the enzyme and one oxygen atom of this phosphate. (It is possible that aspartic acid and glutamic acid residues have essential roles in this process as chemical modification of these residues results in loss of activity (272)). Attack of the terminal 3' OH group of the now growing RNA chain causes subsequent pyrophosphate release.

The events described in the preceding paragraph are also applicable to the elongation mechanism of the RNA chain in a 5'→ 3' direction. Both initiation and elongation are better understood in the prokaryote enzyme than for the eukaryote. Studies with the prokaryote enzyme have suggested roles for the subunits and their involvement in transcription. Early investigations into the subunits functions implicated the σ subunit in promoter recognition (see page 30 of this section) (The subunit dissociates from the bacterial RNA polymerase after the ternary initiation complex has been formed (348)). Since then, methods such as chemical modification, cross-linking by photolysis (150), studies on mutants (116) and the use of subunit-specific antibodies (277) have helped in elucidating the roles of the subunits in the bacterial RNA polymerase. The β subunit is believed to be

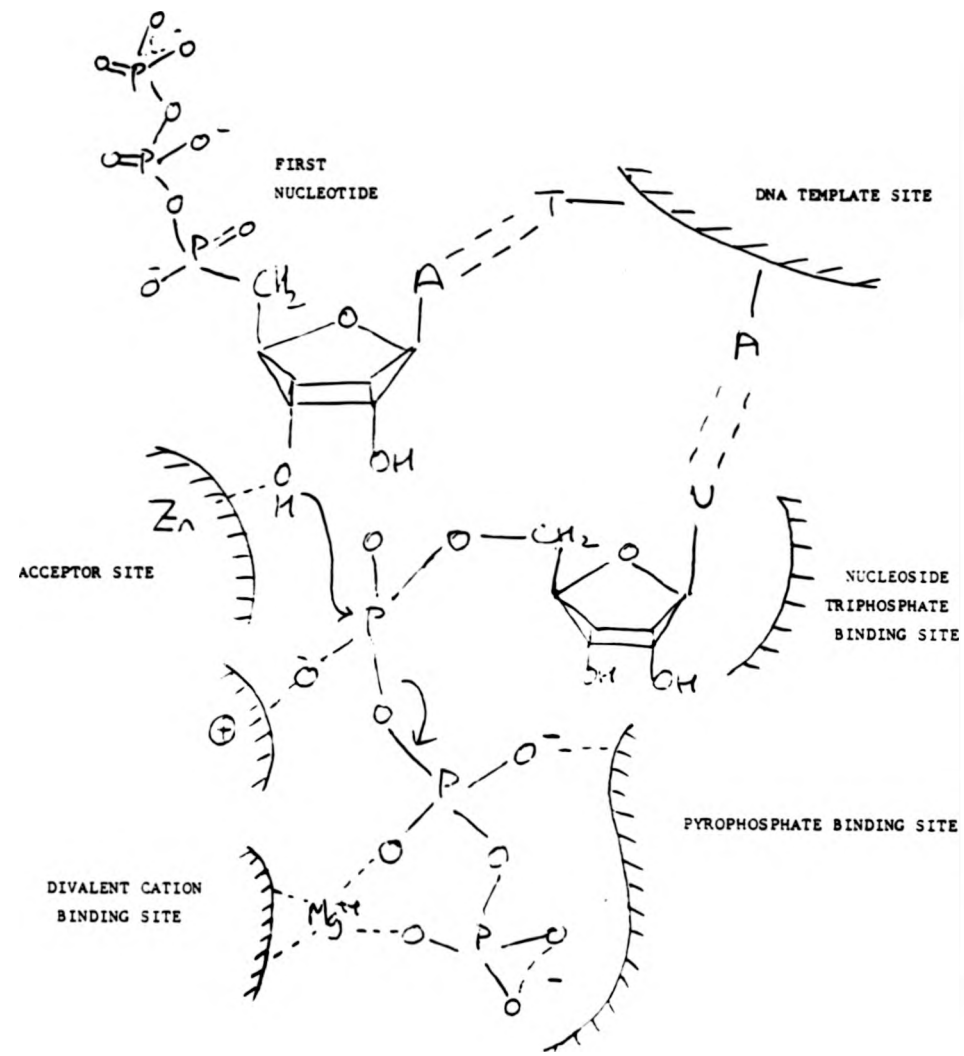


Fig. 1.5 Schematic model of active site of RNA polymerase during RNA chain initiation.

The figure shows a schematic representation of RNA polymerase initiating the first phosphodiester bond between ATP and UTP (based on models proposed by Armstrong et al (10), Lattke and Weser (209) and Mildvan et al (259)).

involved in both the initiation and elongation reactions (116, 150) and to contain a nucleoside triphosphate binding site (230) (It is worthwhile remembering that the β subunit is believed to have at least one, if not both, of the Zn atoms - see 1.3.3). The β' subunit appears to be involved in DNA binding and assisting in promoter selection (150), whilst the two α subunits, also involved in DNA binding, contain a RNA product site (236). Gamper and Hearst(120) postulated an "unwindase" centre, at the "front" and "back" of the RNA polymerase, to uncoil the two DNA strands. However, these functions do not appear to have been assigned to any subunit as yet.

There is evidence to suggest roles for some subunits of eukaryotic RNA polymerases. Research by Huet et al (170), of nucleic acid binding to S. cerevisiae RNA polymerase I, has implied four subunits were bound to the DNA. These had M.W. of 185,000, 48,000, 36,000 and 23,000. Martial et al (241) have reported that pyridoxal-5' phosphate - acting as a nucleotide analogue - could inhibit the activity of S. cerevisiae RNA polymerase I. The subunits the analogue bound to could be identified. Reduction of the Schiff base formed with lysyl residues on the enzyme - by NaB $[^3H]_4$ - revealed the tritium label associated with both large subunits (of M.W. 185,000 and 137,000) and the 48,000 and 36,000 subunits. The addition of nucleotides or DNA protected the enzyme from inactivity; the former protected the large subunits whilst the latter protected all four subunits from binding to pyridoxal-5' phosphate. These studies imply these four subunits are involved with DNA binding, whilst the nucleotide binding sites are located on the large subunits.

Cooper and Quincey (79) reported that the subunits with M.W. of 37,000 and 48,000, from RNA polymerase I, are involved in elongation and RNA synthesis at high ionic strength. It was reported that removal of the 48,000 and 37,000 subunits - to form what this group termed RNA polymerase A* - had altered properties in elongation and RNA synthesis at high ionic strength. In contrast, removal of these subunits by chromatography on blue dextran-Sepharose - a resin that interacts with polypeptides that

have nucleotide binding sites - completely inactivated the enzyme (47). The exact function, as noted in 1.3.3, of all the subunits of the enzyme and their involvement in the stages of transcription still needs to be unravelled.

Another approach to defining subunit function of eukaryotic RNA polymerases is based on genetics. It was suggested in 1.3.3 that the 220,000 M.W. subunit of RNA polymerase II may be functionally equivalent to the β subunit of bacterial RNA polymerase. Further research, using antibodies specific to the largest subunits, has implied these may be functionally equivalent to the β and β' subunits from prokaryote RNA polymerase. Antibodies against the largest subunits of RNA polymerase I and II, from rat hepatoma, inhibit binding of DNA to these subunits (312). Monoclonal antibodies against the 190,000 M.W. subunit inhibit chain elongation by RNA polymerases I and III from the same species. A monoclonal antibody, specific against the 135,000 M.W. subunit of RNA polymerase I, inhibits RNA synthesis by this enzyme. This inhibition may be prevented by preincubation of the enzyme with DNA, which appears to confirm earlier indications that this subunit is involved in DNA binding.

Transcription of the DNA template is concluded when the RNA polymerase dissociates from the DNA and releases the newly synthesised RNA chain. Termination of RNA synthesis has been studied in E. coli. Several lines of investigation have implicated a protein - termed the rho (ρ) factor - in transcription termination in vitro (50). It is now established that there is an A-rich region of the DNA that directs a transcript with 6 to 8 terminal uridines. In addition, there are hairpin loops prior to this region (124,313). It is thought that the instability of the rU-dA duplex is a major force in prokaryotic termination (242). There appear to be few consensus sequences for termination. Some workers (e.g. 378) believe a "TACA" sequence at position -33 from the 3' end and a "CGGG" sequence at -12, from the same relative position, may be important in directing prokaryote termination. Trifonov remarked that the distance between these two sequences

was similar to that of the promoter sequences TTGACA and TATAAT (378).

Termination by eukaryotic RNA polymerases - like so many other aspects of transcription - is not as well documented as it is for prokaryotes. Bogenhagen and Brown have reported that RNA polymerase III recognised a short sequence at the end of the X. laevis 5 S rRNA gene, that signalled the end of transcription (34). Studies have been made on transcription termination with RNA polymerase I using cloned rDNA injected into frog oocytes (18). The results from these studies have revealed that, in addition to a specific DNA sequence, other factors are required for accurate termination. The length of RNA transcribed by RNA polymerase II may be modified by heat stable protein factors (341,342). A protein of M.W. 30,000, purified from rat liver cells, increases the average product size of RNA transcribed from ds DNA. This factor is species specific and has no effect on RNA polymerases I and III. Other protein factors, from lamb and calf thymus (305,359) have been found to specifically stimulate the elongation reaction of RNA polymerases from these species. In order to avoid repetition, further discussions on the modifying influence of some proteins on RNA synthesis, will be deferred until 7.4.1

1.4 RNA synthesis in C. albicans - aims of this thesis

It was noted in 1.2.2 that in contrast to the huge amount of information available on RNA synthesis in brewer's and baker's yeast (Saccharomyces) there was a paucity of information on this process in C. albicans. Although C. albicans shares some characteristics with S. cerevisiae, it would be unwise to believe that all aspects of their metabolism are similar. This thesis will examine some aspects of RNA synthesis in C. albicans and how comparable it is to eukaryotes in general and S. cerevisiae in particular.

The initial research was concerned with the preparation and characterisation of two in vitro systems (protoplasts and nuclei). These were chosen as they would then allow investigations to be made on the mode of action of potential novel inhibitors of C. albicans. The ability of these two

systems to actively synthesise RNA was monitored at each stage of preparation by the incorporation of a labelled substrate into RNA. The methods used to prepare both *in vitro* systems were based on those described for the preparation of protoplasts and nuclei from *S. cerevisiae*. How comparable the RNA species synthesised by nuclei and protoplasts were with that reported by other groups, using different organisms will be discussed.

The three RNA polymerases from eukaryotes are distinguishable by a variety of criteria (see 4.4.2). Those of *S. cerevisiae* have been extensively characterised (see 1.3.3). They differ from the RNA polymerases of higher eukaryotes in at least one important respect - the ∝ amanitin sensitivity of class I and III enzymes. How comparable the RNA polymerases from *C. albicans* were to those from other organisms will be discussed in chapter 4. Methods used to purify, to electrophoretic homogeneity, the RNA polymerases from the yeast form of *C. albicans*, and part purify those from the mycelial form, were based on those described for other fungi.

The action of lomofungin - a known inhibitor of RNA synthesis - on *C. albicans* growth and RNA synthesis was investigated. It was hoped that these studies would eventually lead to novel inhibitors of this organism. Chapter 5 will show the results obtained from such investigation. Included in this chapter are results showing the effects of several potential novel inhibitors of transcription against *in vivo* and *in vitro* RNA synthesis in *C. albicans*. These compounds were chosen from a collection of more than 190,000 available from Imperial Chemical Industries, Pharmaceuticals Division (ICI).

Although there are a number of studies on the changes in the cell wall and membrane of *C. albicans* during the yeast-mycelial transformation, there are few investigations into RNA synthesis during this process. Both *denovo* RNA synthesis and changes in the total levels of RNA during the dimorphic change were monitored for *C. albicans* in buffered media that induced this morphological transition. These findings were compared to studies made by other workers. Studies were also made into the classes of RNA synthesised

by C. albicans in different media that could induce germ tube formation. Results for such investigations would suggest which RNA polymerase enzymes were most active during the transformation.

The final chapter of this thesis (chapter 7) will be concerned with evaluating the results presented in chapters 3 to 6 and suggest further avenues for future research on RNA synthesis in C. albicans.

CHAPTER 2 MATERIALS AND METHODS

2.1 Introduction

This chapter deals with the biochemical methods and microbiological techniques used during the course of this research. It outlines the general techniques used when growing and using a pathogen such as C. albicans. In addition, the various procedures used to investigate RNA synthesis in this organism are described. Some experimental techniques developed in the course of research will be discussed in the relevant chapters.

2.2 Chemicals, enzymes, radiochemicals, scintillation fluids, buffers and media

Calf thymus DNA (type I), Candida utilis RNA (type II), protamine sulphate, α-amanitin, unlabelled nucleotides, bovine serum albumin (BSA), phenylmethyl-sulphonylfluoride (PMSF), agarose (type V), dithiothreitol (dTT) and N-acetyl glucosamine (NAG), penicillin streptomycin, Ficoll, actinomycin D DNase A, Blue dextran 2,000, and poly [d(A-T)] were all obtained from the Sigma chemical Co. Tetramethylethylenediamine (TEMED), RNase free sucrose, Cellex 410 cellulose powder and Biogel Agarose A-5M (pre-swollen) were obtained from Biorad. Acrylamide, N,N' -methylene-bis-acrylamide and Amberlite MB3 ion exchange resin were obtained from BDH. Diethylaminoethyl (DEAE)-Sephadex A-25 was obtained from Pharmacia Fine Chemicals. E coli 16 S and 23 S rRNA markers, for gel electrophoresis of RNA, were purchased from Boehringer Mannheim. Human serum was obtained from Flow Laboratories. Rifamycin AF/021, lomofungin and rapamycin were kindly donated by Nuevo Petrone, Upjohn Chemical Co. and Ayerst Chemical Co. respectively. All other chemicals used were purchased from commercial sources and were of the highest analytical grade.

Zymolyase 60,000 was obtained from the Kirin brewery, Japan. This enzyme - supplied as a lyophilised powder - contained 60,000 units of lytic activity/g dry weight. 1 unit was defined as the amount of enzyme that gave a decrease of ½ in optical density, at 650 nm, when an aliquot of treated cells was diluted in 1.30 in water, compared to that of cells before treatment.

All the radiochemicals used in this research were obtained from Amersham International, i.e. [5,6-^{3}H] uridine (specific activity 47.5 - 52 Ci/mmol, 1758 - 1924 GBq/mmol), [5-^{3}H] uridine (specific activity 23 Ci/mmol, 851 GBq/mmol), [4-^{14}C] uridine (specifc activity 488 mCi/mmol, 18 GBq/mmol), [5-^{3}H] uridine 5' triphosphate, ammonium salt (specific activity 23 - 26 Ci/mmol, 851 - 962 GBq/mmol), [4-^{14}C] uridine 5' triphosphate, ammonium salt (specific activty 48 mCi/mmol), [^{32}P] sodium orthophosphate in aqueous solution, carrier free (activity 10 mCi/ml, 0.37 GBq/ml).

A toluene-based scintillation fluid was used for all liquid scintillation counting. The scintillation "cocktail" consisted of 0.2 g 2,2 - (1,4-phenylene) bis-5-phenyloxazole and 5 g 2,5-diphenyloxazole per L toluene. Solubilizing gel slices, containing either [^{3}H] or [^{14}C], involved incubation of the slice in 0.7 ml N.C.S. (Nuclear Chicago solubilizer) solution (1 vol N.C.S.:6 vol scintillation fluid) for 12 hr at 40° C..

All buffers and solutions used throughout the research were made up in glass double-distilled water. The protoplast buffer consisted of 1.0 M D (-) sorbitol, 10 mM $MgCl_2$ in 50 mM Tris HCl pH 7.2, as described by Harrington and Douglas (152). Buffers A, B and C, as described by Schultz (331), were used in the isolation of nuclei. Buffer A consisted of 18% (w/v) Ficoll, 1 mM $MgCl_2$ in 20 mM potassium phosphate buffer pH 6.6. Buffer B consisted of 7% (w/v) Ficoll, 1 M D (-) sorbitol, 1 mM $MgCl_2$, 20% (w/v) glycerol in 20 mM potassium phosphate buffer pH 6.6. Buffer C consisted of 0.6 M sucrose, 1 mM $MgCl_2$ 1 mM dithothreitol in 50 mM Tris HCl pH 7.5. The purification of RNA polymerases from C. albicans involved the use of buffers described by Hager et al (141). The buffer used to suspend the yeast cells (buffer i) consisted of 85% (w/v) glycerol, 10% (w/v) DMSO, 50 mM $MgCl_2$, 1 mM Na_3EDTA, 5 mM PMSF and 5 mM dTT in 100 mM Tris HCl pH 7.9 (4° C.). Buffer A of Hager et al (141) contained 10% (v/v) glycerol, 5 mM $MgCl_2$, 0.1 mM Na_3EDTA, 0.2 mM dithiothreitol in 50 mM Tris HCl pH 7.9 (4° C.). Buffer B of Hager et al (141) was identical with buffer A except that the glycerol concentration was 25% (v/v).

All media used to grow C. albicans yeast and mycelial phases and

S. cerevisiae were made up in glass double distilled water. Sabourauds dextrose broth (S.D.B.) contained 4% (w/v) D (+) glucose and 1% (w/v) mycological peptone (oxoid). The pH was adjusted to 5.5 by the addition of 1 M NaOH, prior to autoclaving. Sabourauds dextrose agar (S.D.A.) was identical to S.D.B., except that 1.5% (w/v) agar (oxoid) was added. Yeast extract - peptone (YEP) broth consisted of 1% (w/v) yeast extract (Difco), 1% (w/v) Bacto peptone 3% (w/v) glycerol, 0.1% (w/v), adenine sulphate in 50 mM potassium phosphate buffer pH 6.25. Glucose-beef extract (G.B.E.) media contained 0.1% (w/v) D (+) glucose and 2.6% (w/v) beef extract (Lab Lemco). The pH was adjusted to 7 with NaOH. The growth media of Shepherd and Sullivan (S. and S medium) contained 0.005% $(NH_4)_2 S O_4$, 0.2% $KH_2 PO_4$, 0.05% $CaCl_2.2H_2O$, 0.005% $MgSO_4 .7H_2O$ and 1.5% glucose. Biotin was added to a final concentration of 25 µg/L and the pH adjusted to 5.2 (34).

2.3.1 C. albicans - Use of a pathogen in biochemical research

Research on any aspect of a pathogen - such as C. albicans - should be conducted with caution. In addition to the standard aseptic techniques used in microbiology, extra care had to be taken when handling the organism. All solutions, media and buffer used for growing and harvesting the organism were autoclaved at 120° C. for 18 minutes. Glassware, pipettes and plastic tips were sterilised by overnight (17 hours) incubation in an oven at 140° C.. Glassware and equipment which had been in contact with C. albicans were decontaminated by immersion in 25% sodium hypochlorite solution in water.

2.3.2 C. albicans - Strains used

Several strains of C. albicans were used in studies on the inhibitor lomofungin. These were strains of A and B serotypes, i.e. NCPF3153 and NCPF3156 and also strains AB2, B2630, Leeds and 6406/8. All strains were maintained on slopes of Sabaurauds dextrose agar at 25° C. In addition, reference colonies of the strains of C. albicans were kept in sterile double distilled water.

The A serotype - NCPF3153 - was mostfrequently used in this research for two main reasons. As a wild type strain any results pertaining to its mode of RNA synthesis would be applicable to the species as a whole. In addition, the A serotype readily formed mycelia in suitable media. (There have been reports that some clinical isolated strains, particularly the drug resistant strains, did not readily produce mycelia (11, 147)).

2.3.3 C. albicans - Growth conditions

It was indicated in 1.2.1 that the morphological form of C. albicans may be influenced by the media and growth conditions used for cultivation. In order to obtain large quantities of the yeast phase, the organism was grown in Sabourauds dextrose broth at 37^{o} C., in an orbital shaker operating at 150 r.p.m.. Under these conditions all the strains of C. albicans studied had doubling times of 71 $\pm$ 3 min during the expoential phase of growth. The cells were grown to the required cell density and harvested. The cells were collected by centrifugation at 3,000 r.p.m. for 15 min in a M.S.E. Mistral centrifuge (4 x 1L). The cells were washed twice with double distilled water and the pellet suspended in buffer consistent with the yeast cells eventual use.

Cultivation of the mycelial phase C. albicans involved growing a small inoculum (typically 1 x 10^5 - 2 x 10^5 yeast cells/ml media) in glucose - beef extract (G.B.E.) medium. The suspension was incubated for seven days at 37^{o} C. without shaking. This method resulted in a culture consisting of both mycelial and unicellular forms of C. albicans. The mycelia were easily identifiable as large "clumps" floating in the media. Separation of the two forms was achieved by pouring the suspension into a 1L measuring cylinder and allowing the mycelia to precipitate under gravity. The media was siphoned off from the top of the cylinder by vacuum aspiration as the mycelial clumps precipitated. The mycelia were collected on a sintered glass funnel by filtration. They were then resuspended in double distilled water, transferred to a measuring cylinder and the process repeated. A further repetition of

this washing process resulted in preparations which contained predominantly the mycelial form of C. albicans (85 - 95% mycelia as judged by observation of diluted samples under the light microscope (x 200 magnification)).

The cultivation of cells for studies on the yeast to mycelial transformation involved preparing yeast cells of C. albicans A serotype as described by Shepherd and Sullivan (346). The cells were grown in shake cultures in S. and S. medium. After incubation for at least 50 hr the cells were harvested by centrifugation at 3,000 g, for 10 min in a G.S.A. rotor, using a Sorvall RC5B centrifuge. They were then washed three times with double distilled water and finally resuspended in 50 mM potassium phosphate buffer pH 5.6 as a thick slurry (typically 10^{10} - 10^{11} cells/ml buffer). Germ tubes were induced when the cells were diluted in water and treated as described in 6.2.1.

Cell growth of C. albicans was occasionally measured by the absorbance of a cell suspension. Measurements of the O.D., at 650 nm, were made using a Pye-Unicam SP500 spectrophotometer

2.3.4 C. albicans - Identification

Research on any microorganism demands methods for the identification of that species. Apart from the ability of C. albicans to ferment certain sugars and assimilate certain compounds as sole carbon and nitrogen source, there are two widely used methods for identification of C. albicans. These are based on the organisms ability to form chlamydospores and formation of germ tubes in suitable media.

C. albicans forms chlamydospores when inoculated in nutritionally impoverished media containing surfactants. A small inocolum was streaked across a microscope slide embedded in cornmeal-Tween 80 agar, enclosed within a sterile plastic tray. The slides were then incubated for four days at 25° C.. When C. albicans NCPF3153 was applied as an inoculum, observation under the light microscope (x 200 magnification) revealed the presence of chlamydospores. In addition, it was noticed that this strain produced clumps

of blastospores at regular intervals along the pseudo-hyphal shoot. In contrast, it was found NCPF3156 and 6406/8 strains of C. albicans grew as unicellular blastospores in this media. The ability of all the strains to form chlamydospores is not a universal characteristic of this species. In addition, "ritualised" inoculation techniques have been described by many groups attempting to produce this phenomenon (190,329).

The standard procedure used throughout the course of this research to identify C. albicans was the germ tube test. A standard test involved the addition of a very small inoculum (typically 10^5 yeast cells) to 1 ml human serum. The suspension was incubated at 37° C. for 3 hr. A sample of the suspension was removed after this time and observed under the light microscope (x 200 magnification). The presence of mycelia (germ tubes) growing from the yeast cell was judged to be positive presumptive evidence for C. albicans.

2.3.5 C. albicans - Recovery from oral flora

The incidence of C. albicans in the oral flora of university staff, working in the vicinity of the research was measured, as described by Clayton and Noble (76). Mouthwashes were taken from sixteen individuals in the following manner. Participants were asked to swill their mouths with 10 ml of 0.9% NaCl solution. 1 ml of this solution was pipetted over 10 ml of Sabourauds dextrose agar. The agar had been supplemented with a 5% solution of penicillin/streptomycin(20,000 units penicillin and 5 mg streptomycin/ml H_2O). The plates were then left for 48 hr at 30° C. to allow any yeast colonies to develop. The incidence of C. albicans was determined using the germ tube test on sample yeast colonies, as described in the preceding section. It was found that C. albicans colonies were recovered from four of the sixteen individuals working in the department prior to commencement of this research. (Repetition of this experiment six months later revealed that C. albicans was recovered from mouthwashings of only three different individuals out of sixteen).

2.3.6 C. albicans - Preparation of protoplasts

Enzymatic degradation of the cell wall of C. albicans resulted in the formation of protoplasts. The method used to prepare protoplasts was based on that described by Harrington and Douglas (152). A commercially available enzyme preparation (Zymolyase 60,000) was used to remove the cell wall. The protoplasts were identifiable as spherical bodies under the light microscope. In addition, protoplasts are osmotically sensitive. This property provided a useful tool to monitor the formation of protoplasts. The optical density, at 650 nm, of a sample of cells, diluted thirty times in distilled water was greater before enzymatic digestion than after.

Protoplasts were prepared from yeast phase C. albicans growing at 37° C.. The cells were harvested when the cell density corresponded to mid-exponential phase (1 - 5 x 10^7 cells/ml Sabourauds dextrose broth). The yeast cells were then suspended, at 8 mg dry weight cells/ml protoplast buffer and incubated at 37° C.. (It was found that the dry weight was approximately 10% of the wet weight of a sample of cells dried in an oven at 85° C.). A suspension of cells of 8 mg/ml, diluted 30 fold in H_2O was sufficient to give an optical density at 650 nm of approximately 1 O.D. Unit. Protoplast formation commenced on addition of the enzyme. 1 mg of enzyme was used to prepare protoplasts from 100 mg dry weight of mid-exponential phase C. albicans. This ratio of enzyme to dry weight of cells resulted in a good yield of protoplasts after 45 min incubation at 37° C., in an orbital shaker operating at 150 r.p.m. Protoplast formation was monitored by the susceptibility to lysis of 100 µl aliquots, removed at various time intervals, diluted into 2.9 ml water. The optical density at 650 nm was measured using a Pye-Unicam SP600 spectrophotometer. Protoplast formation was judged to be complete when the optical density had fallen to ⅓ of the original value.

The protoplasts were decanted from the suspension of digested cell walls and collected by centrifugation at 3,000 g for 5 min, using a Sorvall S534 rotor in a Sorvall RCB5 centrifuge. The protoplasts were washed twice in

protoplast buffer and stored at -70° C. until required. Typically between 30% to 50% of the original yeast cells of C. albicans were converted into protoplasts, as judged by observation under the light microscope (x 200 magnification).

The viability of the protoplasts - as defined by the ability of the cells to regenerate new cell walls - was found to be between 50 and 70%. Protoplast viability was determined by serial dilution of samples of the cells, in protoplast buffer, to an expected protoplast density of 100 - 200 cells/ml. 1 ml of this suspension was then plated onto 10 ml modified Sabourauds dextrose agar (made up in protoplast buffer, not water) and incubated for three days at 28° C. The percentage viability was found by counting the number of colonies growing on the plate and comparing this value with the expected number.

2.3.7 C. albicans - Preparation of nuclei

Nuclei were prepared from protoplasts by hypotonic stock using a method based on that described by Schultz (331) to prepare nuclei from S. cerevisiae. Protoplasts were first prepared as described in 2.3.6. These were lysed by dilution 1 : 5 in Schultz's buffer A - modified by the exclusion of Ficoll and inclusion of 0.5 mM $CaCl_2$. This procedure lysed the protoplasts, but left nuclei intact. The nuclei, cell debris, contaminating whole cells and any unlysed protoplasts were precipitated by centrifugation at 10,000 g for 10 min, using a Sorvall S534 rotor in a Sorvall RC5B centrifuge, pre-cooled to 4° C.

Several procedures were used to try and achieve an uncontaminated nuclear preparation. The first method was based on that used by Coles to prepare Tetrahymena pyriformis macronuclei (78). This involved resuspending the crude nuclear pellet in Schultz's buffer A, modified by exclusion of Ficoll and by the inclusion of 0.5 mM $CaCl_2$. The suspension was then layered on top of 20 mM potassium phosphate buffer pH 6.6 containing 1 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 2.1 M RNase free sucrose. The nuclei were collected by

centrifugation at 59,000 g for 60 min using a Beckman type 30 rotor in a Spinco L2 centrifuge, pre-cooled to 4° C. An alternative method involved layering the protoplast lysate on 20 mM potassium phosphate buffer pH 6.6 containing 1 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 2.2 M RNase free sucrose. Centrifugation of 30,000 g for 90 min using a Beckman type 40 rotor in a Spinco L2 centrifuge, pre-cooled to 4° C. resulted in precipitating the nuclei. This method was based on that described by Busch and Daskal (53).

The procedure described by Schultz (331) was also used to attempt to separat nuclei from cellular contaminants. This involved lysis of protoplasts by 1 : 5 dilution in Schultz's buffer A, modified by the inclusion of 0.5 mM $CaCl_2$. Whole cells and protoplasts were removed by low speed centrifugation (2,000 g for 5 min, in a Sorvall S534 rotor in a Sorvall RC5B centrifuge, pre-cooled to 4° C.) The nuclei remaining in the supernatant were collected by centrifugation at 30,000 g for 30 min, using a Beckman type 30 rotor in a Spinco L2 centrifuge, pre-cooled to 4° C. Any membranous material at the top of the supernatant was removed by vacuum aspiration and the remaining supernatant decanted. The nuclear pellet was then resuspended in Schultz's buffer B, modified by the inclusion of 0.5 mM $CaCl_2$. A further low speed centrifugation was tried to remove contaminating unlysed protoplasts or whole cells. Centrifugation at 20,000 g, for 25 min in a Beckman type 30 rotor in a Spinco L2 centrifuge, resulted in the precipitation of the nuclei.

The nuclear pellet collected from any of the high speed centrifugation was resuspended in Schultz's buffer C. The integrity of the nuclei was assessed by light microscopy (x 1,000 magnification) and <u>in vitro</u> RNA polymerase activity. The DNA and protein content of the nuclear preparation were determined. The most successful method for preparation of nuclei was judged to be that which had <u>in vitro</u> RNA polymerase activity and was uncontaminated by whole cells or unlysed protoplasts.

2.3.8 C. albicans - Purification of RNA polymerases

The methods used to purify the RNA polymerases from the two morphological forms were based on those described by Hager et al (141) and Kumar et al (203). In order to avoid repetition, the experimental details developed to purify the RNA polymerases from C. albicans will be fully discussed in 4.2.1 - 4.2.7.

2.4 S. cerevisiae - Strain and growth conditions used

Studies on the inhibitor lomofungin involved the use of S. cerevisiae CD40 strain (a wild type strain). This organism was maintained and grown in YEP broth at 28° C., in an orbital shaker operating at 150 r.p.m. Under these conditions this strain of S. cerevisiae had a doubling time, during exponential phase, of approximately 130 min.

2.5 Enzyme assays

2.5.1 Measurement of RNA synthesis by protoplasts and yeast cells

Incorporation of [^{14}C] or [^{3}H] uridine into TCA insoluble material was used as an index of RNA synthesis. The precursor was added to a suspension of whole cells or protoplasts in protoplast buffer, supplemented with 50 mM fructose (see 3.3.1, 5.3.3, 5.3.6 and 6.4.4). The cell suspensions had been pre-incubated for the length of times and temperatures as subsequently described in these sections. After specified times duplicate or triplicate 0.5 ml aliquots were removed. The reaction was terminated by addition of the aliquot to 2 ml ice cold 10% TCA. The insoluble precipitate was collected by filtration onto a Whatman GF/C 25 mm disk over a sintered funnel. The disk was washed three times with 10 ml ice cold 5% TCA containing 10 µg/ml uridine, once with 10 ml ethanol and once with diethyl ether. The disks were dried and the radioactivity incorporated measured by liquid scintillation counting, using a Packard Tricarb scintillation counter. The assay to measure RNA synthesis in vivo by yeast cells or in vitro by protoplasts was based on that described by Kuo et al (205).

2.5.2 Measurement of RNA synthesis *in vitro*

Incorporation of [^{3}H] or [^{14}C] UTP into TCA insoluble material was used as an index of RNA synthesis, in cell free extracts, nuclei, purified and partially purified RNA polymerases. Two incubation media were assessed for their ability to support RNA synthesis by the aforementioned *in vitro* systems. These were based on the assay "cocktails" described by Higashinakagawa *et al* (163) (to support RNA synthesis by *Tetrahymena pyriformis* macronuclei) and Schultz (331) (to support RNA synthesis by *S. cerevisiae* nuclei). The assay media of Higashinakagawa *et al* contained, at final concentrations: 50 mM Tris HCl pH 7.9 (22° C.), 2.25 mM $MnCl_2$, 0.25 mM dTT, 2 mM ATP, 0.5 mM GTP and CTP, 150 mM KCl, 50 nCi (1.85 KBq) of [4 ^{14}C] UTP and 0.1 mM "cold" UTP. The "cocktail" based on that described by Schultz contained, at final concentrations: 50 mM Tris HCl pH 7.9 (22° C.), 1 mM dithiothreitol, 0.5 mM each of GTP, CTP and ATP. The concentration and specific activity of [^{3}H] or [^{14}C] UTP and concentration of KCl, and either $MgCl_2$ or $MnCl_2$ in the "cocktail" were varied during the course of the research, depending upon the aim of the experiment. In appropriate cases, exogenous DNA was added. The DNA was heat denatured according to the method of Aposhian and Kornberg (9). This involved heating a solution of DNA (2 mg DNA/ml in 20 mM Tris HCl (containing 20 mM NaCl), at 100° C. for 10 min.

The reaction was started by the addition of the test suspension (i.e. nuclei, cell free extract, partial purified RNA polymerase) to the assay "cocktail", and in appropriate cases the exogenous DNA. The reaction was allowed to proceed for a given length of time. The reaction was terminated by spotting aliquots (typically 100 µl) onto Whatman GF/C disks and immersing the disks into ice cold 5% TCA containing 1% sodium pyrophosphate In total, the disks were washed three times in ice cold 5% TCA containing 1% sodium pyrophosphate, once in ethanol and once in diethyl ether. The disks were dried and counted by liquid scintillation counting as described in 2.5.1.

2.5.3 Assay for RNase activity

The RNase activity of a solution was estimated by the ability to degrade RNA substrate. The method of McDonald (249) was used to measure the release of acid soluble oligonucleotides from the exogenous RNA substrate.

An appropriate amount of the test sample was suspended in 50 mM Tris HCl pH 7.3 and 0.1 M KCl to 1.5 ml. This was mixed with 1 ml of 1% (w/v) C. utilis RNA and incubated at 30° C. After 10 min 1 ml of 0.75% (w/v) uranylacetate in 25% (v/v) perchloric acid was added to precipitate.the intact RNA. The mixture was cooled in ice for 15 min and centrifuged at maximum speed for 5 min, at 4° C. in a B & T microangle bench centrifuge. Aliquots of the supernatant were diluted with 3.0 ml double distilled water and increased absorbance of 260 nm over a control, which lacked a RNase sample was measured.

2.5.4 DNase treatment of nucleic acids extracted from C. albicans

Section 2.7.1 and 2.7.2 will deal with the procedures that were used to extract RNA synthesised both in vivo and in vitro. Contaminating DNA was degraded using the method of Zaug and Cech (415). The precipitate of nucleic acids was collected, by centrifugation at 3,000 r.p.m., for 10 min in a M.S.E. Mistral centrifuge (12 x 150 ml rotor) cooled to 4° C. The nucleic acid precipitate was then suspended in 1 ml of 50 mM Tris acetate buffer pH 7.5. Sodium acetate and $MgCl_2$, in aqueous stock solutions of 3 M, were added to final concentrations of 0.25 M and 0.01 M respectively. A sufficient volume of 50 mM Tris acetate buffer pH 7.5 was added to give a final volume of 4 ml.

The nucleic acid/Tris acetate suspension was incubated with DNase for 4 min at 25° C. (10 µl of a 10 mg/ml solution of the enzyme was added to the nucleic acid suspension). DNase A is an endonuclease (molecular weight 65,000) which cleaves both double and single stranded DNA. It is not active against RNA (219). The action of the DNase was terminated by cooling in ice for 10 min.

2.5.5 Lactate dehydrogenase (LDH) assay

One of the criteria used for successful homogenisation of C. albicans was L DH activity before and after the two methods for cell suspension had been attempted. The activity of this enzyme was measured using a method based on that described by Williamson and Corkey (399). LDH activity was measured in terms of the decrease in concentration of NADH (substrate), followed by measuring changes in absorbance at 366 nm.

An appropriate amount of sample (typically 100 µl) was added to a cuvette containing 1 ml 0.2 M sodium phosphate buffer pH 7.2, 0.1 ml 4.0 mM sodium cyanide, 0.1 ml NADH (5.2 mM in 10 mM Tris HCl pH 8.5) and 1.6 ml double distilled water. The solutions were carefully mixed and the reaction started by the addition of 100 µl 60 mM sodium pyruvate solution. The change in O.D., at 366 nm, over a 3 min period, was measured using a Pye-Unicam SP1800 spectrophotometer and compared with a blank (i.e. 0.1 ml H_2O added instead of sodium pyruvate).

2.6 Estimation of protein and nucleic acid content

2.6.1 Determination of DNA content

The isolated DNA was quantitated by a colorimetric reaction involving the deoxyribose residues and diphenylamine as described by Burton (52).

The nucleic acid, from a suspension of at least 10^9 nuclei/cells in 0.5 ml water, was precipitated by the addition of 0.5 ml ice cold 10% TCA. The samples were centrifuged, at maximum speed for 5 min at 4° C., in a B & T microangle centrifuge and the supernatant discarded. The pellet was then resuspended in 0.5 ml 0.5 M $HClO_4$, treated for 15 min at 70° C. and centrifuged as previously described. The supernatant from this centrifugation was removed and kept in ice. A further 0.5 ml of 0.5 M $HClO_4$ was added to the pellet and treated as previously described. The supernatants from the first and second centrifugation of 0.5 ml of 0.5 M $HClO_4$ were combined with 2 ml of diphenylamine reagent (1.5 g diphenylamine in 100 ml glacial acetic acid and 1.5 ml 8 M H_2SO_4, and just prior to use, 0.5 ml 1.6% aqueous

acetaldehyde was added to the diphenylamine solution). This assay mixture (total volume 3ml) was mixed and incubated for 17 hr at 28° C.
The optical density at 600 nm of the solution was recorded using a Pye-Unicam SP1800 spectrophotometer. Calf thymus DNA was used to construct a linear standard plot from 10 - 100 µg DNA.

2.6.2 Determination of RNA content

The RNA content of a sample was estimated using the method of Schnieder (330). This utilised the reaction of orcinol with pentose sugars.

Samples were diluted to 1.5 ml in H_2O, mixed with 6 ml of ice cold 10% TCA and incubated for 5 min at 4° C. The tubes were centrifuged, at maximum speed for 5 min at 4° C., using a B & T microangle centrifuge. The pellet was resuspended in 5 ml ice cold 5% TCA and centrifuged as previously described. The pellet was resuspended in 5 ml ice cold 5% TCA, extracted at 90° C. for 20 min, cooled and centrifuged as previously described. 1.5 ml of this solution was then added to 1.5 ml orcinol reagent and heated for 15 min at 90° C. (The orcinol reagent consisted of 1 g purified orcinol dissolved immediately prior to use in 100 ml 12 M HCl containing 0.5 g $FeCl_3$. The orcinol was purified from commercial sources by recrystallizing from boiling hexane). The optical density of the solution at 660 nm was recorded. C. utilis RNA was used to construct a linear standard plot from 10 - 100 µg RNA.

2.6.3 Estimation of protein content

The method used to estimate the protein content of C. albicans was based on that of Lowry et al (224). A colorimetric assay was based on the biuret reaction between protein and copper ion in alkali and the reduction of a phosphomolybdic-phosphotungstic reagent by the tyrosine and tryptophan present in the treated protein.

A test sample (up to 0.6 ml) of C. albicans homogenatewas mixed with 3 ml alkaline copper solution for 10 min at 25° C. (The alkaline copper solution

consisted of 50 ml 2% Na_2CO_3 in 0.1 M NaOH combined with 1 ml of an aqueous solution of 0.5% $CuSO_4 . 5H_2O$ and 1 ml of an aqueous solution of 1% sodium potassium tartrate). 0.3 ml of Folins reagent was then added to the homogenate-alkaline copper solution and immediately mixed. After 30 min, the optical density at 600 nm of the solution was then recorded using a Pye-Unicam SP600 spectrophotometer. A linear standard plot, using 10 - 100 µg bovine serum albumin (BSA), was prepared in a similar manner to the test sample.

Estimates of the protein content of nuclei and during the various stages of RNA polymerase purification were performed on untreated samples. However, prior to estimating the protein content of whole cells the samples were homogenised using a Potter-Elvejhem homogeniser.

2.7.1 Extraction of RNA synthesised in vivo

In vivo synthesised RNA was extracted by the hot phenol—SDS method described by Hermolin and Zimmerman (162). The extraction was performed on a cell suspension (typically 10^6 yeast cells) in 5 ml of extraction buffer. (The extraction buffer consisted of 10 mM EDTA in 10 mM sodium acetate buffer pH 5.0, containing 20 µg polyvinylsulphate/ml buffer. The cells were disrupted by the addition of a 10% solution of SDS to a final concentration of 0.5% and 10 ml of phenol reagent added. (The phenol reagent consisted of a water-saturated solution of 50 ml phenol, and 7 ml m-cresol, containing 50 mg 8-hydroxyquinoline. Both the m-cresol and phenol were purified by re-distillation of the commercially available product).

The disrupted cell and phenol suspension was then incubated for 5 min in a 50° C. water bath. The mixture was then cooled in ice for 10 min. The aqueous and organic layers were separated by centrifugation at 3,000 r.p.m. for 5 min in an M.S.E. centrifuge pre-cooled to 4° C. The nucleic acids, in the aqueous top layer and in the interface, were removed and subjected to an additional extraction with 10 ml of organic phase phenol reagent. After this second extraction with the phenol reagent the nucleic acids in the aqueous

layer and interface with the organic layer were removed.

The nucleic acid solution was subjected to an extraction in 10 ml chloroform reagent. (The chloroform reagent consisted of a solution of 24 volumes chloroform : 1 volume isoamylalcohol. The solvents were redistilled from the commercially available material) The aqueous phase was removed and the nucleic acid precipitated by the addition of 10 ml 95% ethanol containing 0.2 M sodium acetate. A precipitate of nucleic acids was obtained after cooling to -20° C. for 12 hr.

In order to avoid contamination of the extracted RNA, rubber gloves were worn throughout this operation.

2.7.2 Extraction of RNA synthesised *in vitro*

RNA synthesised *in vitro* by protoplasts was extracted by the hot phenol/SDS method of Hermolin and Zimmerman (162) as described in the preceding section. RNA labelled and synthesised *in vitro* by nuclei was extracted by the method of Davies and Walker (86).

A suspension of nuclei (450 µl of nuclei and assay "cocktail") was incubated at 25° C. for the length of time indicated in the figures (typically 15 min). 450 µl of 0.2% SDS solution was added to the nuclear suspension. The 900 µl SDS/nuclear suspension was cooled in ice and 900 µl of organic phase of phenol reagent added. The 1800 µl suspension was mixed for 3 min at 0° C. The aqueous and organic phases were separated by centrifugation at 3,000 r.p.m. for 5 min in a M.S.E. centrifuge cooled to 4° C. The aqueous layer was removed and stored in ice. The interface between organic and aqueous phases was removed and resuspended in 3 ml of 1 mM Tris HCl pH 8.0 buffer. The 3 ml solution was combined with an equal volume of organic phase phenol reagent and heated at 70° C. for 10 min. The aqueous layer was separated by centrifugation of 3,000 r.p.m. for 5 min in a M.S.E. centrifuge cooled to 4° C. and combined with the 900 µl aqueous layer from the previous phenol extraction. RNA in the combined aqueous layers was repeatedly extracted with organic phase phenol reagent until the ratio of

optical densities at 260 and 280 nm of the aqueous layer was between 1 and 2. Two extractions, with organic phase phenol reagent, were usually required to achieve this. The RNA was then precipitated by the addition of 8 ml 95% ethanol containing 0.2 M sodium acetate and cooling to -20° C. for 12 hr.

All glassware used in the extraction of both *in vivo* and *in vitro* synthesised RNA was chromic acid cleaned and sterilised by heating in a 40° C. oven for 17 hr. The solutions used for the extraction of RNA were assayed for RNase activity. RNase activity in all solutions was found to be negligible.

2.8 Analytical techniques

2.8.1 Electrophoretic analysis of RNA

RNA extracted, as described in 2.7.1 and 2.7.2 was analysed by electrophoresis using polacrylamide tube gels and agarose slab gels. The 10% polyacrylamide and 2.5% polyacrylamide/0.5% agarose composite tube gels were prepared as described by Ringborg *et al* (300). The 1.3% agarose slab gel was prepared as described by Lehrach *et al* (213).

The 2.5% polyacrylamide/0.5% agarose composite tube gels were prepared in the following manner: 75 mg of agarose was refluxed in 9.5 ml of water, in a water bath at 90° C., for 20 min. The refluxed agarose solution was cooled to 35° C. and added to 2.5 ml of acrylamide solution (15% acrylamide, 0.75% methylene-bis-acrylamide) and 3 ml gel buffer (180 mM Tris HCl pH 7.8, containing 150 mM NaH_2PO_3 and 5 mM EDTA). The gel buffer and acrylamide solution had been previously "de-gassed" by vacuum aspiration : 25 µl of TEMED solution and 150 µl fresh 10% ammonium persulphate solution were then added to the 15 ml acrylamide/agarose solution. Approximately 3 ml of this solution was quickly pipetted into a 10 cm glass tube (100 mm x 6 mm) and allowed to set.

10% polyacrylamide tube gels were prepared in a similar manner to the 2.5% polyacrylamide/0.5% agarose gels. 10 ml of the acrylamide solution, 2 ml of H_2O and 3 ml of gel buffer were "de-gassed" by vacuum aspiration.

50 µl of TEMED and 150 µl of fresh 10% ammonium persulphate solution were then added to the 15 ml polyacrylamide solution. Approximately 3 ml of this solution was quickly pipetted into a 10 cm glass tube (100 mm x 6mm) and allowed to set.

Extracted RNA to be analysed was layered onto the top of the gel in 25 µl sample buffer. (The sample buffer consisted of 30 mM Tris HCl pH 7.8, containing 30 mM NaH_2PO_3, 5 mM EDTA, 0.2% SDS, 10% sucrose (RNase free) and 0.002% Bromophenol blue) Unlabelled RNA to be analysed was added as a suspension containing 2.5 O.D. units of RNA. One O.D. unit of RNA was defined as the amount of RNA which had an optical density of 1 at 260 nm. In vitro synthesised RNA, either [^{14}C] or [^{3}H] labelled, was suspended in 25 µl sample buffer and layered onto the gel. After 3 hr electrophoresis, at 5 mA/gel, the gels were removed from the glass tubes. Gels containing unlabelled RNA were scanned using a Gilson gel scanner, operating at 260 nm wavelength. Gels containing labelled RNA were cut into 2 mm slices using a gel slicer. The polyacrylamide gel slice was solubilized using 0.7 ml of N.C.S./toluene. solution. (1 volume N.C.S. : 6 volumes toluene) The radioactivity of each slice was measured by liquid scintillation counting - with 20 ml toluene scintillant added to each slice.

Agarose gel electrophoresis was performed using slab gels containing 1.3% agarose. Formamide was used as the denaturing agent for the RNA. The gels were prepared by dissolving 1.3 g of agarose in 10 ml Tris acetate buffer (40 mM Tris acetate pH 7.8 containing 20 mM sodium acetate and 2 mM EDTA) 40 ml H_2O and 50 ml formamide. The formamide was de-ionised by the addition of 5 g amberlite MB 3 resin to 50 ml formamide. The suspension was stirred for 3 hr, the resin removed by filtration and the deionised formamide stored at -20° C.) The agarose was dissolved by heating the agarose - Tris acetate - formamide suspension for 20 min at 90° C. This suspension was cooled to 40° C. and poured, whilst still liquid, to form slabs 5 mm x 100 mm x 200 mm.

RNA was extracted as described in 2.7.1 and suspended in 10 µl H_2O (typically 10 µg/sample). 26 µl de-ionised formamide and 4 µl Tris acetate

buffer were added to the RNA suspension. The 40 µl RNA suspension was denatured by heating for 4 min at 60° C. After cooling in ice, 5 µl of 0.04% bromophenol blue marker in 50% glycerol was added. The test samples of RNA were loaded into the wells in the agarose gels. The RNA suspension was layered on top of 20 µl solution of 2 µl Tris acetate buffer and 60% de-ionised formamide. Electrophoresis was carried out at 180 mA for 5 hr. The buffer used in electrophoresis consisted of 4 mM Tris acetate buffer pH 7.8 containing 2 mM sodium acetate and 0.2 mm EDTA.

2.8.2 Polyethyleneimine (PEI) thinlayer chromatography

The nucleotides used in the in vitro RNA polymerase assay, described in 2.5.2, were stored in aqueous solutions at -20° C.. However, in aqueous solution hydrolysis of the NTP can occur and as a consequence nucleoside di- and monophosphates may be formed as breakdown products. Thinlayer chromatography (TLC) was used as an analytical technique for estimating the extent of hydrolysis. The method was based on that described by Randerath and Randerath (297) using commercially available PEI cellulose plates (Whatman).

The plates were prepared for use by developing in 10% NaCl to 5 cm above the origin and then transferred to double distilled H_2O until the "solvent front" ran off the edge. The plates were air dried for 2 hr and immersed in double distilled H_2O and developed until the "solvent front" ran off the edge. After the plates had been thoroughly dried, 5 µl aliquots of test nucleotides (15 mM in aqueous solutions) were applied. The plates were then run in double distilled water for the first 5 cm and then immediately transferred and developed in a 1:1 mixture of 2 M LiCl and 2 M HCOOH for the next 5 cm. The plates were then air dried and the position the nucleotides had migrated was marked, after observation under a UV lamp (254 nm). Both radioactively labelled and unlabelled nucleotides were chromatographed in this way. The unlabelled nucleotides ATP, UTP, GTP and CTP migrated as single spots visible under the UV light. These nucleotides were found to have rf values of 0.37, 0.24, 0.29 and 0.40 for each NTP respectively. These rf values are

comparable to those reported by Randerath and Randerath of 0.33 for ATP, 0.20 for UTP, 0.24 for GTP and 0.32 for CTP (297).

The radiochemical purity of the labelled UTP was assessed by a similar method, using 2 µl 15 mM UTP and 2 µl 15 mM UDP as carrier material for radioactive UTP. The position of the carrier was visualised under UV light and the radioactivity of the spot counted by liquid scintillation counting. Typically between 97 and 99% of the radioactivity migrated with the UTP.

2.8.3 Gel filtration

Pre-swollen Biogel A-5 M agarose was used for the gel filtration step in the purification of RNA polymerase purification. This agarose gel (wet bead diameter 60 - 200 µm) had an exlusion limit of molecular weight 5×10^6. The gel was de-gassed, packed into a 45 cm x 2.5 cm column and eluted with buffer A of Hager *et al* (141) at 4° C.. The elution volume was calculated by exclusion of Blue dextran 2,000 (a high molecular weight polysaccharide). Samples were applied as between 1 and 5% of the bed volume onto a drained bed surface. A flow rate of 5 -8 cm hr^{-1} enabled rapid separation of high molecular weight RNA polymerase from the lower molecular weight components of the cell. The flow rate was controlled using a peristaltic pump. The eluent was monitored at 280 nm using a Uvicord scanner connected to a chart recorder.

2.8.4 Ion exchange chromatography

This method was used to resolve the three classes of RNA polymerase from *C. albicans*. DEAE-Sephadex A-25 was swollen in buffer B of Hager *et al* (141) containing 0.02 M KCl. The swollen ion exchange material was mixed with the sa buffer and packed into a column 30 cm x 1.5 cm. The column was eluted with at least two bed volumes prior to applying the sample. The sample was applied onto a drained bed surface and eluted with the starting buffer. Elution by a continuous gradient of changing ionic strength was used to desorb the bound RNA polymerases. A flow rate of 2 cm hr^{-1} was controlled

using a peristaltic pump. The eluent was monitored at 280 nm using a Uvicord scanner connected to a chart recorder.

2.8.5. Affinity chromatography

Denatured calf thymus DNA-cellulose was used to retain RNA polymerases as a stage in the enzyme's purification. A DNA-cellulose column was prepared as described by Alberts and Herrick (5). A 2 mg/ml solution of calf thymus DNA, in 10 mM Tris HCl pH 7.9 containing 1 mM Na_3EDTA was added to Cellex 410 cellulose powder (approximately 160 ml of DNA solution was added to 50 g of Cellex). The paste was left in a petri dish and thoroughly dried for five days at 70° C.. The dried powder was suspended in 20 volumes of 10 mM Tris HCl pH 7.9 containing 1 mM Na_3EDTA, and left at 4° C. for one day. After two quick washes to remove free DNA the DNA cellulose was stored as a frozen slurry at -20° C.. The amount of DNA bound was determined by heating at 100° C. for 20 min. Typically 50% of the original DNA was normally bound to the cellulose.

The sample was applied to the column (10 cm x 1.5 cm) and extensively washed with buffer B of Hager et al (141) containing 20 mM KCl to remove unbound proteins. The DNA binding RNA polymerases were recovered by elution with buffer containing a linear salt gradient of 2 - 3 bed volumes. A low flow rate of 0.5 cm hr^{-1} was maintained using a peristaltic pump. The eluent was monitored at 280 nm using a Uvicord scanner connected to a chart recorder.

2.8.6 Glycerol gradient centrifugation

The final stage in the purification of RNA polymerases was a glycerol gradient centrifugation using a method based on that of Kumar et al (20). The sample was layered onto 11 ml linear gradients of 15 - 30% glycerol in buffer B of Hager et al (141). The gradients were then centrifuged in the rotors for the lengths of time stated in 4.2.6.

2.8.7 Non-denaturing polyacrylamide gel electrophoresis

Analytical polacrylamide gel electrophoresis, under non-denaturing conditions, was performed on the purified yeast phase RNA polymerases according to the method of Sklar and Roeder (352). A 5% polyacrylamide tube gel was prepared essentially as described in 2.8.1. However, 7.5 ml of gel buffer (containing 50% v/v, 0.75 M Tris HCl pH 8.9, 0.3 mM dithiothreitol was added to 7.5 ml solution of 10% acrylamide/0.26% methylene-bis-acrylamide and de-gassed prior to polymerisation with 60 µl TEMED and 120 µl fresh 10% (w/v) ammonium persulphate.

Typically 1 - 2 µg of protein was applied to the gel in 25 µl buffer B of Hager et al (141) containing 0.005% bromophenol blue. The gels were subjected to electrophoresis at 1.5 mA/gel until the dye front reached the bottom of the gel (5 mM Tris 38 mM glycine was used as the electrode buffer).

2.8.8 Protein staining

The protein in the gels subjected to non-denaturing electrophoresis, as described in 2.8.1, was visualised using the sensitive protein staining technique described by Merril et al (258). The protein in the tube gels was fixed by suspension for 30 min in 50% methanol/12% acetic acid. The gels were re-expanded by storing in 10% ethanol/5% acetic acid for 2 hr. This was followed by three 5 min washes in 10% ethanol. The gels were soaked in a solution of 4% (w/v) paraformaldehyde/1.43% (w/v) sodium cacodylate - the pH adjusted to 7.3 with HCl - for 30 min. After three further washes in 10% ethanol, the gels were gently agitated for 30 min in $CuNO_3/AgNO_3$ solution. (This solution contained 3.5 g $AgNO_3$ in 100 ml H_2O with 1.5 ml of 0.5% $CuNO_3$. Immediately prior to use 4 ml pyridine and 8 ml ethanol were added to the solution). The gels were then soaked for 5 min in fresh diamine solution. (The diamine solution was made up 5 min prior to use and contained 30 ml of 19.4% (w/v) $AgNO_3$ solution and 22.2 ml of $NaOH/NH_4OH$ stock solution. The $NaOH/NH_4OH$ stock solution contained 0.36% NaOH, 45 ml concentrated NH_4OH and 55 ml 20% (v/v) ethanol). The diamine solution was discarded immediately after

use. The gels were washed twice for 1 min in reducing solution A and then washed several times in reducing solution B until the brown/black lines appeared marking the position of the protein band. Reducing solution A contained 2.5 ml of 10% formaldehyde, 6 ml of 1% citric acid and 10% (v/v) ethanol per L H_2O. Reducing solution B was identical to reducing solution A except 5 ml of 10% formaldehyde and 5 ml of 1% citric acid solutions were used instead . The gels containing the protein bands were stored in water.

2.8.9 Preparation of autoradiographs

The method used to prepare autoradiographs of 1.3% agarose slab gels, containing [^{32}P] labelled RNA, was based on that described by Maniatis et al (233). After electrophoresis, the gel was covered with Kodak X-omat X-ray sensitive film. The plate was exposed for between one and fourteen days at -70° C., depending upon the level of radioactivity in the gel. The photographic plates were developed, in a dark room, by soaking in Kodak liquid X-ray developer for 5 min. This was followed by 1 min in 3% acetic acid bath, to stop development, and 10 min in Kodak rapid fixer. The plates were washed in running water for 15 min and dried at room termperature.

2.9 Data analysis

Correct interpretation of any result demands methods for its statistical analysis, which will enable a conclusion to be drawn. The results obtained from most experiments were usually presented as a mean value ± the standard deviation of that sample. A line of best fit for two variables (e.g. see 2.6.1 standard plot of DNA concentration vs O.D. at 600 nm) was made using a regression line. In calculating the regression line the estimated variable was regressed against the given variable. The Pearson product-moment coefficient of correlation was used as an estimate of the closeness of the relationship between two variables.

Significance tests may be useful in determining whether differences in sample data were significant. In order to test if an observed series of values were statistically significant from expected a χ^2 test was performed.

CHAPTER 3 A COMPARISON OF SYSTEMS USED TO STUDY *in vivo* AND *in vitro* RNA SYNTHESIS BY *C. albicans*

3.1 Introduction

RNA synthesis is a complex process that requires multiple approaches in the elucidation of its mechanism and control. Ever since the ability of eukaryotes to synthesise RNA was demonstrated (398), it has been necessary to prepare suitable *in vitro* and *in vivo* systems for studying this process. *In vitro* studies, with isolated enzymes, have been important in defining the characteristics and kinetics of the individual components (e.g. 51). Other groups have isolated mutant organisms, defective in some aspect of RNA synthesis, and used this method to demonstrate which *in vivo* function is blocked (e.g.317). The rationale behind many studies has been to try and recreate the events that occur *in vivo* using *in vitro* systems of increasing complexity. These systems vary from isolated RNA polymerases (e.g. 310) to protoplasts (e.g. 81).

This chapter will deal with the preparation of two *in vitro* systems used to study RNA synthesis in *C. albicans*, namely protoplasts and nuclei. The ability of protoplasts and nuclei to synthesise RNA and the RNA species formed by these two systems were investigated. The suitability of these systems, as models to investigate *in vivo* RNA synthesis, will also be discussed.

Protoplasts are bacterial or plant cells, from which the cell wall has been removed. Removal of the constraints imposed by the cell wall confers osmotic sensitivity on the protoplasts. As a consequence, the protoplasts ought to be prepared in isotonic buffer or lysis of the cell results. A variety of methods have been used to prepare these osmotically sensitive cells. Pre-treatment of the cells with a solution of mercaptoethanol and EDTA (to reduce surface protein disulphide bands) followed by incubation with *Helix pomatia* digestive juice is a commonly used procedure (6, 195). Lytic enzymes from *Cytophaga* (239), *Streptomyces spp* (107) and *Arthobacter luteus* (191) have also been successfully employed for protoplast preparation. In this project protoplasts were prepared using Zymolyase 60,000. This enzyme

had been successful in the preparation of protoplasts from S. cerevisiae (186). The formation of protoplasts from C. albicans blastospores used a method based on that described by this group.

Experiments dependent upon protoplasts, or indeed whole cells, to investigate RNA synthesis are likely to suffer several disadvantages. The cell membrane is impermeable to the immediate substrates for RNA synthesis, i.e. nucleoside triphosphates. Monitoring nucleic acid synthesis in these systems, therefore, requires the use of labelled nucleosides. There is a complicating aspect to such studies as pools of precursors are present in cell cytoplasm. In addition, any molecules introduced into the cell, via the incubation media, would effectively be diluted by the bulk of the cytoplasm. This would reduce the number of molecules reaching their target sites in the nucleus. The RNA products synthesised in the nucleus may be similarly diluted on entering the cytoplasm. The cytoplasm contains a variety of proteins and enzymes that may modify or degrade any molecules exported from the nucleus (251). Some of these problems may be overcome by the use of nuclei.

Nuclei contain the main genomic information of eukaryotic cells (see 1.3.1). The methods employed to isolate the nuclei from C. albicans were based on those used to prepare nuclei from the yeast S. cerevisiae. It was hoped that techniques which had proved suitable for this latter organism would be applicable to C. albicans, as both systems are similar in many respects (see 1.2.2). Published methods for the isolation of S. cerevisiae nuclei are of two major types. One method is through lysis of yeast cells by various pressure methods (e.g. 32). This has the advantage that nuclei are prepared from physiological "normal" cells. However, yields are often low and microscopic appearance indicates damage during preparation. The second method to prepare nuclei is by osmotic shock or detergent lysis of protoplasts (e.g.221,401). This method apparently results in high yields of nuclei, although their nutritional state is questionable. In addition, the relatively long isolation procedures may adversely affect the physiological state of the nuclei.

Nuclei may be separated from whole cells, unlysed protoplasts and cell debris by a variety of techniques. The procederal step most frequently used is differential centrifugation. The methods described by Coles (78), to prepare T. pyriformis macronuclei and Busch and Daskal (53) for mammalian nuclei were used to try and recover C. albicans nuclei. These procedures involved centrifugation of the disrupted cells in hyperosmotic sucrose solutions. Although such high concentrations of sucrose may shrink the nuclei, these methods may be used to rapidly prepare nuclei. Another differential centrifugation technique, described by Schultz (331), was also tried for the recovery of C. albicans nuclei. This method - used to isolate nuclei from S. cerevisiae - had the advantage that the nuclei could be recovered under isotonic conditions by the use of Ficoll containing solutions. Although an uncontaminated nuclear preparation may result, many time consuming centrifugation steps were required. The criteria for a successful preparation of nuclei from C. albicans was freedom of the preparation from contamination by cell debris, whole cells or unlysed protoplasts and whether these nuclei showed the ability to synthesise RNA.

The ability of protoplasts and nuclei to synthesise RNA was investigated. RNA synthesis was measured by the ability of the system, incubated in suitable media, to incorporate a labelled precursor into TCA insoluble material. Radioactively labelled UTP was used to measure RNA synthesis by nuclei in vitro. The nuclear membrane is freely permeable to nucleotides. In contrast, as noted earlier in this section, the cell membrane is only permeable to nucleosides. As a consequence, radioactively labelled uridine was used as an index for measuring RNA synthesis in protoplasts. RNA synthesis in vivo by blastospores was measured by the same method.

This chapter will also describe the conditions required for optimal nuclear RNA synthesis. The labelled RNA species synthesised under these conditions by nuclei and also by protoplasts were extracted and analysed, by polyacrylamide gel electrophoresis. These were compared to the RNA species extracted from protoplasts.

3.2 Materials and Methods

Chapter 2 outlines the general methods and techniques used to conduct investigations into RNA synthesis by whole cells, protoplasts and nuclei. Specific experimental details are given in the legends of figures and tables.

3.3 Results

3.3.1 RNA synthesis by protoplasts

The ability of protoplasts, prepared from yeast form C. albicans, to synthesise RNA was investigated. A time course of [^{3}H] uridine incorporation into TCA insoluble material by protoplasts is shown in Fig. 3.1. There was a lag period of 20 min, after the addition of the label to the incubation medium, before its appearance in TCA insoluble material. The regression line of [^{3}H] uridine incorporation with time was estimated over the next 60 min period since [^{3}H] uridine incorporation was approximately linear. During this period [^{3}H] uridine was incorporated into RNA at a rate of 11 fmoles min^{-1} for 10^7 protoplasts.

The effect of protoplast density on [^{3}H] uridine incorporation into TCA insoluble material is shown in Fig. 3.2. Incorporation of [^{3}H] uridine per unit time increased linearly with protoplast density. If one assumed a 20 min lag period, with no measurable incorporation of [^{3}H] into RNA, the rate of incorporation over the next 40 min, of a 60 min incubation period, may be found. This was estimated as 13 fmoles [^{3}H] uridine min^{-1} by 10^7 protoplasts.

The incorporation of label into RNA was expressed in terms of 10^7 viable protoplasts. Typically 50 - 70% of the protoplasts in the incubation medium were viable. Viability was defined as the ability to regenerate cell walls and grow as colonies on S.D.B. as described in 2.3.6.

3.3.2 RNA synthesis by C. albicans blastospores

The incorporation of [^{3}H] uridine into RNA was measured for two different growth phases of yeast form C. albicans, using the same incubation conditions used for protoplasts. Blastospores were harvested and washed when cell

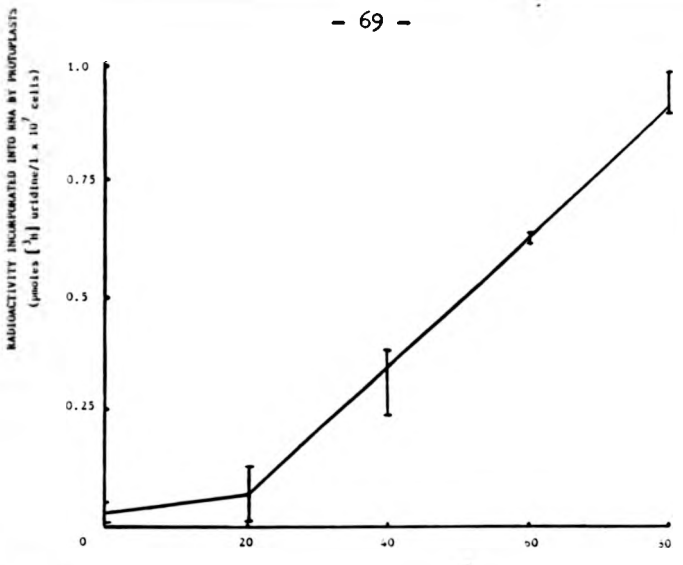


Fig. 3.1 Time course of RNA synthesis by C. albicans protoplasts

Protoplasts were prepared from C. albicans yeast cells, in the exponential phase of growth, as described in 2.3.6. The protoplasts were suspended in protoplast buffer to a cell density of 4 x 10^7 viable cells/ml. The suspension was incubated for 80 min at 37° C. and 10 µCi of [^{3}H] uridine (specific activity 47.5 Ci/mmol) was added per ml suspension. Triplicate 0.5 ml aliquots were removed at the times shown in the figure. The reaction was terminated and radioactivity incorporated into RNA was measured as described in 2.5.1. The error bars refer to the standard deviations of the three values.

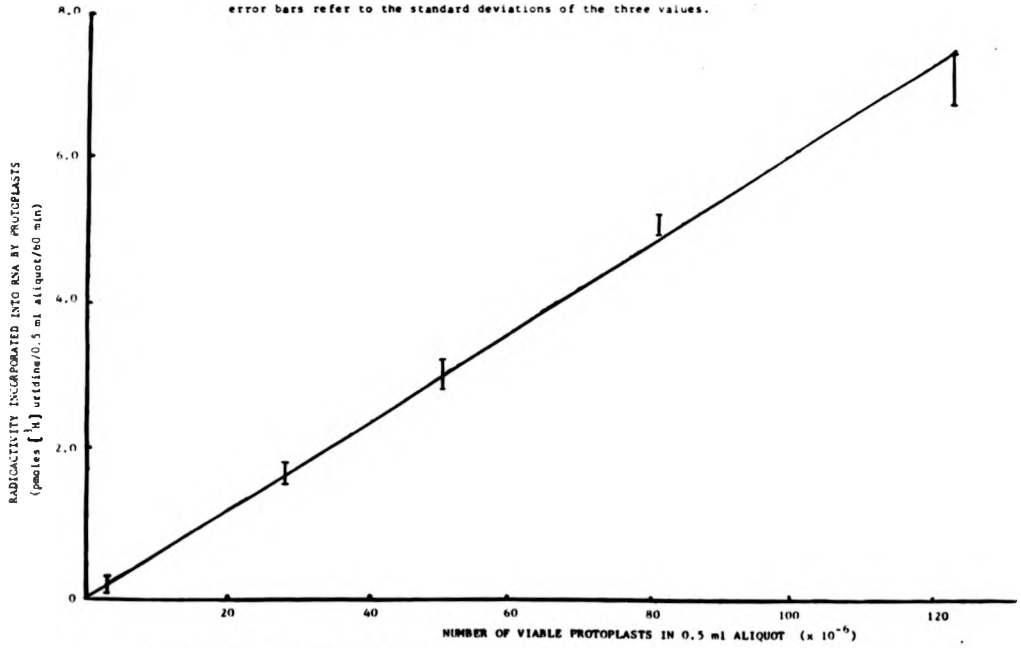


Fig. 3.2 Effect of cell density on RNA synthesis by C. albicans protoplasts

Protoplasts were prepared from C. albicans yeast cells, in the exponential phase of growth, as described in 2.3.6. The protoplasts were suspended in protoplast buffer to a cell density of 124 x 10^8 viable cells/ml. This protoplast suspension was diluted, in the same buffer, to give protoplast suspensions with final cell densities shown in the figure. The diluted protoplast suspensions were pre-incubated for 80 min at 37° C., 10 µCi of [^{3}H] uridine (specific activity 47.5 Ci/mmol) was added per ml protoplast suspension. Triplicate 0.5 ml aliquots were removed after 60 min incubation. The reaction was terminated and radioactivity incorporated into RNA was measured as described in 2.5.1. The error bars refer to the standard deviations of the three values.

densities corresponding to mid-exponential (5×10^7/ml) and stationary (5×10^8/ml) phases were reached. Their ability to synthesise RNA is shown in Fig. 3.3. This time course revealed that, on addition of [^{3}H] uridine to buffer containing mid-exponential phase yeast cells, there was immediate incorporation of label into RNA. A regression line of [^{3}H] uridine incorporation with time was estimated for a 30 min period, when [^{3}H] uridine incorporation was approximately linear with respect to time. Incorporation of [^{3}H] uridine into RNA proceeded at a rate of 49 fmoles min^{-1} by 10^7 yeast cells over this period. Blastospores in the stationary phase of growth had a much slower rate of incorporation of just 0.1 fmoles min^{-1} by 10^7 yeast cells.

3.3.3 Extraction and analysis of RNA synthesised by protoplasts *in vitro*

RNA synthesised by the protoplasts, during a 60 min incubation period, was extracted and analysed by polyacrylamide gel electophoresis. The high and low molecular weight RNA species were analysed by 2.5% polyacrylamide/0.5% agarose and 10% polyacrylamide tube gels respectively (Figs. 3.5 and 3.4 respectively). Most of the [^{3}H] uridine was incorporated into RNA species larger than 4 S. The amount of low molecular weight RNA species (i.e. smaller than 4 S) represented approximately 10 - 15% of the labelled total RNA (based on c.p.m. of gel slices). *T. pyriformis* 25 S, 17 S, 5.8 S and 5 S RNA, *E. coli* 23 S and 16 S RNA and bromophenol blue were the markers used.

3.3.4 Extraction and analysis of RNA synthesised *in vivo* by *C. albicans* yeast cells

RNA synthesised by *C. albicans* yeast form cells was extracted and analysed using 10% polyacrylamide and 2.5% polyacrylamide/0.5% agarose composite tube gels. Unlabelled RNA was extracted from blastospores at a cell density corresponding to the exponential phase of growth (i.e. 5×10^7 cells/ml). It can be seen, from traces made of UV scans of gels containing RNA, that there were slight peaks corresponding to 4 S RNA and 25 S, 17 S and 5 S rRNA (Figs. 3.6 and 3.7). In addition, some RNA species that were smaller than 4 S were found. As a comparison, *T. pyriformis* RNA was analysed using the

Fig. 3.3 Time course of RNA synthesis by C. albicans blastospores

C. albicans yeast cells were grown to cell densities corresponding to mid-exponential (5×10^7 cells/ml) or stationary (5×10^8 cells/ml) phase. The blastospores were harvested and re-suspended in protoplast buffer. The suspensions of exponential (—▪—) and stationary (—●—) phase yeast cells were pre-incubated for 30 min at 37° C.. 10 μCi of [^{3}H] uridine (specific activity 47.5 Ci/mmol) was added per ml suspension. Duplicate 0.5 ml aliquots were removed at the times shown in the figure. The reaction was terminated and radioactivity incorporated into RNA measured as described in 2.5.1. The error bars refer to the standard deviations of the two values.

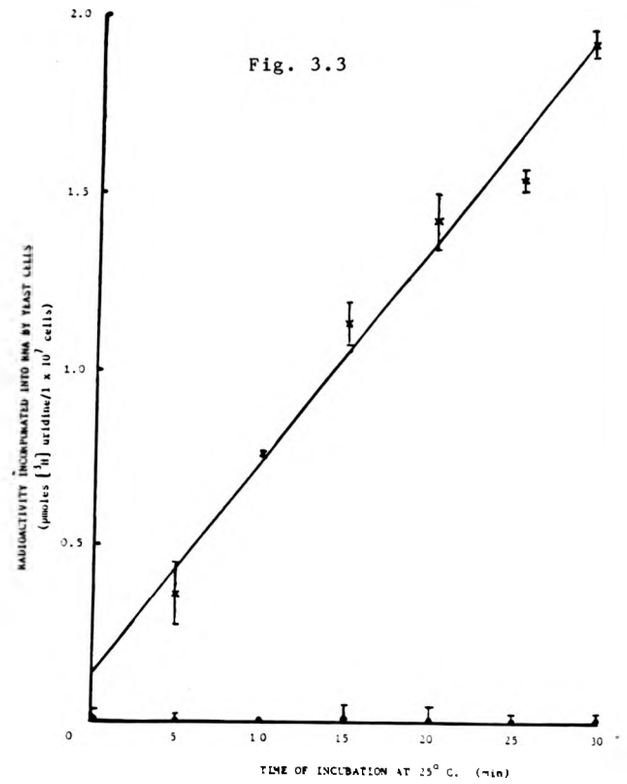
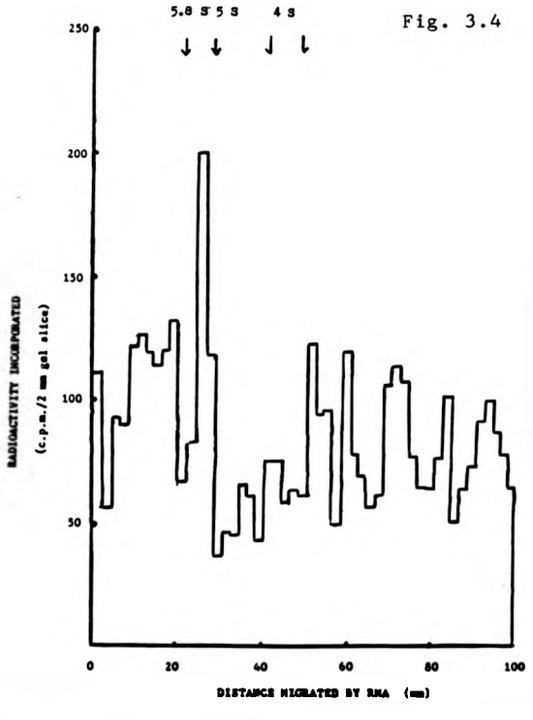


Fig. 3.4

Fig. 3.5

Figs. 3.4 and 3.5 PAGE of labelled RNA synthesised by C. albicans protoplasts

Protoplasts were prepared from exponential phase, C. albicans yeast cells as described in 2.3.6. The protoplasts were suspended in protoplast buffer to a final cell density of 2×10^8 viable cells/ml. The suspension was pre-incubated for 30 min at 37° C.. 10 μCi of [^{3}H] uridine (specific activity 47.5 Ci/mmol) was added per ml suspension. After 60 min incubation the RNA from yeast cells, in a total volume of 5 ml suspension, was extracted as described in 2.7.1. The labelled RNA was analysed by PAGE on 10% polyacrylamide (Fig. 3.4) and 2.5% polyacrylamide/0.5% agarose (Fig. 3.5) tube gels as described in 2.8.1. The position of the bromophenol blue (4 S), T. pyriformis 25 S, 17 S, 5.8 S and 5 S rRNA and E. coli 23 S and 16 S rRNA marker species are indicated in the figure.



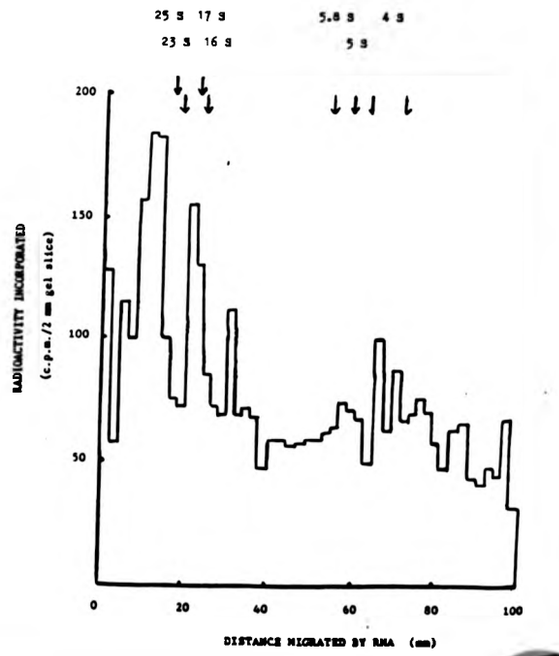


Fig. 3.6

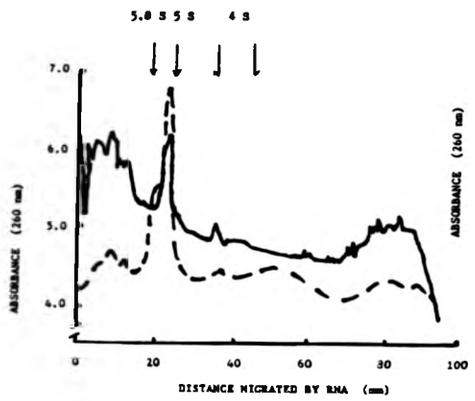
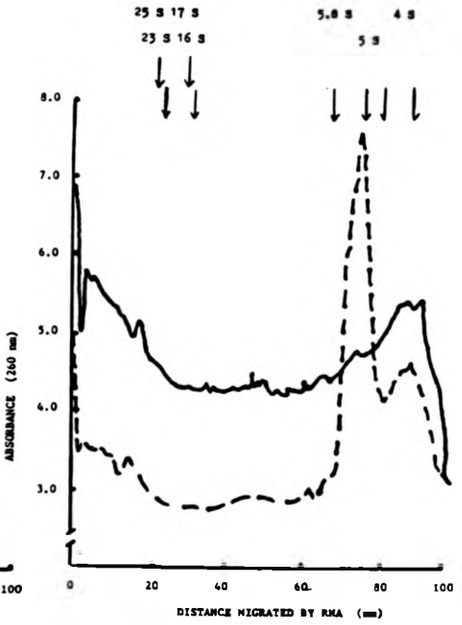


Fig. 3.7



Figs. 3.6 and 3.7 PAGE of RNA synthesised by C. albicans and T. pyriformis in vivo

RNA was extracted from C. albicans yeast cells, in the exponential phase of growth, as described in 2.7.1. Contaminating DNA was degraded by treatment with DNase I as described in 2.5.4. The RNA from C. albicans yeast cells (corresponding to 2.5 O.D. units at 260 nm) was analysed by PAGE on 10% polyacrylamide (Fig. 3.6) and 2.5% polyacrylamide/0.5% agarose (Fig. 3.7) tube gels as described in 2.8.1. The figure shows traces of O.D. scans at 260 nm of RNA from T. pyriformis (— —) -(kindly donated by A.M. Coles) and C. albicans (——). The position of the bromophenol blue (4 S), E. coli 23 S and 16 S rRNA markers is as shown.

	DNA content	Protein content
C. albicans yeast cell (stationary phase)	45 ± 2 fg/cell	7.6 ± 1.3 pg/cell
C. albicans yeast cell (mid-exponential phase)	30 ± 2 fg/cell	8.6 ± 1.0 pg/cell
C. albicans nuclei	60 ± 5 fg/nucleus	0.5 pg/nucleus
S. cerevisiae yeast cell (stationary phase)	47 fg/cell	N/D

Table 3.1 Protein and DNA content of C. albicans yeast cells and nuclei

The DNA and protein content of homogenised C. albicans yeast cells and nuclei was estimated as described in 2.6.1 and 2.6.3 respectively. The DNA content of S. cerevisiae CD60 strain yeast cells was also estimated for a comparison to be made. (N/D - not determined)

same conditions. The traces revealed small peaks due to the 25 S and 5.8 S rRNA and also a high proportion of 4 S tRNA and 5 S rRNA. The distance migrated by these species, and the internal marker (bromophenol blue) and E. coli 23 S and 16 S rRNA is as shown.

3.3.5 Isolation of nuclei

Nuclei were prepared by lysis of protoplasts prepared from exponential phase C. albicans blastospores. The protoplasts were prepared and analysed as cited in 3.2.2. If was found that centrifugation of the protoplast lysate in buffer containing 2.1 M sucrose resulted in a nuclear preparation highly contaminated with unlysed protoplasts and whole cells. This method was based on that described by Coles (78) to successfully prepare T. pyriformis macronuclei. The differential centrifugation technique - described by Schultz (331) - was similarly unsuccessful. Both these methods resulted in nuclear preparations that were contaminated (25 - 50%) by whole cells and protoplasts, as judged by observations under the light microscope (x 1000 magnification). A satisfactory procedure for the separation of nuclei from contaminants was achieved by layering the protoplast lysate onto 20 mM potassium phosphate buffer containing 2.2 M RNase free sucrose and 0.5 mM $CaCl_2$. This was centrifuged, at 4° C. for 90 min at 30,000 g as described in 2.3.7. The resulting nuclear pellet was resuspended in Schultz's buffer C (typically 10^9 nuclei per ml buffer). Nuclei prepared in this manner were approximately 1 µm in diameter when examined using a light microscope (x 1000 magnification). Yields of nuclei from whole cells were estimated to be 5 - 10% based on counts made using an improved Neubauer haemocytometer.

The protein and DNA content of the nuclei were estimated (Table 3.1). For comparison, the DNA and protein content of yeast form C. albicans, harvested in the mid-exponential and stationary phases of growth, were also determined. It was found that the nucleus contained 60 fg of DNA and 0.5 pg of protein. The protein content of stationary and exponential phase C. albicans yeast cells were estimated respectively as 7.6 and 8.6 pg per cell. The DNA

content of C. albicans yeast cells in these two growth phases was determined as 45 fg and 50 . fg for stationary and exponential phase cells respectively. As a comparison the DNA content of S. cerevisiae was measured and estimated at 47 fg per cell.

3.3.6 RNA Synthesis in vitro by nuclei

Two incubation media were assessed for their suitability in supporting in vitro RNA synthesis. A protoplast suspension was completely lysed, as described in the legend of Fig. 3.8, and incubated in assay conditions based on those described by Schultz (331) or Higashinakagawa et al (163). RNA synthesis was measured by the incorporation of [^{14}C] UTP into TCA insoluble material. A time course (Fig. 3.8) revealed that the conditions described by Higashinakagawa et al (163) did not support measurable in vitro RNA synthesis by lysed protoplasts. By contrast, there was marked incorporation of label when assay conditions based on those described by Schultz (331) were used. The amount of label incorporated into RNA was rapid for the first 5 min period and then decreased slightly over the next 5 min. After 10 min incubation the level of [^{14}C] incorporated into the RNA decreased rapidly. These assay conditions were also capable of supporting nuclear RNA synthesis.

A time course (Fig. 3.9) for C. albicans nuclei showed a non-linear increase over the 25 min incubation period. The rate of incorporation of [^{14}C] UTP into RNA decreased the longer the period of incubation. Increasing the number of nuclei, per unit volume, in the assay incubation medium resulted in an increase in [^{14}C] UTP incorporation into RNA (Fig. 3.10). The incorporation of [^{14}C] UTP into RNA was measured over a 10 min incubation period using freshly isolated nuclei. The assay conditions, which supported nuclear RNA synthesis in C. albicans, were also capable of supporting the same process in T. pyriformis nuclei (results not shown).

3.3.7 Effect of Magnesium and salt concentration on nuclear RNA synthesis

The effect of KCl and $MgCl_2$ concentration, in the assay "cocktail", on

Fig. 3.8 RNA synthesis by lysed protoplasts

Protoplasts were prepared from C. albicans yeast cells as described in 2.3.6. A protoplast lysate was prepared from 2×10^{10} cells by dilution 1 : 30 in 20 mM potassium phosphate buffer pH 6.8, containing 0.5 mM $CaCl_2$ and 1 mM $MgCl_2$. A precipitate was collected by centrifugation at 3000 g for 5 min at 4° C. The precipitate was resuspended in Schultz's buffer C to 5 ml. Two assay "cocktails" were tested for the ability to support RNA polymerase activity in 1.1 ml protoplast lysate. The first was based on the medium described by Higashinakagawa et al (—●—), and the second was based on the medium described by Schultz (——). The components of these media are described in 2.5.2. The reaction was started by the addition of protoplast lysate to assay "cocktail". Triplicate 100 µl aliquots were removed after the times of incubation at 25° C. shown in the figure. The reaction was terminated and the radioactivity incorporated into TCA insoluble material measured as described in 2.5.2. The error bars refer to the standard deviations of the three values.

Fig. 3.9 Time course of RNA synthesis by C. albicans nuclei

Nuclei were prepared from C. albicans protoplasts and separated from cellular contaminants by the centrifugation method of Busch, as described in 2.3.7. The nuclei were suspended in Schultz's buffer C to a final density of 8×10^8 nuclei/ml. 0.7 ml of nuclear suspension was incubated, at 25° C., in total volume 1.4 ml of assay medium. The components of the "cocktail" were based on those described by Schultz in 2.5.2. Duplicate 100 µl aliquots were removed at the times indicated in the figure. The reaction was terminated and the radioactivity incorporated into RNA measured as described in 2.5.2. The error bars refer to the standard deviations of the two values.

Fig. 3.10 Effect of number of nuclei in assay medium on RNA synthesis

Nuclei was prepared from C. albicans protoplasts and separated from cellular contaminants by the centrifugation method of Busch, as described in 2.3.7. The nuclei were suspended in Schultz's buffer C to a final density of 1×10^9 nuclei/ml. This nuclear suspension was diluted, in the same buffer, to give nuclear suspension with the final densities shown in the figure. 200 µl of the diluted suspensions were incubated in total volume of 400 µl of assay medium. The components of the "cocktail" were based on those described by Schultz in 2.5.2. Duplicate 100 µl aliquots were removed after 0 and 10 min incubation at 25° C., The reaction was terminated and radioactivity incorporated into RNA measured as described in 2.5.2. The error bars refer to the standard deviations of the two values.

Fig. 3.8

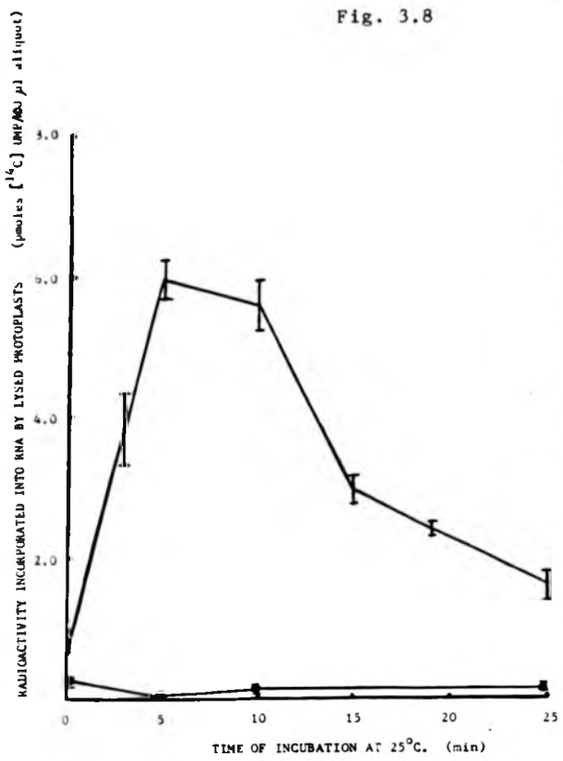


Fig. 3.9

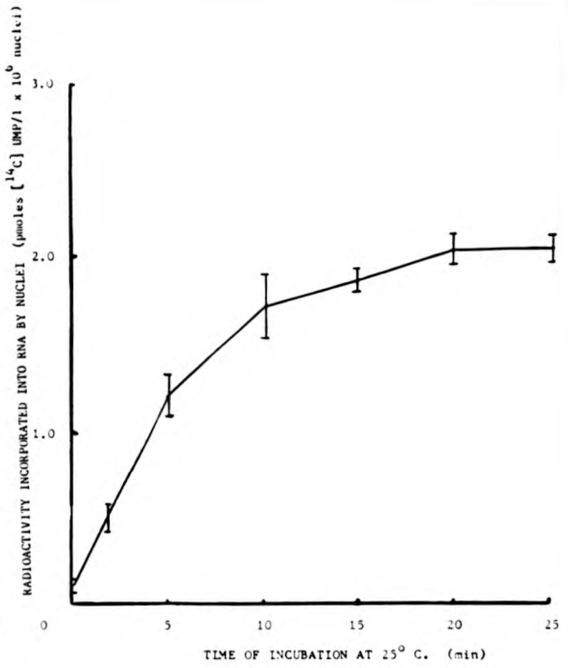
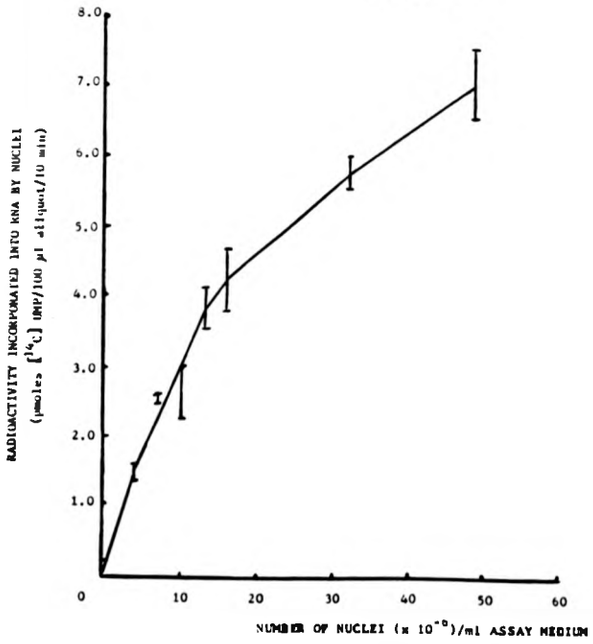


Fig. 3.10



nuclear RNA synthesis is shown in Figs. 3.11 and 3.12 respectively. KCl and $MgCl_2$, each at concentrations of 100 mM, supported the highest level of incorporation of [^{14}C] UTP into RNA by nuclei over a 10 min incubation period. It was found that 39 p moles [^{14}C] UTP were incorporated by 100 µl nuclear suspension in buffer containing 100 mM KCl and 100 mM $MgCl_2$ (see Fig. 3.12).

3.3.8 Analysis of RNA synthesised *in vitro* by nuclei

The RNA species synthesised by *C. albicans* nuclei were analysed by polacrylamide gel electrophoresis. The effect of high (100 mM) and low (10 mM) $MgCl_2$ concentrations, in the assay medium, on the RNA species synthesised by nuclei were investigated. The low and high molecular weight species, synthesised over a 15 min incubation period, were analysed by 10% polyacrylamide and 2.5% polacrylamide/0.5% agarose tube gels respectively (Figs. 3.13 and 3.14). In the assay "cocktail" containing a low concentration of $MgCl_2$, the newly synthesised RNA was smaller than 4 S. Approximately 85 - 90% of the total labelled RNA - based on c.p.m. of gel slices - was found migrating with RNA species smaller than the bromophenol blue marker. A high proportion of labelled RNA, from nuclei incubated in a "cocktail" containing a high $MgCl_2$ concentration, also migrated with RNA species smaller than 4 S. Between 60% and 80% of the [^{14}C] radioactivity - based on c.p.m. of gel slices - was found associated with RNA species smaller than the bromophenol blue marker. Small peaks of [^{14}C] radioactivity were found associated with RNA species that migrated as 25 S and 17 S rRNA.

3.4 Discussion

3.4.1 RNA synthesis in *C. albicans* blastospores and protoplasts

As mentioned in the introduction of this chapter, one approach for elucidating the mechanisms used by a cell to control RNA synthesis is to study this process in models of increasing complexity. It almost appears axiomatic to state that such models should in some way, resemble the *in vivo* system for any applicable conclusions to be made. Protoplasts of *C. albicans*

Fig. 3.11 Effect of KCl concentration on RNA synthesis by C. albicans nuclei

Nuclei were prepared from C. albicans protoplasts and separated from cellular contaminants by the centrifugation method of Busch, as described in 2.3.7. The nuclei were suspended in Schultz's buffer C to a final density of 3.4×10^3 nuclei/ml. 200 µl aliquots of the nuclear suspension were incubated in 400 µl total volume assay medium. The components of the "cocktail" were based on those described by Schultz in 2.5.2. However, KCl was present in the "cocktail" at the final concentrations indicated in the figure. Duplicate 100 µl aliquots were removed after 0 and 10 min incubation at 25° C.. The reaction was stopped and the radioactivity incorporated into RNA measured as described in 2.5.2. The error bars refer to the standard deviations of the two values.

Fig. 3.12 Effect of $MgCl_2$ concentration on RNA synthesis by C. albicans nuclei

Nuclei were prepared from C. albicans protoplasts and separated from cellular contaminants by the centrifugation method of Busch, as described in 2.3.7. Nuclei were suspended in Schultz's buffer C to a final density of 3.4×10^3 nuclei/ml. 200 µl aliquots of the nuclear suspension were incubated in 400 µl total volume assay medium. The components of the "cocktail" were based on those described by Schultz in 2.5.2. However, the KCl concentration of the "cocktail" was 100 mM and $MgCl_2$ was present at the final concentrations indicated in the figure. Duplicate 100 µl aliquots were removed after 0 and 10 min incubation at 25° C. The reaction was stopped and the radioactivity incorporated into RNA measured as described in 2.5.2. The error bars refer to the standard deviations of the two values.

Figs. 3.13 and 3.14 PAGE of RNA synthesised by C. albicans nuclei in media containing high and low $MgCl_2$ concentrations

Nuclei were prepared from C. albicans protoplasts and separated from cellular contaminants by the centrifugation method of Busch, as described in 2.3.7. The nuclei were suspended in Schultz's buffer C to a final density of 1×10^3 nuclei/ml. 400 µl aliquots were incubated in 800 µl total volume assay medium. The components of the "cocktail" were based on those described by Schultz in 2.5.2. However the KCl concentration of the "cocktail" was 100 mM and $MgCl_2$ was present at a final concentration of 10 mM or 100 mM. After 15 min incubation at 25° C. the RNA was extracted from the nuclei as described in 2.7.2. The RNA synthesised by nuclei in the presence of 10 mM (– – –) and 100 mM (———). $MgCl_2$ was analysed by PAGE as described in 2.8.1. The figures show PAGE of the RNA on 10% polyacrylamide (Fig. 3.13) and 2.5% polyacrylamide/0.5% agarose (Fig. 3.14) tube gels. The distance migrated by the bromophenol blue (4 S), E. coli 23 S and 16 S rRNA and T. pyriformis 25 S, 17 S 5.8 S and 5 S rRNA species is shown.

Fig. 3.11

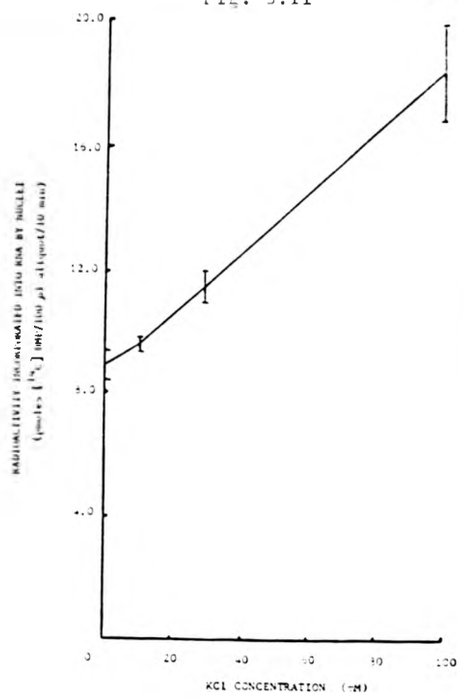


Fig. 3.12

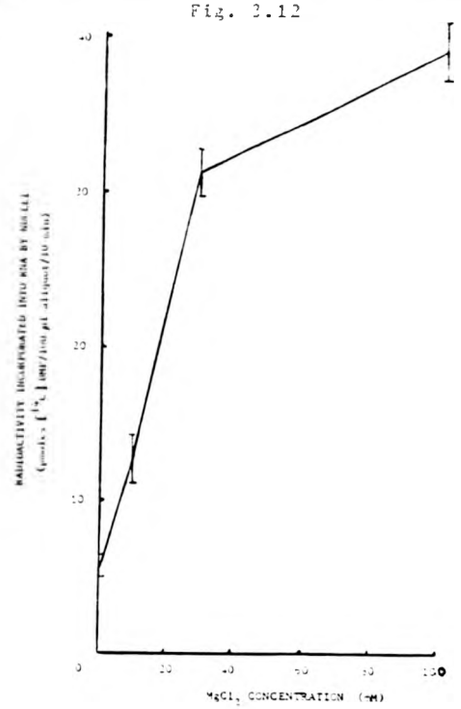


Fig. 3.13

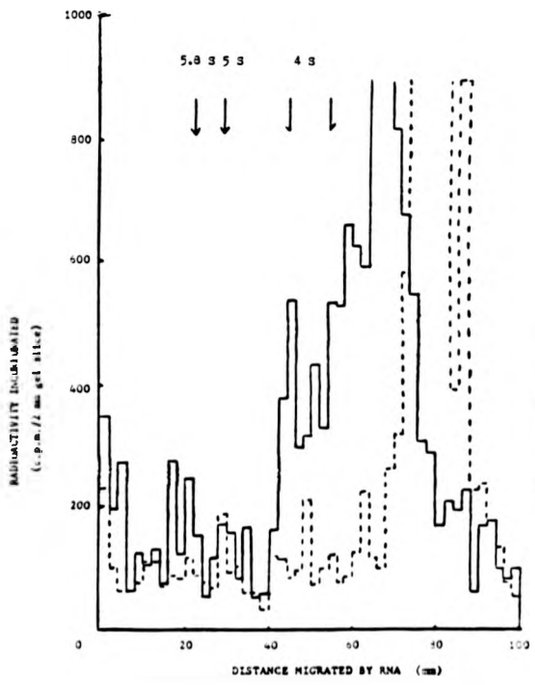
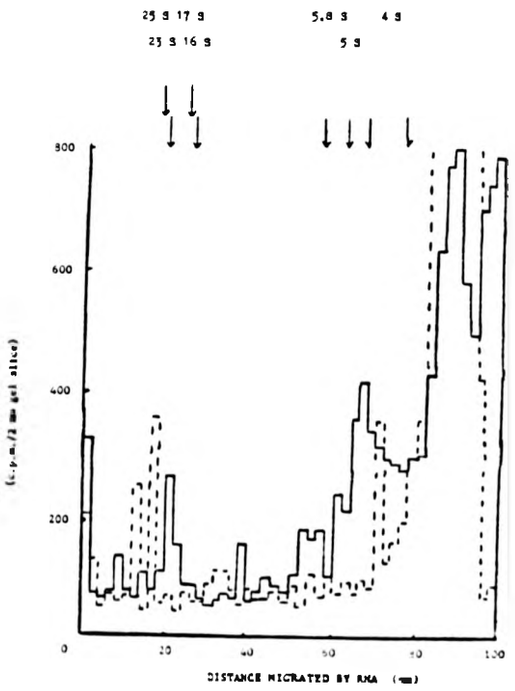


Fig. 3.14



yeast form cells were prepared for two main reasons: 1) protoplasts represent a hightly organised *in vitro* system and so RNA synthesis proceeds in an authentic manner, 2) they may be used as the first stage in the preparation of nuclei. It is the first reason which will be discussed first.

Protoplasts prepared here were shown to be capable of synthesising RNA under suitable conditions (Figs. 3.1 and 3.2). The conditions which supported this process (i.e. incubation in buffer containing sorbitol at 25° C.) also maintained the protoplasts as viable entities. The protoplasts were microscopically distinguishable as spherical bodies, approximately 8 µm in diameter. This indicated successful cell wall removal, since the cell wall maintains the characteristic ovoid shape of *C. albicans* blastospores. However, the susceptibility of the cells to lysis, when diluted in water, was used as the major indicator of protoplast formation. Protoplasts have been prepared by other workers using time consuming methods (e.g. 24 hr incubation period) with highly impure enzyme preparations (e.g. snail digestive juices) (6,195). Protoplast preparation, in this project, involved a short (45 min) incubation of a buffered suspension of blastospores with Zymolyase 60,000 enzyme, as described in 2.3.6. It is interesting to note that protoplast formation was more readily achieved from cells harvested during the exponential growth phase rather than the stationary phase. It is thought the older a cell becomes the greater the degree of cross linking of cell wall components. Some workers believe that this may account for the greater difficulty found when preparing protoplasts from blastospores in the stationary phase of growth (278).

RNA synthesis by protoplasts and blastospores was measured by incorporation of [^{3}H] uridine into TCA insoluble material. The time course (Fig. 3.1 reveals that, after a lag period of 15 - 20 min, linear incorporation of label into RNA ensued, at a rate of 11 fmoles min^{-1} by 10^7 viable protoplasts. In contrast, no such lag period was found when blastospores were incubated under identical conditions (Fig. 3.3). There are a variety of explanations to account for this difference. One reason may be that there was no RNA synthesis

during the first 95 - 100 min after protoplast preparation (i.e. 15 - 20 min after the addition of the [^{3}H] uridine label and 80 min pre-incubation of the protoplasts). This seems unlikely as the protoplasts were prepared from rapidly growing yeast form cells - a growth phase accompanied by a high level of RNA synthesis (see Fig. 3.3). Another possibility could be that the 15 - 20 min lag period was an indication of the time taker for the [^{3}H] uridine to be incorporated into the cell, phosphorylated into [^{3}H] UTP and incorporated into RNA. As protoplasts are perturbed systems, some workers believe they will show different properties from the cells they were prepared from. Burgess (48) has suggested that protoplasts (of plants) do not simply behave as plant cells without walls, but as a stressed system, requiring a recovery period. The lag period found when label was added to protoplasts and the higher rates of incorporation of [^{3}H] uridine shown by blastospores indicates that C. albicans protoplasts behaved as similar stressed systems.

Increasing the number of protoplasts, per unit volume of buffer, resulted in a linear increase in the amount of [^{3}H] incorporated into RNA over a 60 min incubation (Fig. 3.2). Assuming an initial lag period of 20 min when no [^{3}H] was incorporated into RNA, [^{3}H] uridine was incorporated at a rate of 13 fmoles min^{-1} by 10^7 viable protoplasts over the next 40 min. This value was comparable to that obtained from the rate calculated from the time course of Fig. 3.1 (i.e. 11 fmoles min^{-1} by 10^7 viable protoplasts). Incubation of yeast cells - in the exponential phase of growth - under identical conditions resulted in the immediate incorporation of [^{3}H] uridine into RNA at a rate of 49 fmoles min^{-1} by 10^7 yeast cells. In contrast, yeast cells harvested at the stationary phase of growth, incorporated 0.1 fmoles of [^{3}H] uridine min^{-1} by 10^7 yeast cells.

Although the latter values are expressed in terms of 10^7 yeast cells, it should be noted that the cell densities of the blastospore suspensions were different (i.e. 5×10^7 exponential phase yeast cells/ml and 5×10^8 stationary phase yeast cells/ml). If yeast cells from both growth phases were diluted to the same cell density, and pre-incubated for 80 min,

comparable rates of incorporation of label into RNA were found. This indicates that the cell density of the yeast suspension - usually associated with its growth phase - affects the general level of RNA synthesis per unit cell. Such a result should not be unexpected as it is known that periods of rapid growth are associated with high levels of RNA synthesis (139).

Over the past ten to fifteen years protoplasts, from a variety of species, have been used to study RNA synthesis. Protoplasts have been prepared from S. cerevisiae (380), tobacco (174) and cucumber (81) and all these systems have yielded some information about RNA synthesis. The overall picture from these reports is that there are similarities between RNA synthesis in vitro by protoplasts and in vivo by whole cells. In contrast to the results reported here, it has been found that the kinetics of incorporation of label into RNA by Centaurea cyanus protoplasts were similar to whole cells (202). However, this same group reported differences in the metabolism of the 40 S pre-rRNA precursor and also the levels of poly A containing RNA molecules between protoplasts and whole cells. Results obtained using protoplasts prepared from higher plants are not strictly comparable with a lower eukaryote such as C. albicans. Nevertheless, groups using such diverse organisms as cucumber (81) and S. cerevisiae (205) have reported that protoplasts from these species synthesised a high proportion of the high molecular weight rRNA species. A similar result was found when the RNA species synthesised by C. albicans. over a 60 min incubation period, were analysed as described in Figs. 3.4 and 3.5. The results shown in these figures show slight peaks of [^{3}H] associated with 25 S, 17 S, 5.8 S and 5 S rRNA species. Indeed, most (80%) of the label was found associated with RNA species having a higher M.W. than the 4 S marker species. (It is believed that the rRNA species of Candida spp resemble those of S. cerevisiae (408). However, whilst rRNA of C. albicans shows about 80% homology with nucleic acids from other Candida spp. Saccharomyces spp showed a homology of about 25% (339). There are some similarities in the RNA species synthesised by protoplasts from C. albicans and other species. It is possible that these may be required by the cell for cell wall repair.

Analysis of unlabelled RNA extracted from C. albicans blastospores, in the exponential phase of growth, is shown in Figs. 3.6 and 3.7. (These Figures also showed traces made of UV scans of RNA extracted from T. pyriformis as a comparison) The gel scans revealed slight peaks corresponding to 25 S, 5.8 S and 5 S rRNA and 4 S tRNA. It has been reported that a high proportion of RNA synthesised by S. cerevisiae is directed by RNA polymerase I during periods of rapid growth (335). It is, thus, not surprising that peaks are found in RNA, extracted from exponential phase C. albicans, corresponding to the mature RNA species that are directed by this isozyme . Presumably, such high quantities of these RNA species are required to form extra ribosomes and amino acid-tRNA molecules for rapid cellular growth. Although mRNA molecules can direct translation of many copies of a protein, rRNA and tRNA molecules, as the final gene products, are required in large amounts. Thus, during any period of growth the levels of abundant rRNA and tRNA rises (262).

The results discussed above indicate that RNA synthesis does occur in C. albicans protoplasts. However, there do appear to be some differences in the kinetics of RNA synthesis between protoplasts and yeast cells, i.e. there is a lag period before [^{3}H] is incorporated into RNA by protoplasts which is then incorporated at a lower rate compared to blastospores. Thus, preparation of nuclei from protoplasts would involve the preparation of a system that was not physiological "normal" compared to the original blastospore. As protoplasts do synthesise RNA and other methods used to prepare nuclei from physiological "normal" cells require techniques which disrupt the cell, and destroy the nuclear membrane, the preparation of nuclei necessitated protoplast formation.

3.4.2 Preparation of C. albicans nuclei

Several methods were used to try and separate nuclei, prepared by lysis of protoplasts, from contaminants. Two criteria were used to evaluate the success of a preparation were 1) the ability of the nuclei to synthesise RNA

and 2) lack of contamination by cellular debris and whole cells. The former criterion is, of course, of paramount importance when preparing nuclei as a system to study RNA synthesis. Section 3.4.3 will discuss the results obtained from investigations into in vitro nuclear RNA synthesis. This section will consider the techniques and rationale for preparation of nuclei from C. albicans.

It was found that nuclei could be prepared by dilution of a protoplast suspension 1 : 5 in phosphate buffer, as described in 2.3.7. The nuclei could then be collected by a low speed centrifugation. Separation from cellular contaminants and whole cells could be achieved by high speed centrifugation over 20 mM phosphate buffer containing 1 mM $MgCl_2$, 0.5 mM $CaCl_2$ and 2.2 M sucrose. Nuclei prepared by this method had a diameter of 1 - 2 µm, as judged by light microscopy. As a comparison, nuclei isolated from S. cerevisiae are 2 µm in diameter (32). Electron microscopy has shown that the nucleus of C. albicans has a similar dimension in situ (36).

Any medium used in the isolation of nuclei should maintain this organelle, and its components, in, as near as possible, its in vivo condition. C. albicans nuclei were stored in the medium described by Schultz (331) as "buffer C" (see 2.2). This medium was used to successfully store S. cerevisiae nuclei. The basis of any medium is the buffer. Phosphate, HEPES NaOH and Tris HCl are the most commonly used buffers. Nuclei have been maintained in buffer within a pH range of between 6 and 8.5 (251). The osmolality and ionic composition of the medium should be such that nuclear integrity is maintained. The osmolality of the medium is usually determined by the amount of glycerol or sucrose present. The sucrose concentration in "buffer C" was 0.6 M, which is similar to that used by other workers for the storage of nuclei from other organisms (251). Although during the nuclear preparation stages the sucrose concentration was 2.2 M, the effects of this component do appear to be reversible (251). (The ionic conditions employed in the preparation and assaying nuclear activity will be discussed in 3.4.3),

The hypertonic conditions described for the preparation of C. albicans

nuclei did appear to shrink the nuclei. Although the conditions described by Schultz (331) used essentially isotonic conditions (using Ficoll containing buffer) for the centrifugation steps, they had the disadvantage that the nuclear preparation had a high level of yeast cell contaminants. Schultz maintained an uncontaminated nuclear preparation could be achieved by many Ficoll centrifugation steps. However, time consuming methodologies do have a disadvantage in that nuclei need to be rapidly isolated, as a lot of RNA polymerase activity is essentially elongation activity (see 3.4.3). Recently, Ide and Saunders (171) described a technique using polyvinylpyrollidone coated silicia (Percoll) gradients to separate S. cerevisiae nuclei. This method had the advantage that nuclei were prepared rapidly under isotonic conditions, with minimal cytoplasmic contamination. This seems an ideal technique to be used for future nuclear preparations from C. albicans.

The DNA content of C. albicans nuclei was estimated using a colorimetric reaction by the deoxyribose residues and diphenylamine as described in 2.6.1. It was found that the DNA content of C. albicans nuclei, prepared in this thesis, was 60 fg (see Table 3.1). In contrast, the DNA content of S. cerevisiae has been measured as 40 - 46 fg per nucleus (32,279). This difference could arise as a consequence of contamination, in the storage medium, of DNA from broken nuclei. Some authors have suggested using bovine serum albumin in the isolating and storage media to preserve nuclear integrity and minimise lysis. However, many commercial preparations have been shown to be contaminated with a high level of protease and nuclease activity (251).

The DNA content of stationary phase C. albicans yeast cells reported here is 45 fg, which is comparable to the literature value of approximately 40 fg/cell (depending upon the strain used) (321). The DNA content of C. albicans, harvested when the cell density corresponded to the exponential phase of growth, was estimated at 50 fg/cell (see Table 3.1) A χ^2 test revealed that, at the 95% confidence level, any differences in estimated DNA content per cell between stationary and exponential phase cells were not

significant. The DNA content of S. cerevisiae yest cells (wild type CD 40 strain) was measured in order to compare this estimate with published data. The value of 47 fg per cell was comparable to that reported elsewhere (32).

The protein content of the C. albicans nucleus was estimated as 1.3 pg. This value was approximately twice that reported for S. cerevisiae nuclei of 0.7 pg per nucleus (32). The higher protein content of C. albicans nuclei may be due to cytoplasmic contamination or by lysis of nuclei during storage. The protein content of C. albicans yeast cells in the stationary and exponential phases of growth was estimated at 7.6 and 8.6 pg per cell respectively. This value is similar to that reported by other groups working with C. albicans (e.g. 405). However, it is approximately twice the value reported by Bharghava et al of 3.9 pg protein/S. cerevisiae yeast cell (32).

Ideally the preparation of in vitro systems, such as nuclei, should be accompanied by stringent precautions to guard against cytoplasmic contamination. The cytoplasm contains many components which interfere with measurement of RNA synthesis, e.g. proteases and nucleases. An illustration of the effect of such cytoplasmic components, on RNA synthesis, is shown in Fig. 3.8. The results shown in this figure indicate that labelled RNA, synthesised by RNA polymerases on endogenous DNA, in an assay "cocktail" based on that described by Schultz (331), was rapidly degraded within 10 min of the start of the assay. However, the need to prepare nuclei completely free from cytoplasmic contamination must be tempered with achieving this aim rapidly and with minimal perturbation of the nuclear membrane.

3.4.3 Nuclear RNA synthesis in vitro

RNA synthesis in vitro by C. albicans nuclei was the most important parameter considered during their preparation. The ability of the nuclear preparation to incorporate a labelled precursor (i.e. [^{14}C] UTP) into TCA insoluble material was used as a measure of RNA synthesis. Two assay "cocktails", which were capable of supporting RNA synthesis by T. pyriformis (163) and S. cerevisiae (331) nuclei, were tried to show measurable RNA

synthesis by C. albicans in vitro (see Fig. 3.8). It should be realised that the conditions, described in the legend of this figure, lysed both protoplasts and nuclei. The precipitate from the low speed centrifugation was capable of incorporating [^{14}C] UTP into RNA in an assay "cocktail" based on that described by Schultz (331). Those assay conditions described by Higashinakagawa et al (163), which supported nuclear RNA synthesis in T. pyriformis, did not support measurable RNA synthesis by lysed protoplasts and nuclei of C. albicans. As a consequence, the assay conditions based on those described by Schultz (331) were used to monitor RNA synthesis by C. albicans nuclei.

There are almost as many assay conditions described to support in vitro RNA synthesis as there are groups working in the field. However, all assay media described do seem to have common factors in the components that make up the assay "cocktail". Most studies on transcription have used a buffer having a pH in the range 7 - 8.5 (251). The buffers used in the preparation and storage of nuclei are usually adequate for studies on nuclear RNA synthesis (see 3.4.2). Tris HCl was used as the buffer for the assay "cocktail" in this research to maintain a pH of 7.9. The osmolality in the assay "cocktail", in addition to the media used to prepare and store (see 3.4.2), is usually determined by the amount of sucrose or glycerol present. Glycerol is more often used than sucrose to maintain the osmolality of the assay "cocktail", as the latter often contains RNase impurities, when obtained from commercially available sources. However, glycerol has been found to affect the rate of RNA synthesis in vitro (251). RNase free sucrose, at a final concentration of 0.25 M, was used to maintain the osmolality of the assay "cocktail" in this research. Such a sucrose concentration - lower than that in the storage buffer - is believed to retain "normal" chromatin conformation during the assay to measure RNA synthesis (210).

Vital components of any assay "cocktail" are the four ribonucleoside triphosphates necessary for transcription. It was found that omission of one, or more, of the ribonucleotides resulted in negligible incorporation of [^{14}C]

UTP into RNA. This indicated that the pool sizes of nucleotides in the nucleus were negligible and all RNA synthesis was DNA directed and not due to polynucleotide phosphorylase. The concentrations of ATP, GTP and CTP, at 0.5 mM, in the assay "cocktail" was well above the expected Km of 10^{-5} M for RNA polymerases (187) (Estimations of the Km for CTP of the three RNA polymerases are given in 4.3.13). The concentration of [^{14}C] UTP of 5 nmoles/100 μ assay "cocktail" (50 μM) was only slightly above the expected Km. It was found that the addition of 0.1 mM "cold" UTP resulted in dilution of the [^{14}C] UTP to such an extent that there was no measurable incorporation of [^{14}C] into RNA. This may account for the negligible incorporation of [^{14}C] UTP into RNA, which resulted when lysed nuclei were incubated in the assay "cocktail" of Higashinakagawa et al (163). This assay medium contained 0.1 mM "cold" UTP. The assay "cocktail" of Higashinakagawa et al (163) also contained lower concentrations of dithiothreitol (dTT) than that of Schultz (331) (see 2.5.2). This component is often added to media to protect thiol residues on the RNA polymerase enzyme (251).

Long term (i.e. longer than 30 min) nuclear RNA polymerase assays require an energy-conserving system. Transcription is an active process and utilization of ATP in this way leaves less available for incorporation into RNA itself. The assay "cocktail" described by Schultz (331) contained phosphoenolpyruvate/pyruvate kinase as S. cerevisiae nuclei were incubated for periods of 60 min to analyse the RNA polymerase III directed transcripts. As C. albicans nuclei were incubated for relatively short periods of time (i.e. less than 15 min) and as ATP concentration was not limiting, phosphoenolpyruvate and pyruvate kinase were omitted. Other energy conserving systems which have been used include creatine phosphate and creatine phosphokinase (e.g. 232).

The unrestricted action by nucleases and proteases in a nuclear preparation is a problem for which there is no easy answer. Although RNases have an important role to play in the nucleus (e.g. processing (106)), many workers have tried to minimise the otherwise destructive effects of these enzymes.

Most of the direct inhibitors of nuclease or protease activity (e.g. SDS, diethylpyrocarbonate and PMSF) have been found to have a direct effect on the template activity of the nucleus (251). Heparin has been employed as an RNase inhibitor *in vitro*, but has been found to cause gross structural modification of the chromatin (90). In addition, RNA polymerase initiation is inhibited (381). NaF has been added to assay "cocktail" as a potential RNase inhibitor (251). The presence of fluoride will inhibit other enzymes which require the divalent cations Mg^{++} and Mn^{++} such as RNA polymerase. The use of NaF in an *in vitro* system which is measuring the activity of this enzyme is thus questionable.

Potentially the most successful method for inhibiting RNase activity is that of end product inhibition. Crampton and Woodland (83) found that the addition of 1 mM CMP to *Xenopus* cell culture nuclei resulted in an increase in the higher molecular weight rRNA species this system synthesises (i.e. 40 S, 28 S and 18 S). There was a corresponding decrease in the amount of low molecular weight degradation products. However, it was found that the addition of 2 mM CMP, CDP, AMP or ADP to *C. albicans* nuclei resulted in negligible incorporation of [^{14}C] UTP into RNA over a 10 min incubation period in the assay "cocktail" described in the legend of Fig. 3.10. This appears to be a contradictory finding to that described by Crampton and Woodland (83) - inhibition of RNase action ought to be accompanied by an increase in [^{14}C] UMP incorporation into RNA. However, the ratio of rNTP to rNDP has been found to be an important controlling mediator for RNA synthesis (134). The levels of nucleotides have been found to be important in regulating RNA synthesis *in vitro* in nucleoli (270) and RNA polymerases (135). One further measure, taken to reduce the action of nucleases, was incubation of the nuclei from *C. albicans* in an assay medium at a temperature of 25° C.. Incubation at a higher temperature (i.e. 37° C.) has been shown to result in a higher level of RNase activity (381).

A time course for RNA synthesis by *C. albicans* nuclei incubating at 25° C. in the assay "cocktail" based on Schultz (331) was as shown in Fig. 3.9. The

rate of reaction decreased as time progressed with no increase in the incorporation of [^{14}C] UTP into RNA after 20 min. This "levelling off" in the incorporation of [^{14}C] UTP could have arisen as a consequence of protease and RNase action on the proteins involved in RNA synthesis and degradation of the product synthesised. Alternatively, the decrease in the rate of incorporation may have been due to disengagement of the RNA polymerase enzymes from the DNA template. It is unlikely that the decrease in rate of incorporation of label into RNA was due solely to the former factors (i.e. protease and RNase activity). Although these enzymes do cause problems in nuclear preparations, disengagement of the RNA polymerase from the template, without re-initiation, has been commented upon by other workers (e.g. 381).

Endogenous RNA polymerases can exist in two distinct states - the so called "template engaged" and "free" forms (414). In many of the organisms, from which nuclei have been isolated, the majority of template engaged RNA polymerases appear to be retained (82). After transcribing a section of DNA the RNA polymerase will disengage. Although re-initiation of an RNA polymerase can occur in certain circumstances, such as in mouse myeloma nuclei, it is not readily accomplished (356). However, RNA polymerase III - responsible for 5 S rRNA and 4 S tRNA synthesis - does appear to have a greater ability to re-initiate, than either RNA polymerases I and II (251). (It is thought that the factors responsible for accurate transcription initiations may be lost, through the nuclear pores during isolation (251)).

The inability of RNA polymerases to re-initiate successfully in C. albicans nuclei may account for the non-linearity found between incorporation of [^{14}C] into RNA, over a 10 min incubation period, and the number of nuclei in an assay aliquot (Fig. 3.10). When the nuclei were prepared they were suspended in Schultz's buffer C to the density indicated in the legend of Fig. 3.10. Samples of nuclei were taken from this suspension, diluted to the required nuclear density and incubated in the assay "cocktail". If the RNA polymerases in the nuclei in the original suspension did not remain attached to the DNA, and disengaged, the proportion of template engaged enzyme would decrease. This

would be accompanied by a corresponding decrease in the level of [^{14}C] UTP incorporated into RNA by a nucleus. Thus, although increasing the number of nuclei per unit volume assay "cocktail" would be accompanied by increasing [^{14}C] incorporation, the increase in this value would be non-linear when "older" nuclei were used. Changes in the levels of bound and free RNA polymerases in a nucleus over the period they were stored in suspension, could be determined by measurement of template activity with exogenous poly [d(A-T)] as described by Yu (414) and in 4.4.1.

The assay "cocktail" used to support RNA synthesis by C. albicans nuclei was also capable of supporting RNA synthesis by T. pyriformis nuclei. This suggests that assay media, which contain components in common, may direct in vitro RNA synthesis in other systems. The assay "cocktail" described by Higashinakagawa et al (163) did not support measurable RNA synthesis in lysed nuclei of C. albicans (see Fig. 3.8). However, this was probably due to excess "cold" UTP in the assay "cocktail" (see earlier in this section). Another major difference between the assay media described by Higashinakagawa et al (163) and that used in this research, was the ionic composition. This is the most variable component of any of the various media described which support in vitro RNA synthesis.

Inorganic ions have a dramatic effect on gross nuclear morphology (3[illegible]) and on chromatin structure (1). The ionic strength of the assay "cocktail" used in this research was maintained by the KCl concentration. $(NH_4)_2 SO_4$ may also be used to maintain the ionic strength (e.g. 251). At higher ionic strength (i.e. 100 mM KCl) C. albicans nuclei synthesised a greater level of RNA over a 10 min incubation period, compared to lower ionic strength (Fig. 3.11). It is believed that higher KCl concentrations "opens up" the chromatin allowing easier transcription to occur (1,210). Alteration of these ionic conditions has been found to modify in vitro transcription. Laval et al (210) found that, using Mn^{++} as the divalent cation and 0.28 M $(NH_4)_2SO_4$, the RNA synthesised had a A:U/G:C ratio of approximately 1. In comparison, at lower ionic strengths the A:U/G:C ratio of the product RNA decreased to 0.47.

Most preparations of nuclei show slower rates of RNA synthesis *in vitro* than are found *in vivo* (251). As a consequence, many studies have attempted to optimise conditions for *in vitro* transcription by nuclei. The divalent ion present, and the concentration in the media, have been found to markedly affect the general level of RNA synthesis by nuclei. Mn^{++} has been found to generally result in higher rates of RNA synthesis than Mg^{++} in nuclei prepared from various organisms (210). The concentrations of Mn^{++} which have been reported to give highest rates of RNA synthesis (approximately 1 mM) are not normally found in physiological systems (251). The addition of Mn to an assay "cocktail" has been reported as resulting in aberrant transcription and is incapable of maintaining nuclear morphology (387). As a consequence, Mg^{++} was the divalent cation used to stimulate *in vitro* RNA synthesis by *C. albicans* nuclei.

The effect of Mg^{++} concentration, in the assay medium, on nuclear RNA synthesis over 10 min, is shown in Fig. 3.12. Possibly the most interesting result of the research on *C. albicans* nuclei was that the highest level of incorporation of [^{14}C] into RNA was found when the Mg^{++} concentration was 100 mM. This finding seems in complete contrast to Mg^{++} optima reported by other workers for *in vitro* nuclear RNA polymerase activity. These are generally in the range 3 - 20 mM (251). Indeed, such high Mg^{++} concentrations have been found to affect nuclear conformation (1). A change in the molecular environment may have far reaching effects on the ability of RNA polymerase to bind to the DNA template. The higher level of [^{14}C] incorporatior into RNA may arise as a consequence of such nuclear changes. However, it seems unusual that this phenomenon is observed solely in *C. albicans* nuclei.

The higher level of [^{14}C] UTP incorporation into TCA insoluble material may have arisen as an experimental artefact. Divalent cations, such as Mg^{++}, bind to nucleotides (and as such are the substrates for RNA polymerase - see 1.3.4) and at higher concentrations of Mg^{++} could bind non-specifically to GF/C filter disks used to collect the TCA insoluble precipitate. The addition of $MgCl_2$, to a final concentration of 100 mM, after a 10 min

incubation of nuclei in media containing 10 mM $MgCl_2$, did not result in an increase in the level of [^{14}C] incorporated into TCA insoluble material. Thus, the higher level of [^{14}C] incorporation, found when nuclei were incubated in media containing 100 mM $MgCl_2$, did not arise as an artefact of non-specific binding.

RNases present a problem when investigations into in vitro RNA synthesis are made. Some RNases do show a requirement for a divalent cation, such as Mg^{++} However, high Mg^{++} concentrations can inhibit these enzymes. For example RNase H from a basidiomycete (Ustilago maydis (19)) and RNase from S. cerevisiae (211) are both inhibited by Mg^{++} concentrations greater than 10 mM. It was thus possible that an increase in Mg^{++} concentration, from 10 mM to 100 mM, would inhibit C. albicans RNases, and a corresponding increase in the level of recoverable [^{14}C] would be found. The effects of low and high Mg^{++} concentration on the RNA species synthesised by C. albicans nuclei would give an insight into the effects of RNases during an incubation period.

3.4.4 RNA species synthesised in vitro by C. albicans nuclei

The effect of $MgCl_2$, at a concentration of 10 mM or 100 mM, on the classes of RNA synthesised by C. albicans nuclei is shown in Figs. 3.13 and 3.14. The RNA was extracted from nuclei after a 15 min incubation period at 25° C.. A 15 min period was chosen as the rate of incorporation of [^{14}C] into RNA was slowing - indicating possible disengagement of the RNA polymerase from DNA after finishing transcription (see 3.4.3). The figures showed the low (i.e. Fig. 3.13) and high (i.e. Fig. 3.14) molecular weight RNA species synthesised when the $MgCl_2$ concentration of the assay "cocktail" was either 10 mM or 100 mM. These figures showed that the majority of the newly synthesised RNA migrated as a species smaller than 4 S. RNA extracted from nuclei in assay "cocktail" containing 10 mM $MgCl_2$ was 85 - 90% smaller than the 4 S marker species (bromophenol blue). In contrast, only 60 - 80% of the RNA from nuclei incubated in a "cocktail" containing 10 mM $MgCl_2$ was smaller than the

bromophenol blue marker. This may indicate endonuclease activity was responsible for the higher percentage of low molecular weight RNA species found in RNA extracted from nuclei incubated when the $MgCl_2$ concentration was 10 mM.

Fig. 3.14 shows small, but distinct peaks due to [^{14}C] UTP incorporated into 25 S, 17 S and 5.8 S rRNA species. These probably arose from processing of the 37 S precursor rRNA. The 37 S pre-rRNA transcript is synthesised by RNA polymerase I in S. cerevisiae (288). As the ribosomes of Candida spp resemble those of S. cerevisiae (408), C. albicans also presumably synthesised a similar sized RNA. Polyacrylamide gel electrophoresis is not usually a sensitive enough technique to monitor the synthesis of RNA polymerase II directed transcripts - assays for the activity of specific genes often require other techniques (see 7.4.2).

It has been found that in media with high ionic strength, using Mn^{++} as the divalent cation, a greater amount of the RNA synthesised in vitro is RNA polymerase II directed. In contrast, lower ionic strength, plus Mg^{++} as the RNA arising due to RNA polymerase I directed transcription (310). RNA polymerase III is apparently equally active in vitro in nuclei when Mg^{++} or Mn^{++} is used as the divalent cation. These ionic strength optima and divalent cation preferences are two criteria used by Roeder (310) to discriminate between the different RNA polymerase isozymes. Whether the RNA polymerases from C. albicans exhibited similar preferences will be discussed in 4.4.2.

Most of the RNA synthesised in vitro by C. albicans nuclei was RNA polymerase III directed. In contrast, a high proportion of RNA synthesised in vitro by protoplasts, from which the nuclei were isolated, was the higher molecular weight species. Thus, during the preparation of nuclei the level of RNA polymerase I directed synthesis falls. Nuclei and protoplasts from C. albicans share some similarities with these systems from other lower eukaryotes (see 3.4.1 and earlier in this section). However, the nuclei do show unusual Mg^{++} optima for RNA synthesis. This is probably the most unusual result in this chapter.

CHAPTER 4 PURIFICATION OF RNA POLYMERASES FROM YEAST AND PARTIAL PURIFICATION OF MYCELIAL FORM C. ALBICANS

4.1 Introduction

It was established in the late 1960's that eukaryotes possessed three distinct classes of nuclear DNA-dependent RNA polymerases (311). Since then, there has been intensive research into their involvement in the regulation of gene transcription. The sub-cellular localisation, subunit composition and reaction properties of these complex enzymes has been elucidated over the past decade. Numerous reviews have appeared describing the general properties of eukaryotic RNA polymerases (63, 100, 310) Some facets of current knowledge on this subject were outlined in 1.3.3. In this chapter the techniques, and reasons, for the purification of yeast and the partial purification of mycelial forms of C. albicans RNA polymerases will be discussed. Some properties of the yeast RNA polymerases will also be discussed.

It was reported in 3.4.3 that nuclei showed a higher level of incorporation of [^{14}C] when incubated in media containing 100 mM rather than 10 mM $MgCl_2$. In order to investigate whether this finding was due to activation of one or more forms of RNA polymerase, at higher $MgCl_2$ concentrations, or to other factors, the three RNA polymerases were purified. In addition, purification or at least partial purification, would be the first step in allowing studies to be made on the action of specific inhibitors directly on isolated RNA polymerases from C. albicans. The main reason for purification of the RNA polymerases was to ascertain whether the three classes of RNA polymerases, from the two morphological forms possessed distinctly different properties.

It has been known for some time that the major classes of cellular RNA (i.e. ribosomal (r)RNA, transfer (t)RNA and messenger (m)RNA are synthesised at different rates during the many growth and developmental transitions of eukaryotes (310). For instance, during the cell cycle of S. cerevisiae, it has been reported that the relative levels of RNA polymerases I and II - as judged by DEAE-Sephadex profiles from whole cell extracts - differ during G_1, S, early G_2 and late G_2 stages of the cell cycle (58, 336). In addition,

it has been reported that there seems to be a correlation between growth rate, cellular RNA content and the levels of RNA polymerase I activity. It was suggested that RNA polymerases I and II are independently regulated in S. cerevisiae - the level of RNA polymerase I regulating rRNA synthesis (335). RNA polymerase activities has been monitored during developmental transitions in several fungi. Changes in the specific activities and amounts of RNA polymerases I and II, but not III, of the phycomycete Mucor rouxii have been observed (413). RNA polymerases I, II and III from yeast and mycelial phases of the dimorphic fungus Histoplasma capsulatum are reported to differ in their sensitivities to α-amanitin, mono and divalent cations and temperature (203).

Several reviews have dealt with quantitative and qualitative changes in RNA polymerases during various growth and developmental transitions. The general impression, given by these reviews of plant and lower eukaryote (138) and animal (310) RNA polymerases may be summarised as follows. Firstly, there is not necessarily a correlation between the level of activity for a particular class of RNA polymerase and the total amount of that particular isozyme in the cell. In addition, RNA polymerases I, II and III are almost undoubtedly present in both quiescent and dividing cells. Thus other factors, i.e. proteins or chromatin structure, will play a role in regulation of the overall rates of transcription by the individual RNA polymerases. Thirdly, it has been suggested that the evidence for qualitative changes in subunits may be explained by purification artefacts such as proteolysis of susceptible subunits (138). However, some workers believe that there are differences between the RNA polymerases from distinct growth and developmental forms of the same organism. Kumar et al (204) havereported that antisera to RNA polymerase III, from yeast form H. capsulatum, did not completely cross-react with the same isozyme from the mycelial form. These differences in the antigenic determinants suggest there could be differences between the RNA polymerases of the two morphological forms.

The initial breakthrough in eukaryotic RNA polymerase purification resulted

from the efficient solubilization of the enzymes from the DNA template. This was achieved by employing high salt sonication procedures with isolated nuclei or whole cell extracts. The high salt concentration (i.e. greater than 0.3 M $(NH_4)_2SO_4$) disrupts the nuclear chromatin and sonication reduces the viscosity of the high salt extract. This procedure has been generally applicable to the purification of nuclear RNA polymerases from animals, plants and lower eukaryotes. After solubilization of the RNA polymerases, purification of the enzymes may be achieved by conventional techniques. These include ammonium sulphate fractionation, chromatography on cationic (e.g. carboxymethyl-sephadex) or anionic (e.g. diethylaminoethyl-sephadex) exchange columns and sedimentation through glycerol or sucrose density gradients. In addition, as eukaryotic nuclear RNA polymerases are large proteins (approximate molecular weight 500,000) gel filtration may also be used as a step in the purification of these enzymes.

Two common procedures now employed in the purification of enzymes are nucleic acid precipitation and affinity chromatography. Jendrisak and Burgess originally suggested the use of polymin P (polyethylenimine) to precipitate nucleic acids in cell free extracts (178). Another polyamine - protamine sulphate - has also been used to achieve the same end (293). Methods such as these are now widely employed in the purification of RNA polymerases. Affinity chromatography on DNA sepharose or DNA cellulose has also been applied to RNA polymerase purification (127,412). In addition to the use of DNA as an affinity ligand, heparin - as heparin sepharose - has also been successfully used to achieve this aim (e.g.137,143). RNA polymerases bind very tightly to their affinity ligands whilst most contaminating proteins do not. The enzymes may be eluted from the column by buffer containing high salt concentrations.

In order to achieve purification of C. albicans yeast form RNA polymerases to homogeneity, and the partial purification of mycelial form RNA polymerases, some of the above described techniques were employed. The procedures used to achieve this were based on those used by Hager et al (141) and Kumar et al (203) to purify RNA polymerases from S. cerevisiae and H. capsulatum

respectively. In addition, the three RNA polymerases from the yeast form of of C. albicans were characterised with reference to their divalent metal ion preferences and salt optima, Kms for the substrate CTP and α-amanitin sensitivities. These results will be discussed with reference to RNA polymerases from other eukaryotes in general and S. cerevisiae in particular.

4.2. Materials and Methods

4.2.1 Preparation of cell free extracts

C. albicans A serotype (NCPF3153) yeast and mycelial forms were cultivated in Sabouraud's dextrose broth and glucose beef extract medium respectively, as described in 2.3.3. Yeast cells, growing to the cell density required, and mycelia were harvested, washed and resuspended $^{1}/_{10}$ (w/v) in buffer i.

Two methods of cell breakage were tried. The first was based on that used by Hager et al (141) using a press to disrupt the cells. 10 ml of the C. albicans suspension in buffer i were compressed at 100 Nmm^{-2} (15,000 p.s.i.) in a press at 4° C. The second method, as described by Kumar et al (203), involved homogenisation with balotini. The suspension of either yeast or mycelial cells was mixed with twice their weight of ballotini (0.45 - 0.55 mm diameter) and homogenised in a Braun homogeniser. The suspension was kept cool (0 - 5° C.) using CO_2 gas. The criteria for disruption were based on the appearance of lactate dehydrogenase (LDH) and RNA polymerase activity and on cell counts before and after treatment.

After successful disruption of the cells, the homogenate was diluted with an equal volume of buffer A of Hager et al (141), which contained 1.0 M $(NH_4)_2SO_4$, 1% (w/v) DMSO, 0.5 mM dTT, 0.5 mM PMSF and 1 mg bovine serum albumin/ml buffer. The viscous homogenate was sonicated with six 10 sec bursts at maximum power on an M.S.E. sonicator using a 1.3 cm probe. The sonicate was then centrifuged for 20 min at 10,000g, using a Sorvall S.S.34 rotor in a Sorvall RC5B centrifuge, cooled to 4° C.. The resulting supernatant (fraction F_1) was diluted with buffer A of Hager et al (141) to give a suspension with a final $(NH_4)_2SO_4$ concentration of 0.15 M.

4.2.2 Protamine sulphate precipitation of nucleic acids

A 2.5% (w/v) solution of protamine sulphate in buffer A of Hager et al (141), containing 0.15 M $(NH_4)_2SO_4$, was prepared by stirring at 3° C. for 17 hr 21.8 ml of this solutionwas added to every 100 ml of diluted F_1 at 4° C.. The resulting suspension was then stirred for 30 min at 4° C., followed by centrifugation at 8,000g for 20 min, in a Sorvall GSA rotor in a Sorvall RC5B centrifuge, cooled to 4° C.. The supernatant from this centrifugation (F_2) was adjusted to 82% saturation with ammonium sulphate. After stirring for 30 min at 4 ° C. the $F_2/(NH_4)_2SO_4$ suspension was centrifuged at 8,000g for 1 hr, in a Sorvall GSA rotor in a Sorvall RC5B centrifuge, cooled to 4° C. The precipitate from this centrifugation (F_2ASppt) was resuspended in buffer A of Hager et al (141) to give a protein concentration of 30 mg/ml.

4.2.3 Gel filtration

The F_2ASppt suspension was applied, in a total volume of not more than 7 ml, to an agarose A-5 M column (45 cm x 2 cm), which had been previously equilibrated with buffer A containing 0.1 M $(NH_4)_2SO_4$, as described in 2.8.3. The column was then eluted with the same buffer. Fractions which contained significant RNA polymerase activity were pooled, concentrated by $(NH_4)_2SO_4$ salt precipitation (as F_2) and dialysed three times against buffer B of Hager et al (141). The resulting dialysate was termed F_3.

4.2.4 Ion exchange chromatography

The F_3 fraction was layered onto a DEAE-sephadex A-25 column (30 x 1. cm), which had been previously equilibrated with buffer B of Hager et al (141) containing 20 mM KCl, as described in 2.8.4. The bound protein was eluted with a linear gradient, as described in the legends of Figs. 4.4 and 4.20 The fractions were collected and assayed for RNA polymerase activity. The resulting peaks of activity from the yeast or mycelial form that were found were each separately combined. The three separate pools from the yeast form

were concentrated by $(NH_4)_2SO_4$ precipitation (as F_2) and dialysed three times against buffer B of Hager et al (141). The three fractions were termed fractions F_4, F_5 and F_6.

4.2.5 Affinity chromatography

Dialysed fractions F_4 and F_5 were applied to two DNA cellulose columns, which had been equilibrated with buffer B of Hager et al (141) containing 20 mM KCl. (The DNA cellulose was prepared as described in 2.8.5). The columns were washed with the same buffer and the proteins eluted as described in the legends of Figs. 4.5 and 4.6. The fractions were collected and assayed for RNA polymerase activity. The resulting peaks of activity - termed F_7 and F_8 arising from fractions F_4 and F_5 respectively - were concentrated by $(NH_4)_2SO_4$ precipitation (as F_2).

4.2.6 Glycerol gradient centrifugation

The ammonium sulphate precipitates of fractions F_7, F_8 and F_6 were applied onto 11 ml linear 15 - 30% glycerol gradients, as described in 2.8.6. The gradients were then centrifuged in the rotors and for the lengths of time stated in the legends of Figs. 4.7, 4.8 and 4.9.

4.2.7 Polyacrylamide gel electrophoresis

Analytical PAGE, under non-denaturing conditions, was performed on the purified yeast phase RNA polymerases according to the method of Sklar and Roeder (352), as described in 2.8.7. Fractions from the glycerol gradient, showing RNA polymerase activity, were separately pooled. Aliquots of these pooled fractions were analysed on 5% tube gels (0.8 x 10 cm). The gels were stained for protein using the silver staining technique of Merril et al (258), as described in 2.8.8.

RNA synthesised by yeast, in the presence of α-amanitin, was labelled with [^{32}P] sodium orthophosphate, as described in the legend of Fig. 4.15. The

[^{32}P] labelled RNA was extracted, as described in 2.7.1, and analysed by agarose gel electrophoresis, as described in 2.8.1.

4.3 Results

4.3.1 Purification of RNA polymerases from C. albicans

An outline of the procedure used to purify the RNA polymerases from yeast and mycelial forms of C. albicans is shown in Tables 4.1 and 4.2. The total RNA polymerase activities, protein, specific activities and class of isozyme present at each stage of the purification of the enzymes, from both morphological forms, are shown. One unit of RNA polymerase activity was defined as the incorporation of 1 pmole [^{3}H] UMP into TCA insoluble material, per 5 min incubation at 25^{o} C..

The specific activities of RNA polymerases, from the yeast form, increased from 0.18 to 1.5 units mg protein^{-1} from the preparation of homogenate to after the gel filtration step. The ion exchange step resolved the yeast RNA polymerases into three peaks, with specific activities of 3.57, 3.8 and 0.32 units mg protein^{-1}. DNA cellulose chromatography resulted in further purification, to 7.52 and 15.61 units mg protein^{-1} for RNA polymerases I and II. Glycerol gradient centrifugation resulted in a decrease in the specific activities of RNA polymerase I and II, to 0.68 and 1.17. Although RNA polymerase III showed an increase in specific activity, to 0.75 units mg protein^{-1}, the total activities of this and the other isozymyes fell markedly. This may be illustrated by 110 total units of activity, for the three RNA polymerases prior to glycerol gradient centrifugation, decreasing to 0.32 after this step.

The specific activity of RNA polymerases from the mycelial form increased from 0.22 units mg protein^{-1} in homogenate to 1.3 units mg protein^{-1} after gel filtration.

4.3.2 Initial stages in the purification of RNA polymerases from yeast C. albicans: preparation of homogenate

The suitability of the two methods, i.e. compression and the use of

	Fraction	RNA Polymerases present	Total Polymerase activity (units)	Total Protein concentration (mg)	Specific activity (units/mg)
	Homogenate	I,II,III	473	2560	0.18
	Sonicate	I,II,III	296	2720	0.11
	F_1	I,II,III	216	1450	0.15
	F_1ppt	I,II,III	97	518	0.19
	F_2	I,II,III	31	1260	0.02
	F_2ppt	I,II,III	14	144	0.10
	F_3	I,II,III	427	372	1.15
DEAE sephadex A25 chromatography	F_4	I	50	14	3.57
	F_5	II	93	24	3.88
	F_6	III	2.2	6.9	0.32
DNA cellulose chromatography	F_7	I	18.8	2.5	7.52
	F_8	II	89	5.7	15.61
Glycerol gradient centrifugation	F_9	I	0.19	0.28	0.68
	F_{10}	II	0.07	0.06	1.17
	F_{11}	III	0.06	0.08	0.75

Table 4.1 Summary of purification of RNA polymerases from yeast form C. albicans

C. albicans yeast from cells were grown in S.D.B. at 37° C. to a cell density corresponding to late exponential phase. The cells were homogenized and fractions prepared as described in 4.2.1 to 4.2.6. RNA polymerase activity was measured using the assay conditions described in 2.5.2. The table shows results obtained from 80 g (wet weight) of C. albicans. Protein was measured as described 2.6.3.

Fraction	Total RNA Polymerase activity	Total Protein concentration (mg)	Specific activity (units/mg)
Homogenate	94	422	0.22
Sonicate	73	427	0.17
F_1	70	238	0.29
F_1ppt	7	125	0.06
F_2	10	184	0.05
F_2ppt	3	46	0.06
F_3	13	10	1.3

Table 4.2 Summary of partial purification of RNA polymerase from mycelial form C. albicans

C. albicans mycelial form cells were grown in G.B.H. medium at 37° C.. The mycelia were collected after 7 days growth as described in 2.3.3. The cells were homogenized and fractions prepared as described in 4.2.1 to 4.2.6. RNA polymerase activity at each stage was measured using the assay conditions described in 2.5.2. The value shows the results obtained from 12 g (wet weight) of C. albicans. Protein was measured as described in 2.6.3.

balotini to homogenise the cell suspension, is shown in Table 4.3. There was no decrease in the cell density of samples of cell suspension before or after compression. In addition, the negligible RNA polymerase activity and the low LDH activity (i.e. 0.8 - 1.0 μmoles NADH oxidised min^{-1} mg $protein^{-1}$) did not change markedly on compression. There was a marked decrease in cell count of samples of cell suspension removed after 1 min homogenisation with ballotini. After 3 min homogenisation the cell count of a sample fell to approximately 0.5% of that prior to treatment. In addition, the measurable activity of RNA polymerase of a sample, increased from 0 to 0.33 pmoles of [^{14}C] UMP incorporated min^{-1} mg $protein^{-1}$ after 3 min homogensation with balotini. The LDH activity of samples removed after treatment with balotini was greater than that prior to homogenisation (i.e. rising from 0.8 to 12 - 14 μmoles NADH oxidised min^{-1} mg $protein^{-1}$). RNA polymerase activity of samples before and after the two methods of cell disruption attempted, was measured in the assay "cocktail", described in 3.3.7. This resulted in optimal RNA synthesis <u>in vitro</u> in nuclei. The components of the "cocktail" were the same as described in 2.5.2, with the exception that 250 nCi of [^{14}C] UTP was added and the $MgCl_2$ concentration was 100 mM. The effect of altering the Mg^{++} concentration, in the "cocktail", of homogenates prepared from yeast and mycelia, is shown in Fig. 4.1. Optimal RNA polymerase activity occurred in an assay "cocktail" containing 10 mM $MgCl_2$. Optimal specific activity (i.e. 0.2 units mg $protein^{-1}$) was found in RNA polymerases from yeast, harvested in the exponential phase of growth.

Table 4.4 shows the specific activity (units mg $protein^{-1}$) for RNA polymerases in crude homogenate prepared from <u>C. albicans</u> harvested at different cell densities. Similar specific activities (of between 0.178 - 0.219 units mg $protein^{-1}$) were found for RNA polymerases in homogenates prepared from early (2×10^7 yeast cells/ml), mid (5×10^7 yeast cells/ml) and late (1×10^8 yeast cells/ml) exponential phase cells. ($A\chi^2$ value of 0.057, with five degrees of freedom, for the specific activity of RNA polymerases in crude homogenates prepared from yeast cells in any period of growth, indicated that there were no significant differences between these values). RNA polymerases in a crude homogenate prepared from stationary phase yeast cells

Table 4.3 Cell breakage of C. albicans

C. albicans yeast form cells were grown in S.D.B. to a cell density corresponding to late exponential phase. The cells were harvested and washed as described in 2.3.3. and resuspended 1/10 (w/v) in buffer i. Two methods were then used to try and disrupt the cells: a) compression at 90 - 100 Nmm^{-2} in a press, cooled to 4° C., b) homogenisation of 10 ml yeast suspension with twice the weight of balotini added (0.45 - 0.55 mm diameter), at low temperature, for the lenghs of time indicated in the table. RNA polymerase activity was measured as described in 3.4.2. L.D.H. activity was measured as described in 2.5.5. Protein was measured as described in 2.6.3. Cell counts were made using an improved Neubauer haemocytometer, on samples removed before and after treatment, and diluted 1/20 in buffer i.

Fig. 4.1 Effect of $MgCl_2$ concentration in assay "cocktail" on RNA polymerase activity in homogenates prepared from yeast and mycelial form C. albicans

Yeast form C. albicans was grown in S.D.B. at 37° C. to cell densities corresponding to the stationary (5×10^8 cells/ml media) and late exponential (10^8 cells/ml media) phases of growth. Mycelial form cells were grown in G.B.E. medium at 37° C.. The cells from both growth forms were harvested and washed as described in 2.3.3. and homogenates prepared as described in 4.2.1. The RNA polymerase activities of 200 µl homogenate, prepared from stationary (——) and late exponential (—■—) phases of growth, of yeast and mycelial (—●···) forms of C. albicans were measured using the conditions described in 2.5.1. The error bars refer to the standard deviations of duplicate measurments. Protein was measured as described in 2.6.1.

Table 4.3

Method of cell disruption	Duration of treatment	RNA polymerase activity (pmoles [^{14}C] UMP incorporated into RNA/min/mg protein)	L.D.H. activity (µmoles NADH oxidised/ min/mg protein)	Cell count of sample (number of cells ($\times 10^{-6}$)/ml)
Homogenisation with balotini	0 min	0	0.8	215
	1 min	0.03	5	64
	2 min	0.12	7	28
	3 min	0.33	12	1
	4 min	0.31	11	1
	5 min	0.27	14	1
Compression in press	before compression	0	0.8	220
	after compression	0	1.0	210

Fig. 4.1

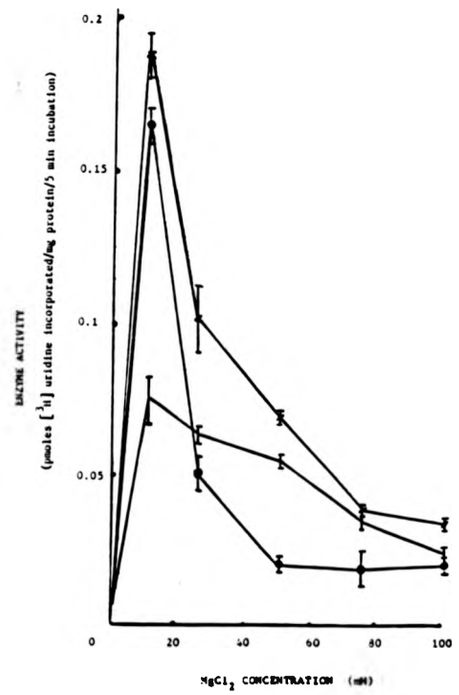


Table 4.3 Cell breakage of C. albicans

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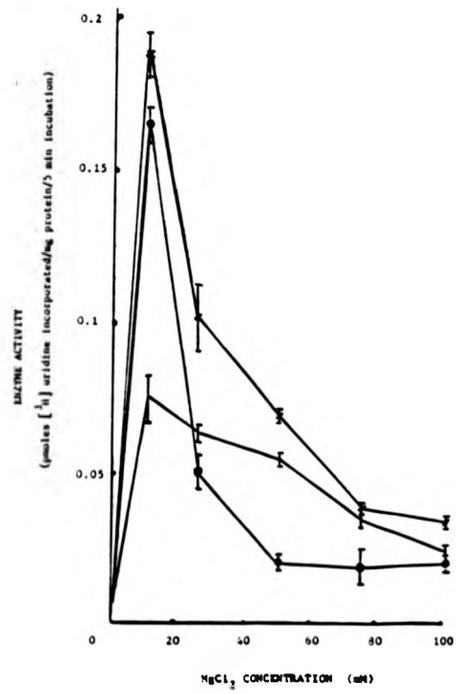
Fig. 4.1 Effect of $MgCl_2$ concentration in assay "cocktail" on RNA polymerase activity in homogenates prepared from yeast and mycelial form C. albicans

Yeast form C. albicans was grown in S.D.B. at 37° C. to cell densities corresponding to the stationary (5×10^8 cells/ml media) and late exponential (10^8 cells/ml media) phases of growth. Mycelial form cells were grown in G.B.E. medium at 37° C.. The cells from both growth forms were harvested and washed as described in 2.3.3. and homogenates prepared as described in 4.2.1. The RNA polymerase activities of 200 µl homogenate, prepared from stationary (—•—) and late exponential (—○—) phases of growth, of yeast and mycelial (—●—) forms of C. albicans were measured using the conditions described in 2.8.2. The error bars refer to the standard deviations of duplicate measurments. Protein was measured as described in 2.6.3.

Table 4.3

Method of cell disruption	Duration of treatment	RNA polymerase activity (pmoles [^{14}C] IMP incorporated into RNA/min/mg protein)	L.D.H. activity (µmoles NADH oxidised/ min/mg protein)	Cell count of sample (number of cells ($\times 10^{-6}$)/ml)
Homogenisation with balotini	0 min	0	0.8	215
	1 min	0.03	5	64
	2 min	0.12	7	28
	3 min	0.33	12	1
	4 min	0.31	11	1
	5 min	0.27	14	1
Compression in press	before compression	0	0.8	220
	after compression	0	1.0	210

Fig. 4.1



(5×10^8/ml) had a much lower specific activity of 0.035 units/mg protein.

4.3.3 Initial stages of RNA polymerase purification: preparation of sonicate

The effect of sonication on the percentage of bound and free RNA polymerase in homogenates, prepared from yeast in the exponential phase of growth, is shown in Table 4.5. The addition of exogenous DNA template to the homogenate, prepared from yeast harvested at 1×10^8 cells/ml, resulted in an increase in the amount of [^{3}H] UMP incorporated (i.e. from 41.3 to 70.6 fmoles) Sonication, of the same homogenate, resulted in a decrease of RNA polymerase activity, on endogenous DNA, to 11.6 fmoles. The addition of exogenous DNA, to the sonicate, resulted in an increase in incorporation of [^{3}H] UMP into RNA to 24.6 fmoles. The RNA polymerase activity, using endogenous DNA, of homogenate and sonicate corresponds approximately to the amount of "bound" RNA polymerase in the sample. The activity of the enzyme in the presence of exogenous DNA approximates to the level of "bound" plus "free" RNA polymerase activity. The level of "free" RNA polymerase may be estimated by measurement of the activity in the presence of actinomycin D and exogenous poly [d(A-T)] template. It was found that the addition of both these components to the "cocktail" resulted in 28.3 and 17.3 fmoles [^{3}H] UMP incorporated into RNA by homogenate and sonicate respectively. A low level of [^{3}H] UMP incorporation into RNA by homogenate and sonicate, with endogenous DNA plus actinomycin D, of 5.3 and 1.7 fmoles respectively, indicated the residual amount of RNA polymerase activity.

The percentage of "bound" and "free" RNA polymerases in homogenates, prepared from yeast at two different cell densities, before and after sonication, may be estimated as described above. The percentage of "bound" RNA polymerase in the homogenate of yeast grown to 1×10^8 cells/ml was 58%. The level of "bound" enzyme in the homogenate prepared from 3×10^8 yeast/ml was less (44%). Sonication slightly reduced the percentage of "bound" enzymes in homogenates from 1×10^8 and 3×10^8 yeast/ml (i.e. to 47 and 33% respectively).

Table 4.4 RNA polymerase activity of homogenates prepared from yeast form C. albicans harvested at various cell densities

C. albicans yeast cells were grown in 1 L S.D.B. to the cell densities shown in the table. Cell densities, of aliquots removed from suspension, were measured using an improved Neubauer haemocytometer. The cells were harvested and homogenates prepared with balotini as described in 4.2.1. The RNA polymerase activity of 200 µl homogenate over a 5 min period was estimated as described in 2.5.2.

Table 4.5 Template free and engaged forms of RNA polymerase in homogenates of C. albicans before and after sonication

Homogenates were prepared from yeast form C. albicans - grown to the cell densities indicated in the table - as described in 4.2.1. The RNA polymerase activity of 200 µl homogenate, before and after the sonication step of 4.2.1, was estimated over a 5 min period. The activities were measured as described in 2.5.2 with the exception that no calf thymus DNA was added. When required 120 µg of Actinomycin D and 15 µg poly [d(A-T)] were added to 400 µl assay mixture. (The asterisks refer to homogenate before * and after sonication **).

Fig. 4.2 RNA polymerase activity in homogenates left for 90 min at 0° C. and 25° C.

A homogenate was prepared from yeast form C. albicans - grown to exponential phase in S.D.B. - as described in 4.2.1. Aliquots of homogenate were kept on ice at 0° C. or in a water bath at 25° C. The RNA polymerase activity, after the lengths of time indicated in the figure, was measured using the assay conditions described in 3.4.2. The figure shows activity in the homogenate left at 0° C. (—▪—), 25° C. (—●—) and at 0° C. with the addition of bovine serum albumin (1 mg/ml) to the homogenate (·—·—·). The error bars refer to the standard deviations of duplicate measurements.

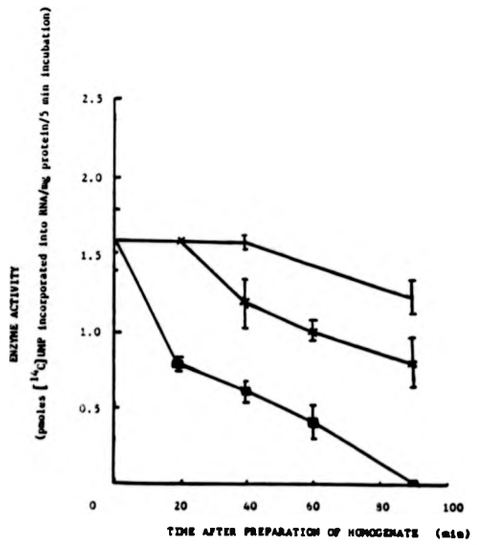
Table 4.4

Cell density of yeast on harvesting	Dry weight of yeast from 1L S.D.B.	RNA polymerase activity of homogenate (units/mg)
2×10^7/ml	0.82 g	0.178 ± 0.04
5×10^7/ml	1.23 g	0.193 ± 0.04
1×10^8/ml	3.75 g	0.219 ± 0.04
5×10^8/ml (stationary phase)	17.85 g	0.035

Table 4.5

Cell density of yeast on harvesting	RNA polymerase activity of cell free extract (fmoles [^{3}H] UMP incorporated into RNA/50 µl aliquot/5 min)				
	Cell free extract only	Cell free extract + actinomycin D	Cell free extract + poly [d(A-T)]	Cell free extract + actinomycin D + poly [d(A-T)]	Percentage "bound" enzyme
1×10^9/ml	41.3*	5.3	70.6	28.3	58%
	11.6**	1.7	24.6	17.3	47%
3×10^8/ml	34.2*	6.3	78.6	48.3	44%
	15.8**	3.0	47.8	25.7	33%

Fig. 4.2



4.3.4 RNA polymerase activity at various times after preparation of homogenate

The decrease in activity of RNA polymerases in freshly prepared homogenate, over a period of time, is shown in Fig. 4.2. Samples of the homogenate were kept at 0° C. and at 25° C.. Using the conditions that resulted in optimal nuclear RNA synthesis, aliquots of the homogenate were assayed for RNA polymerase activity after various periods of time. The figure also shows the effect of adding bovine serum albumin to the "cocktail". RNA polymerase activity, of a 50 µl aliquot of homogenate was negligible after 90 min at 25° C., whilst at 0° C. approximately 50% of initial activity was detectable. The addition of BSA increased the level of detectable RNA polymerase activity.

4.3.5 Gel filtration of yeast form RNA polymerases

The fraction F_2ASppt suspension was applied to an agarose A-5 M column, as described in 2.8.3. Fig. 4.3 shows a typical result obtained with gel filtration of this fraction. The RNA polymerase activity of selected fractions, assayed as described in 2.5.2, was found to be optimal in fractions eluting from the column just behind the void volume (i.e. having a M.W. smaller than the exclusion M.W. of 5×10^6). The fractions containing significant activity were pooled and concentrated by ammonium sulphate precipitation.

4.3.6 Ion exchange chromatography of yeast RNA polymerase

The ammonium sulphate precipitate derived from fraction F_3 was dialysed, as described in 4.2.4. Fig. 4.4 shows a typical result obtained when the F_3 fraction was chromatographed on DEAE-sephadex. Three peaks of RNA polymerase activity were found when fractions eluted from the column were assayed for RNA polymerase activity. The activities were eluted when the salt concentration of the buffer B was 0.06 - 0.09 M KCl, 0.09 - 0.2 M KCl, and 0.24 - 0.29 M. The three enzyme activities were named RNA polymerases I, II and III respectively according to the procedure of Roeder and Rutter (311).

Fig. 4.3

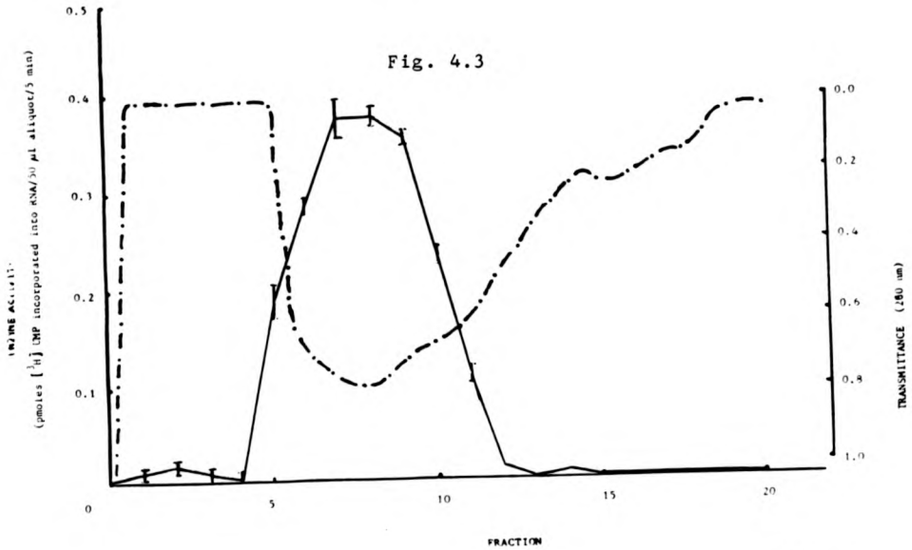


Fig. 4.3 Gel filtration of fraction F_2 from yeast form C. albicans

The ammonium sulphate precipitate of fraction 2, containing 30 mg protein/ml, was applied to an agarose A-5 M column. The column was eluted with Hager's buffer A containing 0.1 M $(NH_4)_2SO_4$. The transmittance, at 280 nm, of the eluent (— • —) was recorded using a Uvicord scanner. Twenty two 5 ml fractions were collected and alternate fractions were assayed for RNA polymerase activity in a "cocktail" containing 10 mM $MgCl_2$ and salt to a final concentration of 100 mM as described in 2.5.2. The activity of duplicate 50 µl aliquots was measured by the incorporation of [^{3}H] UMP into TCA insoluble material over a 5 min incubation period at 25° C. (——) The error bars refer to the standard deviations of duplicate measurements.

Fig. 4.4

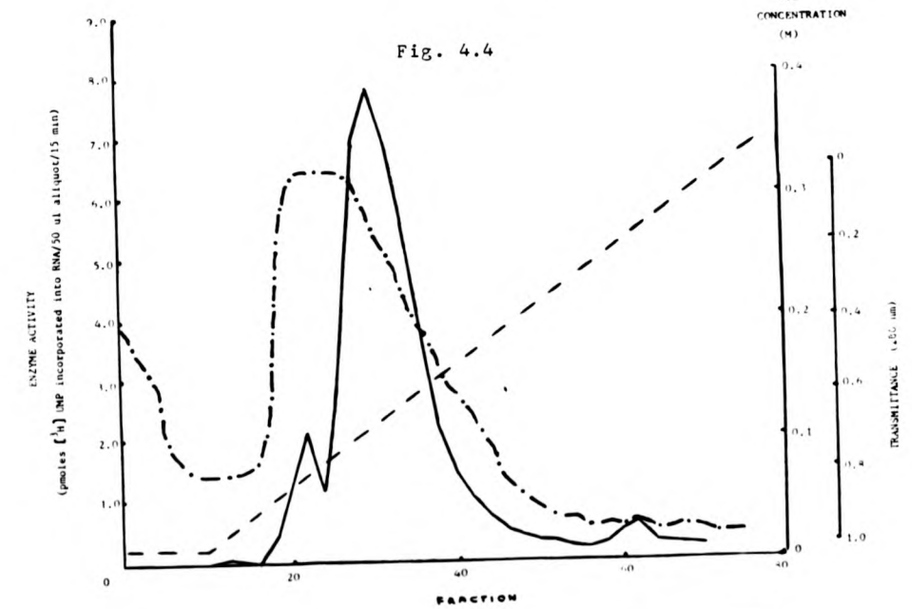


Fig. 4.4 Ion exchange chromatography of fraction F_3 from yeast form C. albicans

The ammonium sulphate precipitate of fraction 3, containing 63 mg protein (dialysed as described in 4.2.4), was applied to a DEAE-Sephadex A-25 column. The column was washed with Hager's buffer B containing 0.02 M KCl, followed by elution with a 100 ml gradient of 0.02 M - 0.42 M KCl (— • —). Eighty 1 ml fractions were collected and selected fractions assayed for RNA polymerase activity in a "cocktail" containing 10 mM $MgCl_2$ and 100 mM KCl, as described in 2.5.2. The activity of duplicate 50 µl aliquots was measured by the incorporation of [^{3}H] UMP into TCA insoluble material over a 15 min incubation period at 25° C. (——) The error bars refer to the standard deviations of duplicate measurements. The transmittance at 280 nm of the eluent (— • —) was recorded using a Uvicord scanner.

Table 4.6 Adsorption and elution of RNA polymerases I, II and III from DNA cellulose

The 3 RNA polymerases from yeast form C. albicans were separated as described in 4.2.4, by ion exchange chromatography. 100 µl aliquots of fractions F_{1a}, F_3 and F_4 were mixed with 0.5 ml (bed volume) DNA cellulose slurry. The slurry was washed with 100 µl of buffer B containing the increasing concentrations of KCl shown in the table. The DNA cellulose was separated from the "eluting" buffer by centrifugation at 0-4° C, in a B and T micro-angle bench centrifuge, for 10 min at maximum speed. The RNA polymerase activity in the supernatant was measured over a 10 min incubation period using the conditions described in 2.5.2. (N/D = not determined)

RNA Polymerase isozyme	RNA polymerase activity (pmoles [^{3}H] UMP incorporated into RNA/ 50 µl aliquot/10 min)					
	Before application to DNA cellulose	KCl conc^n of buffer used to wash DNA cellulose				
		0.02 M (before washing)	0.1 M	0.3 M	1.0 M	2.0 M
I	0.50	0.37	0.078	0.047	0.002	N/D
II	0.54	0.40	0.089	0.029	0.002	N/D
III	0.017	0.001	0.002	0.001	0.001	0.020

4.3.7 Affinity chromatography of RNA polymerases from yeast form C. albicans

The next stage of enzyme purification was affinity chromatography, using DNA as the ligand. However, before this stage was performed, aliquots of the three enzymes were titrated against DNA cellulose (Table 4.6). This was done in order to determine whether any of the enzymes had too strong or too weak an affinity for the ligand. Aliquots of each class of enzyme were mixed with 0.5 ml (bed volume) of DNA cellulose slurry and "washed" with buffer B containing increasing amounts of KCl. After each "washing" the RNA polymerase activity in the buffer was measured. The buffer was separated from the cellulose by centrifugation. It was found that RNA polymerases I and II were "eluted" from the DNA cellulose when the KCl concentration was 0.1 M. RNA polymerase III activity was eluted from the DNA cellulose slurry when the salt concentration of the buffer was 2.0 M. As a consequence, only fractions F_4 and F_5 were chromatographed on DNA cellulose.

Figs. 4.5 and 4.6 show typical results obtained when fractions F_4 and F_5 were chromatographed on DNA cellulose. Peaks of RNA polymerase activity were eluted when the buffer contained 0.1 - 0.2 M and 0.05 - 0.1 M KCl, when fractions F_4 and F_5 respectively were chromatographed. To avoid cross contamination, only the leading fractions showing activity of RNA polymerase II and the latter of RNA polymerase I were pooled and concentrated by $(NH_4)_2SO_4$ precipitation as described in the methods section. These two fractions were termed fraction F_7 (RNA polymerase I) and fraction F_8 (RNA polymerase II).

4.3.8 Glycerol gradient centrifugation of yeast RNA polymerases

Fractions F_7, F_8 and F_6 were centrifuged on 15 - 30% glycerol gradients at the speeds and for the lengths of time indicated in Figs. 4.7, 4.8 and 4.9 respectively. Three peaks of low activity were observed.

4.3.9 Non-denaturing PAGE of purified yeast RNA polymerases

Analysis, by non-denaturing PAGE of the purified enzymes was performed.

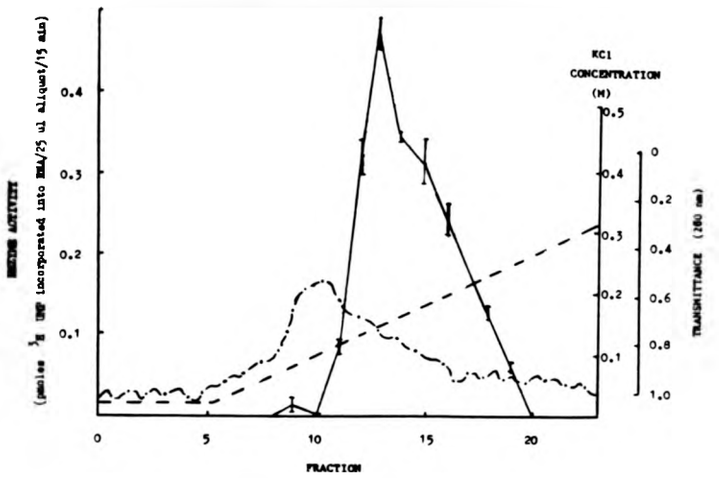


Fig. 4.5 DNA cellulose chromatography of fraction F_4

The dialysed ammonium sulphate precipitate of fraction F_4, containing 7 mg protein was applied to a DNA cellulose column. The column was washed with 5 ml of Hager's buffer B containing 0.02 M KCl, and eluted with a 18 ml gradient of 0.02 - 0.4 M KCl (— — —). Twenty five 0.75 ml fractions were collected and selected fractions assayed for RNA polymerase activity in a "cocktail" containing 10 mM $MgCl_2$ and KCl to a final concentration of 100 mM, as described in 2.5.2. The activity of 25 µl aliquots was measured by the incorporation of [^{3}H] UMP into TCA insoluble material over a 15 min incubation period at 25° C. (———). The error bars refer to the standard deviations of duplicate measurements. The transmittance, at 280 nm, of the eluant was recorded using a Uvicord scanner (— • —).

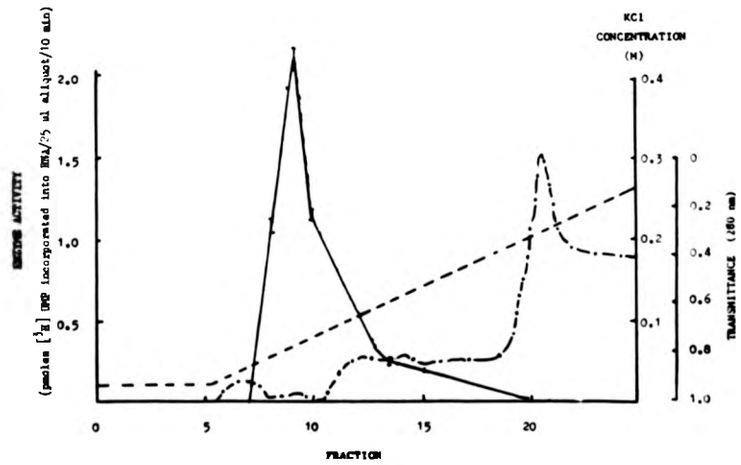
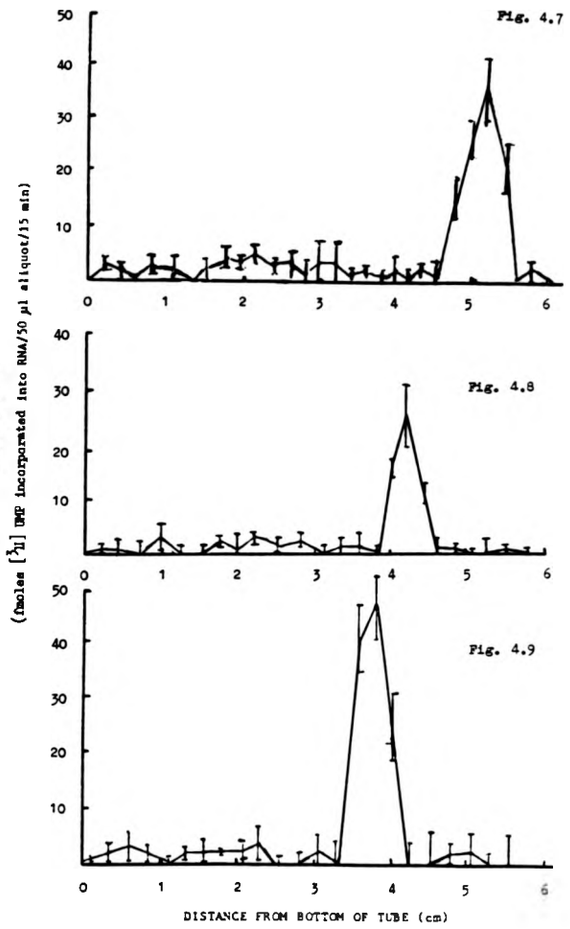


Fig. 4.6 DNA cellulose chromatography of fraction F_5

The dialysed ammonium sulphate precipitate of fraction F_5, containing 11 mg protein, was applied to a DNA cellulose column. The column was washed with 5 ml of Hager's buffer B containing 0.02 M KCl, and eluted with a 20 ml linear gradient of 0.02 - 0.5 M KCl (— — —). Twenty five 0.7 ml fractions were collected and selected fractions assayed for RNA polymerase activity in a "cocktail" containing 10 mM $MgCl_2$ and KCl to a final concentration of 100 mM, as described in 2.5.2. The activity of 25 µl aliquots was measured by the incorporation of [^{3}H] UMP into TCA insoluble material over a 15 min incubation period at 25° C. (———). The error bars refer to the standard deviations of duplicate measurements. The transmittance, at 280 nm, of the eluant was recorded using a Uvicord scanner (— • —).



Figs. 4.7 - 4.9 Glycerol gradient centrifugation of fractions F_1, F_3 and F_5

The ammonium sulphate precipitate of fractions F_1, F_3 and F_5 were layered onto 11 ml 15% - 30% glycerol gradients in Hager's buffer B, containing 0.1 M $(NH_4)_2SO_4$. The gradients were then centrifuged for 19 hr in either a Beckman 50 Ti rotor, at 100,000 g (Fig. 4.7), or Beckman 40 rotor, at 95,000 g (Figs. 4.8 and 4.9). Fractions of 0.5 ml (Fig. 4.7), 0.4 ml (Fig. 4.8) or 0.45 ml (Fig. 4.9) were collected. Duplicate 50 µl aliquots were assayed for RNA polymerase activity in the assay "cocktail" described in 2.5.2. The activity was measured by the incorporation of [^{3}H] UMP into TCA insoluble material over a 15 min incubation period at 25° C. (——). The error bars refer to the standard deviations of duplicate measurements.

Fractions from the glycerol gradient centrifugation, having the highest activity for each of the three RNA polymerases, were analysed as described in 2.8.7. Each of the purified enzymes showed only one band when stained using the technique based on that of Merril et al (258), as described in 2.8.8.

4.3.10 Divalent cation optima for partially purified RNA polymerases

The effect of $MgCl_2$ concentration in an assay "cocktail" on the three RNA polymerases from yeast form C. albicans is shown in Fig. 4.10. Optimal activity, for all three classes of enzyme, was found when the assay medium contained $MgCl_2$ at a concentration of 10 mM.

The effect of $MnCl_2$ concentration on partially purified RNA polymerases in "cocktails" containing either 10 or 100 mM KCl is shown in Figs. 4.11 and 4.12. In a "cocktail" containing 10 mM KCl, optimal RNA polymerase II and III activities were found using a $MnCl_2$ concentration of 1 mM. Optimal RNA polymerase I activity resulted using 0.5 mM $MnCl_2$. Activity of RNA polymerases II and III in an assay "cocktail" containing 100 mM KCl (Fig. 4.12) was optimal for RNA polymerase III at 0.5 mM $MnCl_2$ and optimal for RNA polymerase II at 0.75 mM. In addition, a smaller peak of activity was observed for RNA polymerase II incubating in a "cocktail" containing 1.5 mM $MnCl_2$ and 100 mM KCl.

4.3.11 Salt optima for partially purified RNA polymerases

The effect of KCl concentration on the partially purified RNA polymerases from yeast phase C. albicans, in a "cocktail" containing 10 mM $MgCl_2$, is shown in Fig. 4.13. Optimal activity for RNA polymerases I and II occurred when the KCl concentration was 40 mM and 100 mM respectively. Two peaks of optimal RNA polymerase III activity were found when the assay "cocktail" contained 100 and 240 mM KCl.

4.3.12 α-Amanitin sensitivities of partially purified RNA polymerases

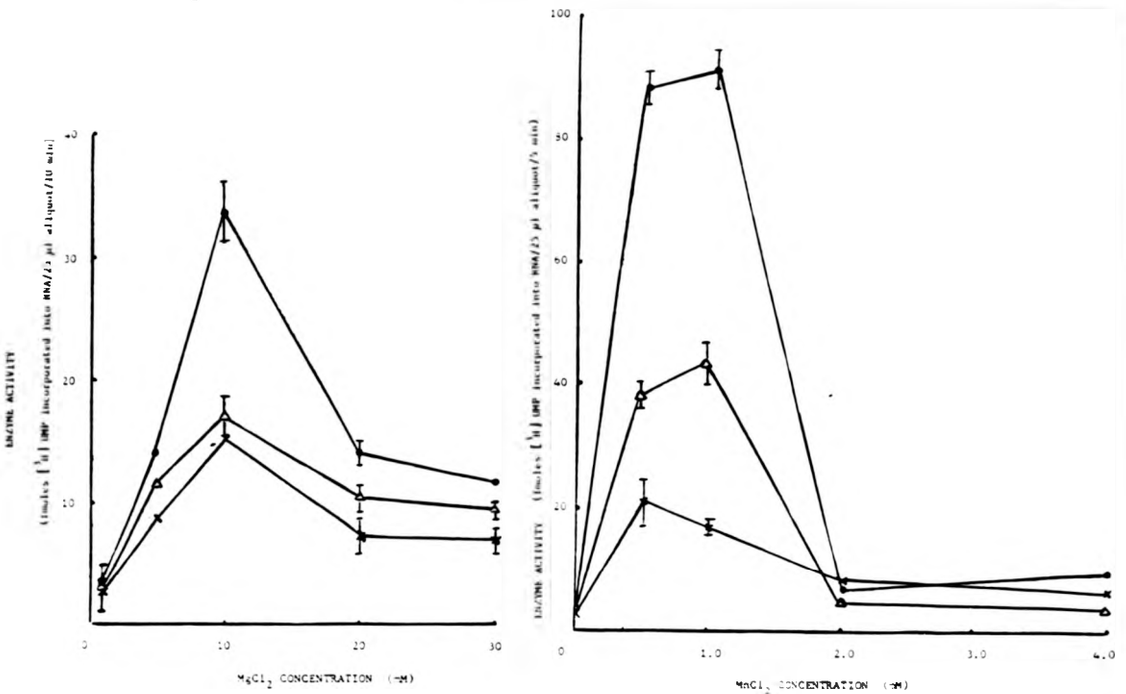
The effect of addition of α-amanitin on the activity of partially purified

Fig. 4.10

Fig. 4.10 Effect of $MgCl_2$ concentration on partially purified RNA polymerases from yeast form C. albicans

The 3 classes of RNA polymerase from yeast form C. albicans were separated by DEAE-Sephadex chromatography, as described in 4.2. . 50 µl aliquots of partially purified RNA polymerase I (containing approximately 6 µg protein —x—), RNA polymerase II (containing approximately 10 µg protein —●—) and RNA polymerase III (containing approximately 70 µg protein —△—) were incubated in 100 µl total volume of assay "cocktail" containing $MgCl_2$ to the final concentrations shown in the figure. The other components of the "cocktail" were as described in 2.5.2. The activity was measured by the incorporation of [^{3}H] UMP into TCA insoluble material over a 15 min incubation period at 25° C. The error bars refer to the standard deviations of duplicate measurements.

Fig. 4.11



Figs. 4.11 and 4.12 Effect of $MnCl_2$ concentration on partially purified RNA polymerases from yeast form C. albicans

The 3 classes of RNA polymerase from yeast form of C. albicans were separated by DEAE-Sephadex chromatography, as described in 4.2. . 50 µl aliquots of partially purified RNA polymerase I (containing approximately 6 µg protein —x—), RNA polymerase II (containing approximately 10 µg protein —●—) and RNA polymerase III (containing approximately 70 µg protein —△—) were incubated in total volume of 100 µl assay "cocktail" containing $MnCl_2$ at the final concentration shown in the figure. The components of the "cocktail" were as described in 2.5.2. section, except KCl was present at final concentrations of 10 mM (Fig. 4.11) or 100 mM (Fig. 4.12). Incorporation of [^{3}H] UMP into TCA insoluble material over a 15 min incubation period at 25° C. was used as a measure of RNA polymerase activity. The error bars refer to the standard deviations of duplicate measurements.

Fig. 4.12

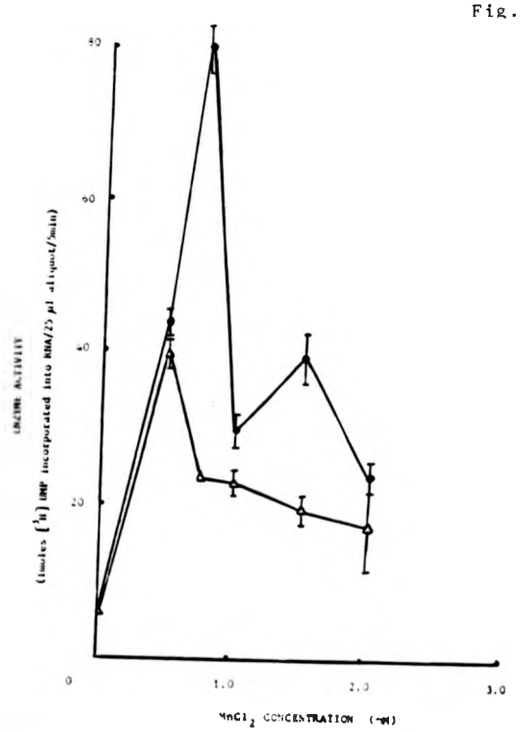


Fig. 4.13

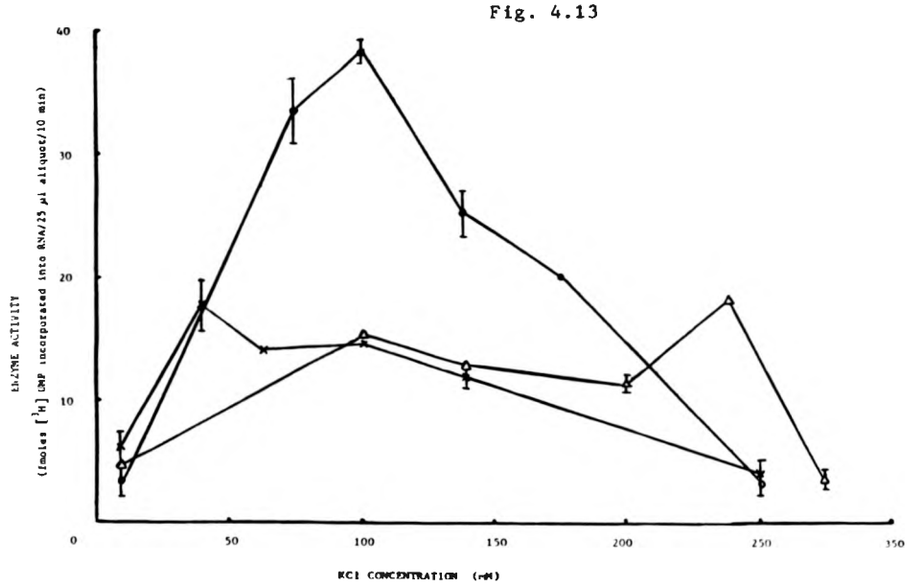


Fig. 4.13 *Effect of KCl concentration on partially purified RNA polymerases from yeast form C. albicans*

The 3 classes of RNA polymerase from yeast form C. albicans were separated by DEAE-Sephadex chromatography, as described in Fig. 4.4. 50 µl aliquots of partially purified RNA polymerase I (containing approximately 6 µg protein —●—), RNA polymerase II (containing approximately 10 µg protein —■—) and RNA polymerase III (containing approximately 70 µg protein —△—) were incubated in total volume of 100 µl assay "cocktail" containing 10 mM $MgCl_2$ and KCl to the final concentrations shown. The other components of the "cocktail" were as described in 4.5.2. section. RNA polymerase activity was measured by the incorporation of [³H] UMP into TCA insoluble material over a 15 min incubation period of 25° C. The error bars refer to the standard deviations for duplicate measurements.

Fig. 4.14

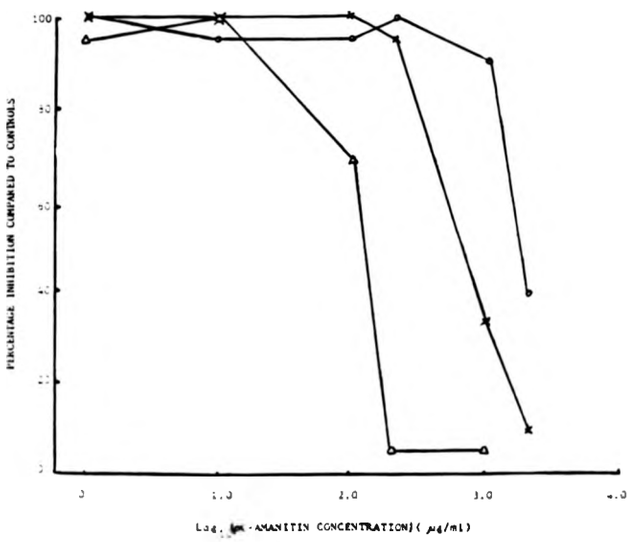


Fig. 4.14 *Effect of α-amanitin concentration on partially purified RNA polymerases from yeast form C. albicans*

The 3 classes of RNA polymerase from yeast form C. albicans were separated by DEAE-Sephadex chromatography, as described in 4.2.4. Duplicate 5 µl aliquots of partially purified RNA polymerase I (containing 17 µg protein —●—), RNA polymerase II (containing 23 µg protein —○—) and RNA polymerase III (containing 180 µg protein —□—) were incubated in a total volume 20 µl assay "cocktail" as described in 4.5.2. α-Amanitin was present in the "cocktail" at the final concentrations indicated in the figure. RNA polymerase activity was measured by the incorporation of [³H] UMP into TCA insoluble material over a 10 min incubation period at 25° C. 100% activity of partially purified RNA polymerases I, II and III was 127, 181 and 118 f moles [³H] UMP incorporated/ 10 µl aliquots/ 10 min incubation at 25° C.

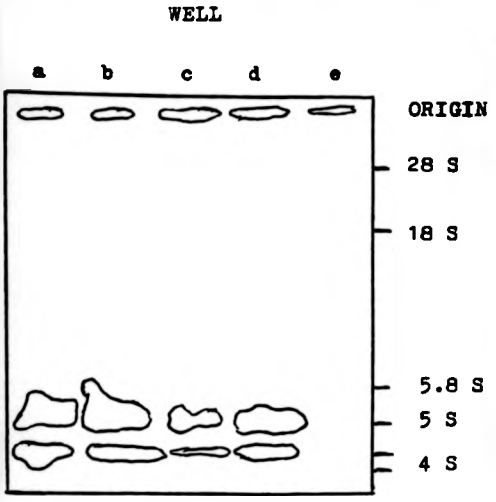


Fig. 4.15 Analysis of RNA synthesised by C. albicans yeast cells, by agarose gel electrophoresis, showing the effects of α-amanitin at 20 and 200 μg/ml

Yeast cells of C. albicans were grown to a cell density corresponding to the stationary phase of growth, harvested and re-suspended in protoplast buffer. The cells were incubated for 10 min at 25° C. prior to the addition of 10 μCi $NaH_2[^{32}P]O_4$ and either 20 μg or 200 μg of α-amanitin per ml yeast suspension. No α-amanitin was added to a control yeast suspension. The three yeast cell suspensions were then incubated at 25° C. for 90 min. The RNA was extracted and DNase treated as described in 2.7.1. and 2.5.4. respectively. The extracted, $[^{32}P]$ labelled RNA was suspended in sample buffer for agarose gel electrophoresis, as described in 2.8.1, to a final volume of 50 μl. The different lanes contained: a) 10 μl and b) 20 μl of $[^{32}P]$ labelled RNA extracted from controls, c) 10 μl and d) 20 μl of $[^{32}P]$ labelled RNA extracted from yeast in the presence of 20 μg α-amanitin/ml, e) 50 μl of $[^{32}P]$ labelled RNA extracted from yeast in the presence of 200 μg α-amanitin/ml. The position migrated by the $[^{32}P]$ labelled RNA was visualised as described in 2.8.9. The position migrated by the 4 S (bromophenol blue) 28 S, 18 S, 5.8 S and 5 S Pisum sativum marker RNA species are shown.

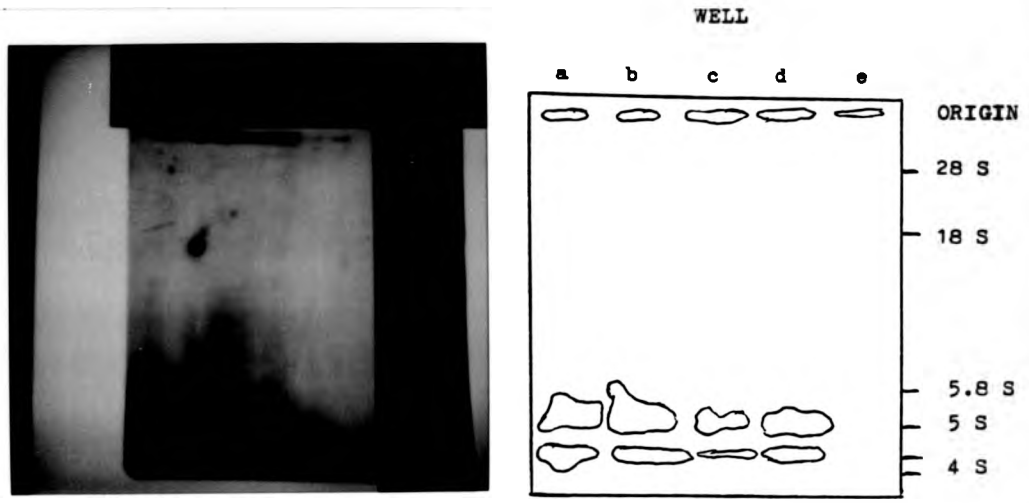


Fig. 4.25 Analysis of RNA synthesised by C. albicans yeast cells, by agarose gel electrophoresis, showing the effects of α-amanitin at 20 and 200 μg/ml

Yeast cells of C. albicans were grown to a cell density corresponding to the stationary phase of growth, harvested and re-suspended in protoplast buffer. The cells were incubated for 10 min at 25° C. prior to the addition of 10 μCi $NaH_2[^{32}P]O_4$ and either 20 μg or 200 μg of α-amanitin per ml yeast suspension. No α-amanitin was added to a control yeast suspension. The three yeast cell suspensions were then incubated at 25° C. for 90 min. The RNA was extracted and DNase treated as described in 2.7.1. and 2.5.4. respectively. The extracted, $[^{32}P]$ labelled RNA was suspended in sample buffer for agarose gel electrophoresis, as described in 2.8.1, to a final volume of 50 μl. The different lanes contained: a) 10 μl and b) 20 μl of $[^{32}P]$ labelled RNA extracted from controls, c) 10 μl and d) 20 μl of $[^{32}P]$ labelled RNA extracted from yeast in the presence of 20 μg α-amanitin/ml, e) 50 μl of $[^{32}P]$ labelled RNA extracted from yeast in the presence of 200 μg α-amanitin/ml. The position migrated by the $[^{32}P]$ labelled RNA was visualised as described in 2.8.9. The position migrated by the 28 S (bromophenol blue) 28 S, 18 S, 5.8 S and 5 S Pisum sativum marker RNA species are shown.

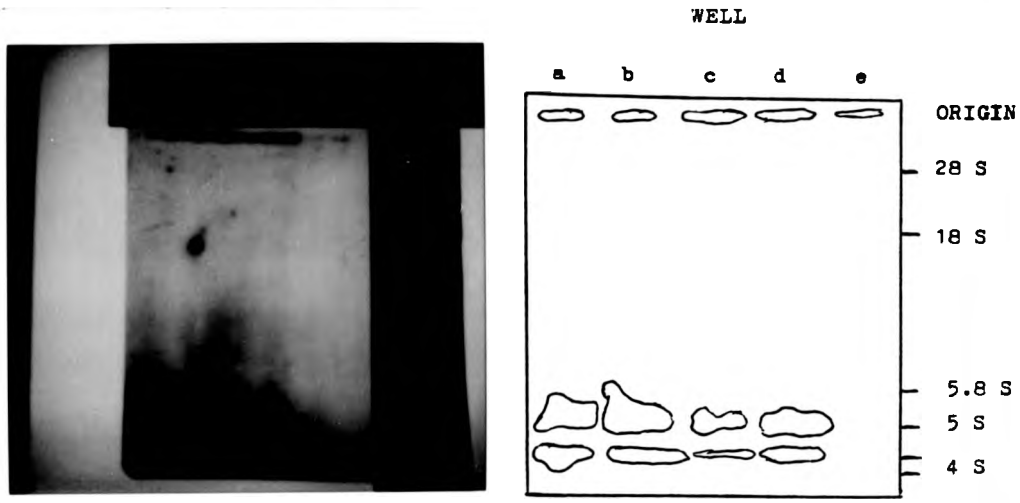


Fig. 4.13 Analysis of RNA synthesised by C. albicans yeast cells, by agarose gel electrophoresis, showing the effects of α-amanitin at 20 and 200 μg/ml

Yeast cells of C. albicans were grown to a cell density corresponding to the stationary phase of growth, harvested and re-suspended in protoplast buffer. The cells were incubated for 10 min at 25° C. prior to the addition of 10 μCi $NaH_2[^{32}P]O_4$ and either 20 μg or 200 μg of α-amanitin per ml yeast suspension. No α-amanitin was added to a control yeast suspension. The three yeast cell suspensions were then incubated at 25° C. for 90 min. The RNA was extracted and DNase treated as described in 2.7.1. and 2.5.4. respectively. The extracted, $[^{32}P]$ labelled RNA was suspended in sample buffer for agarose gel electrophoresis, as described in 2.8.1, to a final volume of 50 μl. The different lanes contained: a) 10 μl and b) 20 μl of $[^{32}P]$ labelled RNA extracted from controls, c) 10 μl and d) 20 μl of $[^{32}P]$ labelled RNA extracted from yeast in the presence of 20 μg α-amanitin/ml, e) 50 μl of $[^{32}P]$ labelled RNA extracted from yeast in the presence of 200 μg α-amanitin/ml. The position migrated by the $[^{32}P]$ labelled RNA was visualised as described in 2.8.9. The position migrated by the 4 S (bromophenol blue) 28 S, 18 S, 5.8 S and 5 S Pisum sativum marker RNA species are shown.

RNA polymerases is shown in Fig. 4.14. At a concentration of 2 mg ∝-amanitin/ml RNA polymerase I was completely inhibited, whereas RNA polymerase II was inhibited 40% by ∝-amanitin at this concentration. RNA polymerase III activity was almost completely inhibited at concentrations of 200 µg/ml.

The effect of ∝-amanitin, at a concentration of 20 and 200 µg/ml, on the RNA species synthesised in vivo by C. albicans, is shown in Fig. 4.15. The [^{32}P] labelled RNA, extracted from yeast cell suspensions containing no ∝-amanitin, has bands migrating to the same positions as the 5 S and 4 S marker species (lanes a and b). Bands due to the presence of [^{32}P] labelled RNA migrating with the mobility of 5 S and 4 S species are evident in lanes c and d. These lanes contained RNA extracted from yeast cells incubated in a suspension containing 20 µg ∝-amanitin/ml. The wells of lanes a and c, and b and d, contained 10 µl and 20 µl of extracted RNA solution respectively. This represented 20% and 40% of the labelled RNA that was extracted from C. albicans incubating in a suspension containing none or 20 µg ∝-amanitin/ml. The well of lane e contained all the RNA that was extracted from yeast cells incubating with 200 µg ∝-amanitin/ml suspension. There are no bands of [^{32}P] labelled RNA migrating with the mobility of 5 S and 4 S species.

4.3.13 Apparent Km for CTP for yeast form partially purified RNA polymerases

The affinity of the partially purified RNA polymerases for one of the nucleotide substrates was investigated. The apparent Km values for the three classes of enzyme was estimated using the Eadie-Hofstee method (Figs. 4.16, 4.17 and 4.18 for RNA polymerases I, II and III respectively). The Km values were estimated using regression lines of "enzyme activity/CTP concentration" on "enzyme activity". These revealed Km values for 0.01 mM, 0.02 mM and 0.06 mM for RNA polymerases I, II and III respectively. The Pearson coefficient of correlation values for these three regression lines of -0.68, -0.73 and -0.84 for RNA polymerases I, II and III, indicated that there was high negative correlation between "enzyme activity" and "enzyme activity/CTP concentration".

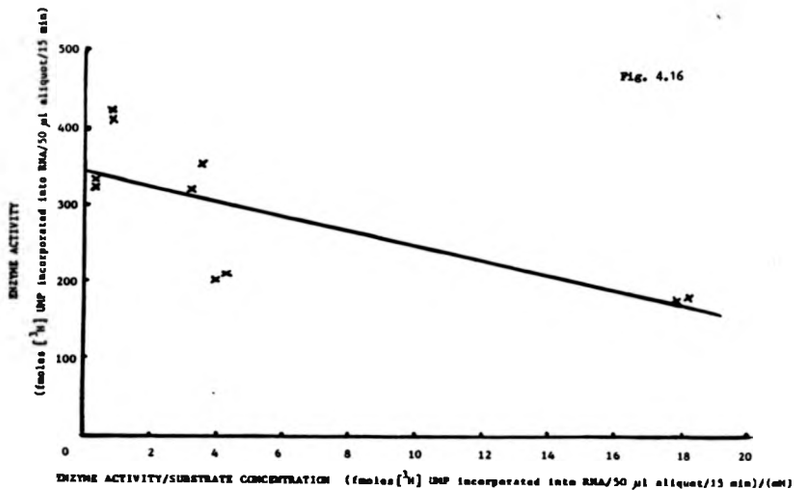


Fig. 4.16

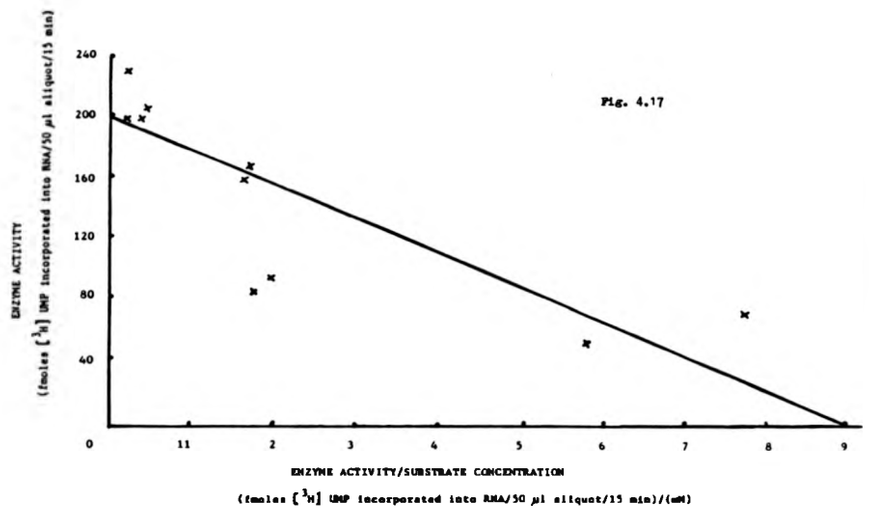


Fig. 4.17

Figs. 4.16 - 4.18 Apparent Km values for CTP of partially purified RNA polymerases from yeast form C. albicans

The 3 classes of RNA polymerase from yeast form C. albicans were partially purified, as described in 4.2.4. Duplicate 25 µl aliquots of RNA polymerase I (containing approximately 6 µg protein) (Fig. 4.16), RNA polymerase II (containing approximately 10 µg protein) (Fig. 4.17) and RNA polymerase III (containing approximately 70 µg protein) (Fig. 4.18. were incubated in 100 µl total volume of assay "cocktail" were as described in 2.9.2. section, with the exception that "cold" UTP was present at a concentration of 0.01 mM and CTP at final concentration of 1 mM, 0.5 mM, 0.1 mM, 0.05 mM and 0.01 mM. RNA polymerase activity was measured by the incorporation of [3H] UMP into TCA insoluble material over a 15 min incubation period at 25° C.. The figures show the Eadie Hofstee plot method used to determine the Km. Regression lines were determined using the statistical methods described in 2.9.1.

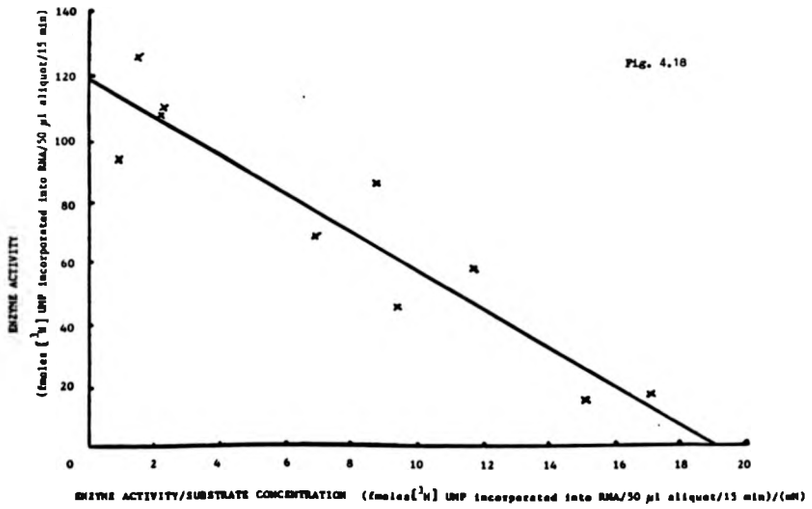


Fig. 4.18

4.3.14 Partial purification of mycelial form. RNA polymerase

Essentially similar procedures were used to partially purify RNA polymerase from mycelial form C. albicans. As noted in section 4.3.1, Table 4.2 shows the total activity, protein and specific activity of the RNA polymerase to the stage after gel filtration.

4.3.15 Gel filtration of mycelial form RNA polymerase

The fraction F_2AS precipitate was layered onto an agarose A-5 M column as described in 2.8.3. Fig. 4.19 shows a typical result obtained of gel filtration of the F_2AS precipitate fraction. Optimal RNA polymerase activity, assayed as described in the legend of Fig. 4.19, was found in fractions eluting after the void volume. This implied the polymerase had a slightly smaller molecular weight than the exclusion molecular weight of 5×10^6.

4.3.16 Ion exchange chromatography of mycelial form RNA polymerase

The ammoniun sulphate precipitate, derived from Fraction F_3, was dialysed as described in 2.8.4. Fig. 4.20 shows the result obtained when this fraction was chromatographed on DEAE-sephadex. One peak, of low activity, was found eluting from the column when the salt concentration of the buffer was 0.225 - 0.25 M KCl.

4.4 Discussion

4.4.1 Purification of RNA polymerases from yeast form C. albicans

The three RNA polymerases from yeast form C. albicans were purified to electrophoretic homogeneity for the first time. The combination of techniques used to achieve this had previously been successfully employed to purify RNA polymerases from S. cerevisiae and H. capsulatum (see 4.1). The enzyme activity at each stage of the purification (see Tables 4.1 and 4.2).

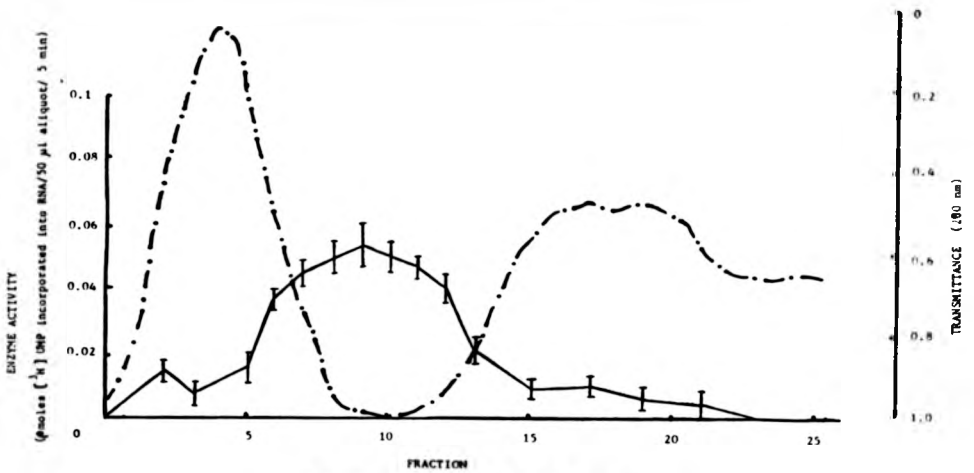


Fig. 4.19 Gel filtration of fraction F_2 from mycelial form C. albicans

The ammonium sulphate precipitate of fraction F_2, containing 30 mg protein/ml, was applied to an agarose A-5 M column. The column was then eluted with Hager's buffer A containing 0.1 M $(NH_4)_2SO_4$. The transmittance, at 280 nm, of the eluent (—•—) was recorded using a Uvicord scanner. Twenty two 5 ml fractions were collected. Selected fractions were assayed for RNA polymerase activity in a "cocktail" containing 10 mM $MgCl_2$ and 100 mM KCl, as described in 2.5.2. The activity of duplicate 50 ul aliquots was measured by incorporation of [^{3}H] UMP into TCA insoluble material over a 5 min incubation period at 25° C. (——). The error bars refer to the standard deviations of duplicate measurements.

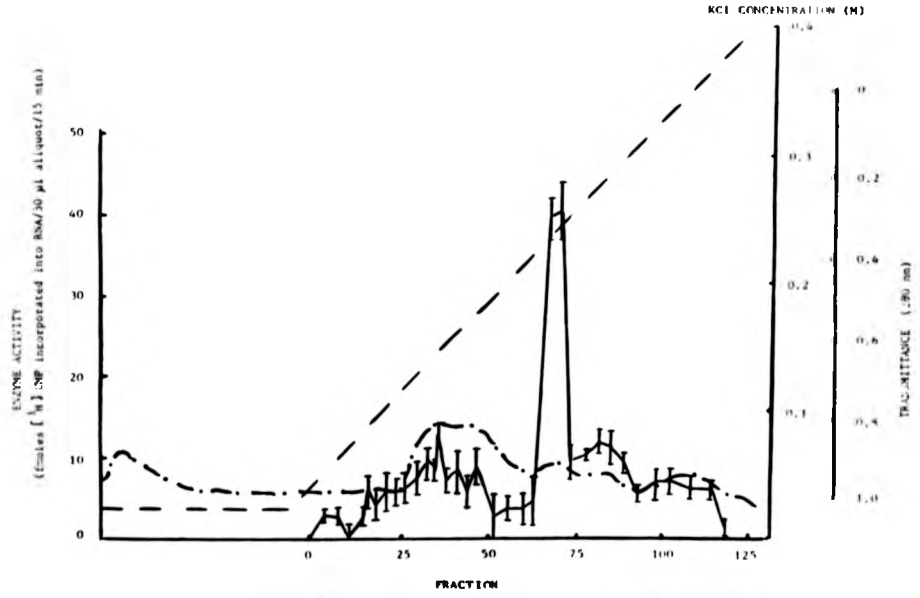


Fig. 4.20 Ion exchange chromatography of fraction F_3 from mycelial form C. albicans

The ammonium sulphate precipitate of fraction F_3^*, containing 40 mg protein (dialysed as described in 4.2.3$_g$), was applied to a DEAE-Sephadex A-25 column. The column was washed with Hager's buffer B, containing 0.02 M KCl, followed by elution with a 100 ml gradient of 0.02 - 0.4 M KCl (- - -). One hundred and fifty 0.5 ml fractions were collected. Selected fractions were assayed for RNA polymerase activity in a "cocktail" containing 10 mM $MgCl_2$ and KCl at a concentration of 100 mM, as described in 2.5.2. The activity of duplicate 50 µl aliquots was measured by the incorporation of [^{3}H] UMP into TCA insoluble material over a 15 min incubation period at 25° C. (——). The error bars refer to the standard deviations of duplicate measurements. The transmittance of the eluent was recorded using a Uvicord scanner (—•—).

was measured using different assay conditions to those found to give optimal activity *in vitro* in nuclei (3.3.7). Higher levels of [^{14}C] UMP incorporation into RNA were found when nuclei were incubated in a "cocktail" containing 100 mM compared to 10 mM $MgCl_2$. Cell-free extracts, prepared from either morphological form of *C. albicans*, showed optimal *in vitro* RNA polymerase activity at a $MgCl_2$ concentration of 10 mM (Fig. 4.1). This finding is in agreement with other workers, with $MgCl_2$ concentrations of 3 - 20 mM required for optimal *in vitro* activity in RNA polymerases, from a wide range of prokaryotic and eukaryotic sources (310).

It is almost axiomatic to state that the first step in the purification of any nuclear protein from such an organism as *C. albicans* requires methods to disrupt the fungal cell wall and membrane. The results shown in Table 4.3 indicate that the most suitable method for disruption of *C. albicans* cells was that described by Kumar *et al* (203) i.e. homogenisation with ballotini. Although compression by 90 - 100 Nmm^{-2} is believed to result in disruption of *S. cerevisiae* (105), it is evident that greater pressure than that used was required to disrupt *C. albicans* cells. Thus, cell-free homogenates of *C. albicans* were prepared by 3 min treatment with ballotini. Homogenisation for longer lengths of time resulted in less recoverable RNA polymerase activity. This presumably arose as a consequence of heating the homogenate by friction of the ballotini on shaking and the disruptive influence of proteases. (Although a jet of CO_2 was used to cool the apparatus, some skill was required to prevent the homogenate freezing or overheating).

Proteases and RNases are two enzymes which interfere, in the initial stages, with the RNA polymerase and hence the measurement of enzyme activity. The action of proteases should be minimised by the addition of PMSF to the buffer (PMSF is a potent inhibitor of serine proteases, but it is believed to interfere with transcription - see 3.4.3). The addition of calf thymus RNA to the "cocktail" used to measure RNA polymerase activity, resulted in an increase in the amount of label incorporated into RNA, over an incubation

period of 5 min (results not shown). However, this practice was abandoned when it was realised that, especially in cruder preparations of commercially available RNA, other ill-defined contaminants would be present. A probable example of the destructive effects of the proteases on the enzyme activity is illustrated in Fig. 4.2. This figure revealed that RNA polymerase activity in the homogenate, kept at 25° C. for 90 min, was negligible, and reduced by half when kept at 0 - 4° C. for the same period. The addition of bovine serum albumin to the homogenate also appeared to enhance the stability of RNA polymerase activity. Gel filtration presumably removed the destructive protease and RNase enzymes. Generally these proteins have much lower molecular weights than the RNA polymerase containing fractions - typical molecular weights of RNases and serine proteases are 15,000 to 30,000 (219,245). Thus, the initial steps in the purification, i.e. from the preparation of homogenate to gel filtration, needed to be carried out as quickly as possible.

RNA polymerases represent a small percentage of the total protein of a cell. Approximately 8 - 15 mg of RNA polymerase I, 20 mg of RNA polymerase II and 3 - 10 mg of RNA polymerase III may be purified from 1 Kg of yeast cells of S. cerevisiae (see 1.3.3). As a comparison, the results shown in Table 4.1 indicate 3.5 mg, 0.75 mg and 1.0 mg of RNA polymerases I, II and III respectively would be purified from 1 Kg (wet weight) of C. albicans yeast cells. Thus, obtaining sufficient quantities of RNA polymerase for characterisation experiments, and other studies, on the enzyme required cultivation of large amounts of the organism.

An attempt was made to optimise the yield of total RNA polymerase activity available from C. albicans growing in a given volume of growth media. Homogenates prepared from yeast cells in the late exponential phase of growth (1×10^8 cells/ml medium) had the greatest amount of total RNA polymerase activity of cells harvested from 1 L of medium (Table 4.4). Measurement of the specific activity of the RNA polymerases present in crude homogenate prepared from the early (2×10^7 cells/ml), mid (5×10^7 cells/ml) and late (1×10^8 cells/ml) exponential phase cells were not significantly different

from one another. This could indicate that there were similar levels of total (i.e. "bound" and "free") RNA polymerase activity, per unit cell, during the growth period. In contrast, the specific activity of RNA polymerase in crude homogenate prepared from yeast cells in the stationary phase of growth.(i.e. 5 x 10^8 cells/ml) was much lower. This finding indicates that, during the stationary phase of growth, RNA polymerase activity per unit cell, was lower than that of growing cells. This confirms the results shown earlier in this thesis (see 3.3.2). It was reported that, in vivo, a greater level of RNA synthesis, per unit cell, was found in exponential phase cells, compared to those in the stationary phase of growth.

The lower specific activity in homogenate prepared from yeast cells in the stationary phase of growth could be accounted for by three factors. The lower specific activity may reflect lower levels of RNA polymerase enzymes per cell. Another possibility was that the assay conditions used to measure RNA synthesis did not measure optimal RNA polymerase activity in homogenates prepared from stationary phase cells. The third reason could be that in the stationary phase of growth specific classes of RNA polymerase could be inactivated (such a phenomenon is not unknown and was briefly commented on in the introduction of this chapter).

An interesting finding reported in this chapter was that the specific activities of RNA polymerases in crude homogenate, prepared from mycelia and exponential phase yeast cells, were comparable (i.e. 0.22 units/mg protein and 0.18 units/mg protein for mycelia and exponential phase yeast cells respectively - see Tables 4.1 and 4.2 and Fig. 4.1). This indicates that the activity of the enzyme, as a proportion of total protein, was similar in mycelia to that in yeast cells growing in exponential phase. This statement, of course, assumes that in the homogenate a given quantity of RNA polymerase directs the synthesis of a given quantity of labelled RNA. (It should be noted that levels of proteases and RNases which may interfere with the perceived levels of RNA polymerase activity, may be different in homogenates prepared from exponential phase yeast cells and mycelial cells)

Sonication of homogenate, in a high salt environment is now a favoured technique for the large scale purification of RNA polymerases. This is believed to result in dissociation of the RNA polymerase(s) from the DNA template. Measurement of the extent of "bound" and "free" enzyme, before and after this treatment, was measured as described by Yu (414). The rationale behind the assay was that the RNA polymerase activity that could be measured in the homogenate and/or sonicate using endogenous DNA template, represents the bound form of the enzyme. The activity in the presence of a defined template (i.e. poly [d(A-T)]) represented the combined activity of both "bound" and "free" forms of the enzyme. A measure of only the "free" form of the enzyme in the homogenate/sonicate was provided by inhibition of the "bound" RNA polymerase using actinomycin D. This compound intercalates with the DNA in G-C rich regions (284). RNA polymerase activity, which was poly [d(A-T)] template directed, would thus be unaffected. Table 4.5 shows that the addition of the drug to homogenate/sonicate transcribing endogenous DNA resulted in 75 - 80% inhibition compared to that untreated control. C. albicans has a G-C content of the DNA of 35% (362). The low level of activity found when actinomycin D was added to either homogenate or sonicate probably represented transcription of A-T rich regions of DNA.

The proportion of "bound" enzyme did not markedly change after sonication (Table 4.5). However, "bound" RNA polymerase activity was much lower after high salt sonication than prior to treatment. This finding could be accounted for by two explanations. Firstly, loss of RNA polymerase activity could arise as either protease activity or through heating as a direct consequence of sonication. Secondly, there could be less affinity for the exogenous template shown by the previously "bound" enzyme. One of the assumptions made for the "bound" and "free" assay was that both forms of the RNA polymerase had equal affinity for endogenous template and poly [d(A-T)]. The high salt conditions employed to dissociate the RNA polymerase from the endogenous DNA may not have allowed the enzyme to bind to the artificial template. Thus, the RNA polymerase dissociated from the endogenous DNA by high salt sonication

may not be measured in an assay due to failure to bind and re-initiate to the same extent on the poly [d(A-T)].

After sonication the homogenate was centrifuged to precipitate cell debris and unlysed cells. Although the bulk of RNA polymerase activity was found in the supernatant (i.e. fraction F_1), a small amount of activity was found in association with the precipitate (i.e. F_1 ppt). This probably represented mitochondrial RNA polymerase and chromatin associated RNA polymerase that had not been dissociated by high salt sonication. Mitochondrial RNA polymerases are very tightly associated with membrane bound DNA. (Solubilisation of these complexes often requires treatment of the homogenate with a detergent (e.g. Triton X-100) with metal chelators(e.g. EDTA), high salt concentrations and, in some cases, DNase (215,333). As a consequence, one may expect the centrifugation step to remove most of the mitochondrial RNA polymerases.

Nucleic acid was precipitated by the addition of the polyamine, protamine sulphate. It has been reported that $(NH_4)_2SO_4$ concentration is critical in this stage of the purification for efficient recovery of the RNA polymerases. Below a concentration of 0.15 M Hager et al (141) reported that RNA polymerase and DNA re-associated. This reduced the RNA polymerase recoverable after the protamine sulphate precipitation step. At concentrations greater than 0.16 M, the insolubility of the protamine sulphate led to entrapment of RNA polymerase molecules (141). After this stage only template-directed RNA synthesis could be measured, i.e. the assay "cocktail" required the addition of exogenous DNA for measurable incorporation of label into TCA insoluble material.

The gel filtration step (see 4.3.4) resulted in the separation of the high molecular weight RNA polymerases from other components of the cell with different molecular weights. An analysis of scans made of UV transmittance indicated that most RNA polymerase activity was associated with those relatively few proteins of high molecular weights - slightly smaller than the exclusion molecular weight. This stage resulted in a fifty five fold increase in the specific activity of the RNA polymerase (i.e. from 0.02 to 1.15 units/mg protein). A small amount of RNA polymerase activity was associated with

fractions eluting in the void volume. This may have represented small amounts of enzyme re-associating with the residual level of nucleic acid. After this stage the RNA polymerases were a good deal more stable than in the earlier stages of purification. Evidence for this is shown in the stability of the enzymes during long periods of dialysis when little loss of activity was observed.

Resolution of multiple nuclear RNA polymerases into three classes was achieved by ion exchange chromatography (see Fig. 4.4). The three peaks of RNA polymerase activity eluted from DEAE-sephadex were termed RNA polymerases I, II and III according to the procedure of Roeder and Rutter (311) and Blatti et al (33). The three peaks of activity were eluted when the buffer contained KCl concentrations of 60 - 90, 90 - 200 and 240 - 290 mM for RNA polymerases I, II and III respectively. These values are similar to those reported for RNA polymerases from a wide variety of plants, animals and lower eukaryotes. If DEAE-sephadex is used to resolve RNA polymerases I, II and III from other eukaryotes, they are eluted from the column when the KCl concentration is 0.1 - 0.15 M, 0.17 to 0.25 M and 0.2 - 0.35 M respectively (138). Although three peaks of RNA polymerase activity were found, complete resolution was not achieved using the methods described in the legend of Fig. 4.5. Re-chromatographing fractions showing activity, after dialysis, on DEAE-sephadex, using a less steep KCl gradient, resulted in negligible RNA polymerase activity eluting from the column. After ion exchange chromatography most (64%) of the activity was RNA polymerase II associated with smaller amounts (34% and 2%) due to RNA polymerases I and III respectively (based on proportion of total activity after ion exchange chromatography).

Affinity chromatography was the next stage in the purification of RNA polymerases. This involved further purification of the RNA polymerases based on their affinity for DNA. The affinity shown by each isozyme was established as described in Table 4.6. Washing the bound RNA polymerase with buffer containing increasing KCl concentration resulted in the "elution" of polymerases I and II at fairly low KCl concentrations. In contrast, RNA polymerase

III was very tightly bound, eluting only when the buffer contained a KCl concentration of 2.0 M. Hager et al (141) reported that S. cerevisiae RNA polymerase III also appeared to bind very tightly to DNA cellulose. However, this group reported that this isozyme was eluted at a much lower KCl concentration of 0.45 M. This tight binding is thought to be a characteristic of the class III isozyme that is reflected in the resistance of the enzyme to high salt inhibition of activity, measured on native DNA (138).

When RNA polymerases I and II (Fractions F_4 and F_5) were chromatographed on DNA cellulose, the relative position of elution for the enzymes was confirmed by α-amanitin sensitivity of fractions. It was found that α-amanitin resistance - at a concentration of 1 mg/ml - was associated with lead fractions (results not shown). (Surprisingly - see 4.4.2.- α-amanitin insensitivity to this concentration of amatoxin was associated with RNA polymerase II). This indicated that RNA polymerase II was eluted from the DNA cellulose prior to RNA polymerase I. Figs. 4.5 and 4.6 show RNA polymerases I and II were eluted from DNA cellulose when the buffer contained 0.1 - 0.2 and 0.05 - 0.1 M KCl respectively. Hager et al have also commented upon a similar finding for RNA polymerases from S. cerevisiae (141). This group reported that DNA cellulose chromatography resulted in two peaks of RNA polymerase activity. The second peak - as earlier mentioned - consisted of RNA polymerase III. The leading fractions of the first peak were enriched with RNA polymerase II, whilst the latter were mostly RNA polymerase I. RNA polymerases I and II from C. albicans seen to resemble those of S. cerevisiae in their elution from DNA cellulose.

The final stage in the purification of the three RNA polymerases from yeast form C. albicans. to electrophoretic homogeneity, was glycerol gradient centrifugation (see Figs. 4.7, 4.8 and 4.9). As noted in 4.3.1, the specific activities of RNA polymerases I and II fell drastically after this step (i.e. from 7.52 to 0.68 units mg protein^{-1} and 15.61 to 1.17 units mg protein^{-1} for specific activities of RNA polymerases I and II respectively). Although the specific activity of RNA polymerase III did increase slightly (i.e. from 0.32 to

0.75 units mg protein^{-1}) measurable total activity had fallen dramatically after this purification step (i.e. from 110 units to 0.32 units). Kumar et al (203) reported that the RNA polymerases from H. capsulatum were very labile after the last step of purification, RNA polymerase I being the most labile. In addition, the low specific activities reported may have occurred on account of the high glycerol concentrations in the incubation medium. High glycerol concentrations (as much as 30% (w/v) in some assays shown in Figs. 4.7, 4.8 and 4.9) have been found to affect in vitro RNA polymerase activity (251). However, this stage resulted in the purification of the three RNA polymerases to electrophoretic homogeneity as judged by single bands migrating by non-denaturing polacrylamide gel electrophoresis. The protein bands were stained using the very sensitive technique of Merril et al as only between 1 and 2 µg of protein were applied to each tube gel. In addition, fractions either side of the peaks of RNA polymerase activity, after the glycerol gradient centrifugation step, had similar specific activities - indicating that the peaks of activity corresponded to single proteins.

4.4.2 Characterisation of RNA polymerases from yeast form C. albicans

In order to catalyse the in vitro synthesis of RNA, eukaryotic RNA polymerases show absolute requirement for a DNA template, the four ribonucleoside triphosphates and a divalent cation. Most reports use Mg^{++} or Mn^{++} salt as the divalent cation source. (The requirement for these metals is believed to reflect binding of the cation to β and δ phosphates of the nucleotide during the initiation and elongation reaction (25) and see 1.3.4) The choice of divalent cation has been shown to affect the fidelity of RNA synthesis by all three classes of RNA polymerase. Sony and Hunt reported that the length of RNA transcribed by RNA polymerases I and II, from rabbit bone marrow erythroid cells, was shorter when Mg^{++} was used in preference to Mn^{++} (357). It has also been reported that RNA polymerase III directed synthesis of 5 S rRNA in vitro results in different lengths of transcript, depending upon the choice of divalent cation (2). The divalent cation may also influence the structure

of the DNA. It has been reported (306) that the conversion of B DNA to Z DNA may be influenced by the concentrations of divalent cations Mn^{++} and Mg^{++} in the buffer. Other groups have also noted that the stability of nucleic acids and the stacking of the bases may be affected by these divalent cations (e.g. 57,226). In addition to binding Mn^{++} at the elongation site, it has been reported (198) that prokaryotic RNA polymerases have binding sites for divalent cations. These may play a role in stabilising the protein. As such, eukaryotic RNA polymerases almost certainly would have similar binding sites.

There have been some reports that other divalent cations may be used in the assay "cocktail" to support RNA synthesis by RNA polymerases. Vaisius and Horgen (382) found that RNA polymerase II from calf thymus and the mushroom Agaricus bisporus may use Co^{++} as well as Mn^{++} and Mg^{++} as activating divalent cations. In an in vitro system such as T. pyriformis nucleoli it was recently reported that Co^{++} may support transcription of chromatin (181). These divalent cations, in addition with Cd^{++} and Zn^{++} (10), have also been reported to be capable of supporting transcription of poly [d(A-T)] by E. coli RNA polymerase.

The optimum divalent cation concentrations for eukaryotic RNA polymerases are generally in the range of 5 - 10 mM for Mg^{++} and 1.0 - 2.0 mM for Mn^{++} (138). The partially purified RNA polymerases from yeast form C. albicans all had optima of 10 mM for $MgCl_2$ (see Fig. 4.10). In "cocktails" containing 10 mM KCl, optimal concentrations of Mn^{++} of 0.5, 0.75 and 0.75 mM were found for RNA polymerases I, II and III respectively (see Fig. 4.11). At higher KCl concentrations (100 mM) RNA polymerases II and III had optima for Mn^{++} of 0.75 and 0.5 mM respectively (see Fig. 4.12). It should be noted that higher rates of in vitro activity - as measured by levels of [^{3}H] UMP incorporated into RNA over 15 min at 25^{o} C. - were found when the RNA polymerases were incubated in "cocktails" containing Mn^{++} compared to Mg^{++}. This result is similar to that reported by Adman et al for RNA polymerases from S. cerevisiae (3). The divalent cation present, and the ionic strength, are thus important factors in determining the activity of the RNA polymerase isozymes

It has been suggested that the higher levels of RNA polymerase activity in the presence of Mn^{++} compared to Mg^{++} are due to Mn^{++} stimulating initiation to a greater extent than Mg^{++} (269). This group reported that Mn^{++} preferentially activated the initiation step of RNA polymerase I from Ehrlich ascites tumour cells whilst Mn^{++} or Mg^{++} was necessary for the elongation step. The inhibition by high concentrations of Mn^{++} is thought to be due to inhibition of chain elongation.

In the presence of a divalent cation such as $MgCl_2$, purified RNA polymerase II from eukaryotic sources initiates non-specific RNA synthesis with low efficiency (104). The purified enzyme lacks one or more factors required for promoter recognition and efficient formation of an "open" complex with the DNA (see 1.3.4). In the absence of these factors, RNA polymerase initiation of RNA synthesis may occur preferentially at "nicks" (216) and at single stranded regions (217). Supercoiled DNA may also provide a good substrate for RNA polymerase II (214) (Torsional strain in the molecule is believed to facilitate "open complex" formation by the enzyme). As earlier noted, the presence of Mn^{++} enables non-specific initiation to occur. This may be enhanced by the presence of a salt such as $(NH_4)_2SO_4$ (231).

In addition to the requirement for a divalent cation, RNA polymerase activity is stimulated by inclusion of a monovalent ion. This is supplied in the form of salts such as $(NH_4)_2SO_4$, KCl and NaCl. The effect of inclusion of KCl in the assay "cocktail" on activity of RNA polymerases I, II and III from yeast form C. albicans is shown in Fig. 4.13. RNA polymerases I and II showed optimal activity when the KCl concentration was 40 mM and 100 mM respectively. In contrast, RNA polymerase III showed a biphasic optima at 100 mM and 240 mM. The RNA polymerases were assayed using heat denatured calf thymus DNA as a template. RNA polymerases I, II and III, purified from S. cerevisiae, displayed optimal activity at 0.03 - 0.05 M, 0.05 - 0.1 M and a biphasic optima of 0.04 - 0.1 and 0.18 - 0.3 M salt concentration for the three enzymes respectively, when assayed using native DNA templates. The effects of ionic composition on RNA polymerase activity on

naked DNA is markedly different from that on chromatin or in nuclei. The latter cases require higher salt concentrations to achieve optimum RNA polymerase activity. This is believed to be a property of the protein associated with the DNA template - possibly as a consequence of the chromatin "opening up" at such high salt concentrations (210) (see also 3.4.3).

The salt concentration of the assay "cocktail", like that of the divalent cation present, has been shown to modify transcription (411). The salt concentration and choice of template for RNA polymerase has been used as a method for quantitating the levels of the three classes of "free" polymerase enzyme (316). This group reported an assay specific for RNA polymerase II from S. cerevisiae based on that enzyme's ability, in the presence of $MnCl_2$, to transcribe the ribohomopolymer poly[r(C)]. The levels of RNA polymerases I and II could be distinguished by transcription of poly [d(I-C)], using Mg^{++} as the divalent cation. RNA polymerase III directed transcription of poly [d(I-C)] occurs solely at high ionic strength (0.1 M $(NH_4)_2SO_4$). This assay is thus able to discriminate between, and measure, the relative levels of each class of "free" RNA polymerases. Possibly the most well known method for discrimination between total activities of each class of RNA polymerase is by each isozyme's sensitivity to the fungal toxin α-amanitin.

It is accepted that, usually, the three classes of eukaryotic RNA polymerase exhibit differential sensitivities to α-amanitin. This bicyclic octapeptide interacts directly with the RNA polymerase, inhibiting elongation of the RNA chain (397). In general, animal, plant and lower eukaryotes show similar sensitivities to inhibition. RNA polymerase II from sources such as mice (310), wheat (179) and Dictyostelium discoidium (292) are inhibited by very low concentrations of α-amanitin (i.e. in the range 0.01 - 0.1 μg/ml). It has been found that the site of very strong binding is the large subunit of RNA polymerase II - an analogue of which is present in all the isozymes found (397). RNA polymerase I is usually not inhibited even by α-amanitin concentrations as high as 1 mg/ml, certainly when isolated from X. laevis (310), Rye (179) and Bombyx mori (silkworm) (310). Eukaryote RNA polymerase III is usuall

inhibited by "intermediate" concentrations of α-amanitin. Typical concentrations for total inhibition of this class of enzyme from calf thymus (395). Triticum aestivum (wheatgerm) (179) and Acanthamoeba castellani (91) all lie within the range of 5 - 20 μg/ml.

In contrast to the general patterns of α-amanitin sensitivities found for animal, plant and most lower eukaryotes, fungal RNA polymerases I, II and III display a number of uncharacteristic sensitivities to the toxin. For example, concentrations required for complete inhibition of S. cerevisiae RNA polymerase II of 1 μg/ml are ten fold higher than those for this isozyme from other eukaryotes (332). However, the main difference lies in the sensitivity of RNA polymerases I and III, from this organism, to α-amanitin. These isozymes are respectively inhibited by "intermediary" (i.e. 100 - 300 μg/ml) and "high" (i.e. greater than 1 mg/ml) concentration of the fungal toxin (331). Such unusual findings are common in fungi. Unsurprisingly, RNA polymerases from basidiomycetes that are able to synthesise α-amanitin (e.g. Amanita suballiacea and A. hygroscopica) are inhibited only by very high concentrations of the drug. The RNA polymerase activities are 190 - 340 fold more resistant than those of S. cerevisiae (182).

The α-amanitin sensitivities of RNA polymerases purified from fungi appear to be the most unusual so far studied. RNA polymerases I and II from Aspergillus nidulans were not inhibited by α-amanitin concentrations of 400 μg/ml (366,367) RNA polymerases from both phases of H. capsulatum, another dimorphic fungus , have been reported to have unusual α-amanitin sensitivities (203). Yeast form RNA polymerase III was the most sensitive isozyme to the drug, requiring 1 μg/ml for 50% inhibition. In contrast, RNA polymerase II was the most insensitive class requiring 1 mg/ml for 50% inhibition. RNA polymerase I required "intermediary" concentrations (i.e. 16 μg/ml) for 50% inhibition. It was found that the mycelial phase RNA polymerases were ten fold less sensitive to α-amanitin than the corresponding yeast form enzymes. The α-amanitin sensitivities of RNA polymerases from yeast form C. albicans reported here are similar to the "unusual" findings for

other deuteromycetes (i.e. RNA polymerases I and II requiring 1 and 2 mg α-amanitin/ml respectively for 50% inhibition with RNA polymerase III inhibited at concentrations of 200 μg/ml) (see Fig. 4.14).

Evidence that α-amanitin, at a concentration of 200 μg/ml, affects RNA polymerase III directed transcription, in vivo, was provided in Fig. 4.15. It is apparent that, at the highest concentration of α-amanitin tested, no 5 S and 4 S RNA species were synthesised by C. albicans. These are the transcripts synthesised by RNA polymerase III in eukaryote cells. Stationary phase yeast form C. albicans were chosen for this experiment as it is believed most RNA, synthesised by quiescent S. cerevisiae, is mostly 5 S rRNA and tRNA (see 273). The overall level of RNA synthesis is very low in stationary phase C. albicans. Thus, in order to obtain adequate amounts of [^{32}P] labelled RNA, for agarose gel electrophoresis, the yeast cells were incubated for a long period of time (90 min) at 25^{o} C.

A measure of the affinity of any enzyme for it's substrate may be given by the apparent Michaelis constant for that substrate (Km). The apparent Km for CTP of yeast form RNA polymerases I, II and III were found using the Eadie-Hofstee method (see Figs. 4.16, 4.17 and 4.18). Enzyme activity of the RNA polymerase was measured in a "cocktail" containing the substrates 0.5 mM ATP and GTP, 0.9 μM [^{3}H] UTP and 0.01 mM "cold" UTP. It has been reported (187) that RNA polymerases have Km for substrate nucleotides in the range of 0.01 - 0.08 mM. The levels of UTP in the assay "cocktail" were thus limiting. Low concentrations of UTP were used as dilution of the radio-labelled UTP with "cold" UTP resulted in a decrease in the level of incorporation of [^{3}H] into RNA, thus the "measurable" RNA polymerase activity appeared to decrease.

The Km for CTP of RNA polymerases I, II and III from yeast form C. albicans were estimated as 0.01, 0.02 and 0.06 mM respectively. The values reported here for RNA polymerases I and II were comparable to values of 0.015 mM reported for RNA polymerases from other eukaryotes (187). The value of Km for CTP of RNA polymerase III was slightly greater. It should be noted that very low c.p.m. were recorded when RNA polymerase III was incubated in the assay

"cocktail" with limiting concentrations of CTP. The values for the Pearson coefficient of correlation for the Eadie-Hofstee plots indicated that there was quite high negative correlation between "enzyme activity" and "enzyme activity/substrate concentration" (i.e. values of -0.68, -0.73 and -0.84 for plots of RNA polymerases I, II and III respectively). Keshgegian (187) reported that higher concentrations of purine nucleoside triphosphates (i.e. ATP and GTP) were required for half maximal RNA polymerase activity compared to the pyrimidine nucleoside triphosphates (i.e. CTP and UTP). (Values for Km of ATP and GTP of approximately 0.08 mM and for CTP and UTP of approximately 0.015 mM were reported by this author) It has been found that the Km for initiating nucleotides is higher than the Km for nucleotides involved in elongation (8). The higher apparent Kms observed for purine nucleoside triphosphates may thus be related to their roles in initiation (188).

4.4.3 Preparation of RNA polymerase from mycelial form C. albicans

Only a partial purification of mycelial form RNA polymerase was achieved. Table 4.2 showed the total RNA polymerase activity, protein content and specific activities of the fractions from cell disruption to gel filtration. An interesting result from the initial stages in the purification (i.e. homogenisation with balotini) was that only 94 units of RNA polymerase activity were recovered. This probably arose as a consequence of the low yields of mycelia when C. albicans was grown in G.B.E. medium. A measure of the low yields may be illustrated by the fact that 7.9 g of mycelia were harvested from 8 L of media.

It was noted that the specific activity of the RNA polymerases in the homogenate prepared from mycelia was similar to that of homogenate prepared from yeast cells in the exponential phase of growth (see 4.4.1). As purification of the RNA polymerases proceeded through high salt sonication, centrifugation and protamine sulphate precipitation, the measurable total RNA polymerase activity decreased. This result was similar to that achieved during the initial stages of purification of yeast form RNA polymerases (see Table 4.1)

Gel filtration of the ammonium sulphate precipitate of F_2 revealed a peak of low activity was eluted from the column just after the void volume (see Fig. 4.19). This indicated that mycelial RNA polymerase(s) had a similar M.W. to those from the yeast form. The specific activity of the RNA polymerase after this step, i.e. 1.3 units/mg, was similar to that of RNA polymerase. It was found that omission of the protamine sulphate-nucleic acid precipitation step with the cell-free extract (i.e. gel filtration of fraction F_1) resulted in approximately 30% of total activity eluting in the void volume. This could indicate that the conditions for precipitation of the nucleic acid resulted in entrapment of the mycelial RNA polymerase molecules. Hence, a large amount of RNA polymerase activity could be lost if the conditions for nucleic acid precipitation were not ideal. An alternative explanation may be that the enzyme could re-associate with nucleic acid as fraction F_1 was eluted through the agarose column. The buffer contained a $(NH_4)_2SO_4$ concentration of 0.1 M. Hager et al (141) noted that at concentrations below 0.15 M $(NH_4)_2SO_4$ RNA polymerases and DNA were able to re-associate (see page 122).

Possibly the most surprising result obtained from the partial purification of mycelial form RNA polymerases was that only one peak, of very low activity, was found after ion exchange chromatography (see 4.3.16). This seems to be in complete contrast with assertions made earlier in this thesis (see 1.3.3) i.e. that eukaryotes possess three different classes of RNA polymerase. Boguslawski et al (35), working with H. capsulatum yeast, reported that only one class of RNA polymerase - of low activity - was eluted from DEAE-Sephadex. In contrast, three classes of RNA polymerase were resolved by ion exchange chromatography of the mycelial form. Kumar et al (203) later reported that treatment of cell extracts from yeast H. capsulatum with Polymin P enabled three classes of RNA polymerase to be resolved. This group presumed that Polymin P precipitated the "factor(s)" responsible for concealing RNA polymerase activity. The presence of such "factors" in mycelial form C. albicans could explain the low RNA polymerase activity eluted. It should be noted that scans of fractions eluted from the DEAE-sephadex contained UV absorbing material. However, these fractions contained no activity significantly different from the background.

Alternatively, the low total activity eluting from the DEAE-sephadex could arise as a consequence of the lability of the RNA polymerases.

It was noted at the beginning of this section that low yields of mycelia were obtained when C. albicans was grown in G.B.E. medium. This illustrates one of the major limiting factors on research with C. albicans mycelial form. Large volumes of media, with low cell densities are required, in order to cultivate mycelia. To obtain large quantities of mycelia - sufficient for further studies on RNA polymerases - a method for the large scale cultivation is needed. However, such large scale preparations must entail stringent safety precautions to guard against the much greater risk of contamination with the pathogen.

CHAPTER 5 SOME STUDIES ON INHIBITORS OF RNA SYNTHESIS IN C. albicans

5.1 Introduction

The eventual aim of the investigations into RNA synthesis in C. albicans was to develop ideas for novel potential inhibitors of this fungus that had no effect on mammalian cells. Originally, these studies were to investigate the effect of a putative inhibitor on RNA synthesis by C. albicans in vitro (in nuclei and protoplasts) and in vivo (in whole cells). This compound - lomofungin - has been shown to affect RNA synthesis in S. cerevisiae (205), probably by direct interaction with the RNA polymerase (55). Studies were made on the effect of this drug against exponential phase growth of C. albicans yeast against RNA synthesis by protoplasts. The results and conclusions of this investigation are described in this chapter.

The effects of several novel potential inhibitors, from the ICI collection, against RNA synthesis in vivo and in vitro were investigated. RNA synthesis is a complex process that may be inhibited at several sites or stages. Indeed, the known inhibitors of RNA synthesis can, and do, act by a variety of mechanisms (e.g. intercalation by actinomycin D with G-C rich regions of the DNA (31) or interaction with the RNA polymerase by various rifamycin derivatives (255)). One possible method for inhibition is by the use of substrate analogues. There are four nucleotides which are used as substrates by RNA polymerase (i.e. ATP, GTP, CTP and UTP). It has been noted that some substrate analogues are effective inhibitors of in vitro RNA synthesis (e.g. Seibert et al reported that Tubercidin triphosphate inhibited RNA polymerases I, II and III from mouse cells (340)). However, nucleoside triphosphate analogues would be unable to cross the cell wall and membrane. In order to overcome this difficulty nucleoside analogues, which can cross the cell membrane via specific permeases, (291) were chosen. The efficacy of 25 of these derivatives against in vitro and in vivo RNA synthesis was compared with other, known RNA synthesis inhibitors.

5.2 Materials and Methods

C. albicans A and B serotype (strains NCPF3153 and NCPF3156 respectively) and strains AB2, Leeds B2630 and 6406/8 were maintained and grown on Sabourauds dextrose broth as described in 2.3.3. S. cerevisiae strain CD40 was grown and maintained on yeast extract peptone (YEP) broth, as previously described (2.4.1).

Protoplasts of yeast form C. albicans were prepared as described in 2.3.6, using Zymolyase 60,000 enzyme to remove the cell wall. RNA synthesis by protoplasts was measured by incorporation of [^{3}H] uridine into TCA insoluble material, as described in 2.5.1. When required, lomofungin was added from a stock solution of 10 mg/ml (32 mM) in 5 mM NaOH. Measurement of RNA synthesis in vivo, using yeast form C. albicans was measured in the same manner as described for protoplasts. The effect of some known transcription inhibitors o nucleoside analogues on RNA synthesis by yeast cells in the exponential phase of growth, was investigated. When required, the test compound was added to the suspension of yeast cells from a stock solution of 12 mM in DMSO. RNA synthesis in vitro was measured in cell free homogenates of C. albicans NCPF3153. Homogenates were prepared, from yeast cells in the exponential phase of growth, using ballotini, as described in 4.2.1. RNA synthesis in vitro was measured as described in 2.5.2. When required the test compound was added, from a stock solution of 12 mM in DMSO, to the homogenate.

5.3 Results

5.3.1 Effect of lomofungin on C. albicans exponential phase growth

The effect of lomofungin, at various concentrations, on cellular growth by C. albicans is shown in Fig. 5.1. Lomofungin, at final concentrations of 15 μg (48 μM), 30 μg (96 μm) or 100 μg (320 μM)/ml media, had no effect on the rate of exponential phase growth in Sabourauds dextrose broth at 37° C.. Under these conditions the yeast cells had a doubling time of 71 min as judged by the regression line of Log_{10} (cell density) against time of incubation at 37° C The Pearson coefficient of correlation (r) was estimated at 0.95, indicating

very high positive correlation between Log_{10}(cell density) and time of incubation at 37° C.. The Log_{10}(cell density) of aliquots of cell suspensions in the presence of the drug was not significantly different from that of the controls.

5.3.2 Effect of lomofungin on growth by several strains of C. albicans

The effect of lomofungin, at a concentration of 100 µg/ml media, on growth of several strains of C. albicans is shown in Fig. 5.2. Under these conditions, all the strains of C. albicans exhibited doubling times of 71 ± 3 min, as estimated by the regression line of Log_{10}(cell density) against time of incubation at 37° C.. The values for r, estimated at approximately 0.9 for all 6 strains, indicated very high positive correlation between Log_{10}(cell density and time of incubation at 37° C. . The error bars refer to the standard deviations for the six strains. Lomofungin, at a concentration of 100 µg/ml media, did not have an effect on exponential phase growth by C. albicans NCPF 3153, AB2, Leeds B2630, 6406/8 and NCPF3156 strains.

5.3.3 Effect of various concentrations of lomofungin on RNA synthesis by protoplasts of C. albicans

The effect of lomofungin, at various concentrations, on RNA synthesis by C. albicans protoplasts is shown in Fig. 5.3. RNA synthesis over an 80 min period was monitored by incorporation of [^{3}H] uridine into TCA insoluble material. Incorporation of label into RNA was linear over the 60 min period of 20 - 80 min after the addition of [^{3}H] uridine to the pre-incubated protoplast suspension. The rate of incorporation of 11 fmoles [^{3}H] uridine min^{-1} by 10^7 viable protoplasts was estimated using the regression line of "pmoles of [^{3}H] uridine incorporated by 10^7 viable protoplasts" against "time of incubation" over the period incorporation of label into RNA was linear The value for r, of 0.85, indicated there was high positive correlation between the two variables. The addition of lomofungin at concentrations of 25 µg (80 µM) on 100 µg (320 µM) per ml suspension did not have any effect on

Fig. 5.1 Effect of lomofungin at various concentrations on exponential phase growth by C. albicans

Inocula of C. albicans NCPF3153 were grown in 5 ml of S.D.B., as described in 2.3.3. At the cell density indicated (↓), lomofungin, as a solution in 5 mM NaOH, was added to final concentrations of 15 μg (48 μM □), 30 μg (96 μM ▲) and 100 μg (320 μM ⊙) per ml media. The cell density of duplicate aliquots was measured at various times before and after the addition of the drug using an improved Neubauer haemocytometer. The error bars refer to the standard deviations for the control (no drug added ✕).

Fig. 5.2 Effect of lomofungin at a concentration of 100 μg/ml on various strains of C. albicans growing in the exponential phase of growth

Inocula of C. albicans strains NCPF3153, NCPF3156, AB2, Leeds, B2630 and 6406/8 strains were grown in 5 ml of S.D.B., as described in 2.3.3. At the cell densities indicated (↓), lomofungin, as a solution in 5 mM NaOH, was added to a final concentration of 100 μg/ml media (0.31 mM) to C. albicans strains NCPF3153 (△), NCPF3156 (□), AB2 (⊙), B2630 (✣), 6406/8 (⊕) and Leeds (✕). The cell densities of duplicate aliquots, before and after the addition of the drug, was measured using an improved Neubauer haemocytometer. The error bars refer to the standard deviations of the controls (no drug added).

Fig. 5.3 Effect of lomofungin at various concentrations on RNA synthesis by C. albicans protoplasts.

Protoplasts of C. albicans NCPF3153 were prepared as described in 2.3.6. The suspension was pre-incubated in protoplast buffer (total volume 8 ml with protoplast density of 7 x 10^7 viable protoplasts/ml) at 37° C. for 80 min. Lomofungin, as a solution in 5 mM NaOH, was added to final concentrations of 25 μg/ml (80 μM —●—) and 100 μg/ml (320 μM —○—) or directly to 1 mg/ml (3.2 mM —○—), 40 μCi [^{3}H] uridine (specific activty 47.5 Ci/mmol) was added to each protoplast suspension and triplicate 0.5 ml aliquots removed after the times of incubation shown. Radioactivity incorporated into TCA insoluble material was measured as described in 2.5.1. The error bars refer to the standard deviations of the control (no lomofungin added).

Fig. 5.1

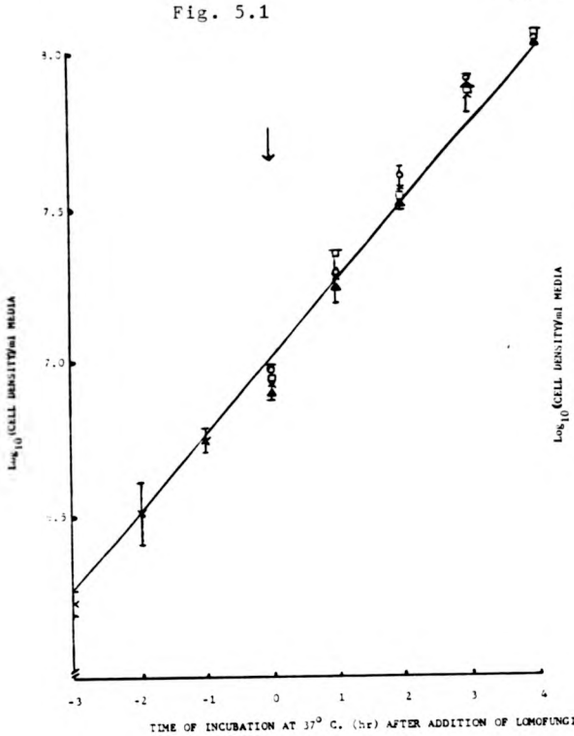


Fig. 5.2

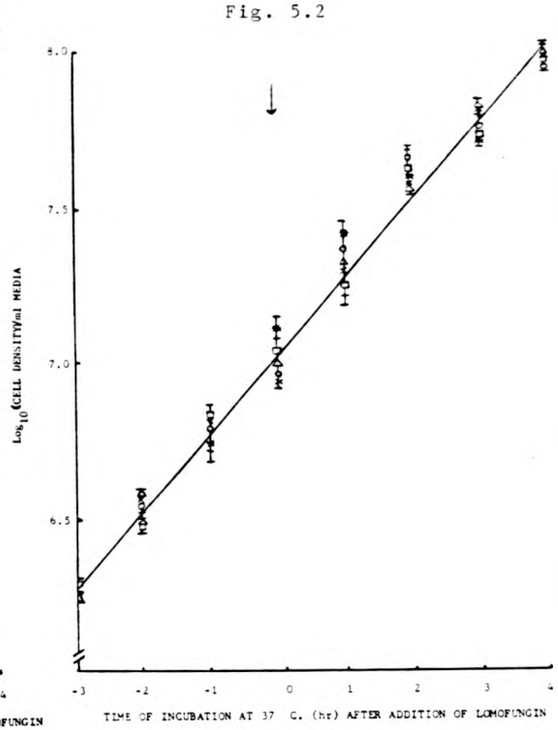
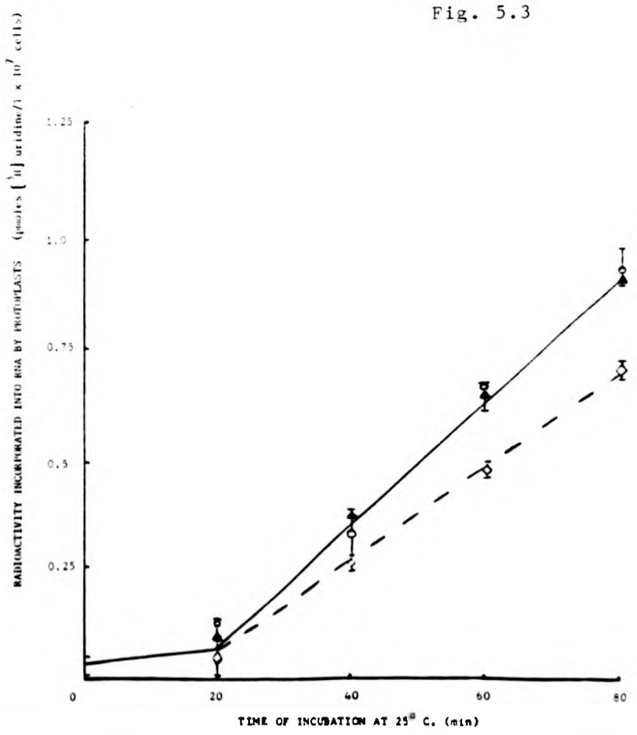


Fig. 5.3



incorporation of [^{3}H] uridine into RNA, compared to the control. Lomofungin, at a concentration of 1 mg/ml suspension (3.2 mM), had some effect on the rate of RNA synthesis by the protoplasts as 7 fmoles [^{3}H] uridine were incorporated by 10^7 viable protoplasts min^{-1}.

5.3.4 Effect of lomofungin at a concentration of 20 µg/ml on growth of S. cerevisiae

The effect of lomofungin at a concentration of 20 µg/ml YEP medium (64 µM) on growth of S. cerevisiae CD40 strain - as measured by increase in Log (cell density) - is shown in Fig. 5.4. The figure shows regression lines of Log_{10} (cell density) against time of incubation for control cultures of S. cerevisiae, with a doubling time of 134 min. The value for r of 0.95 indicated high positive correlation between the two variables . Lomofungin, at a concentration of 20 µg/ml media, completely inhibited cell growth by S. cerevisiae.

5.3.5 Effect of some known inhibitors of transcription and nucleoside analogues on *in vitro* RNA synthesis by C. albicans

The effect of some known RNA synthesis inhibitors and nucleoside analogues o *in vitro* RNA synthesis is shown in Table 5.1. The test compound was added, in a solution of DMSO, to the assay "cocktail" so that the final concentration of the drug would be 1 mM. RNA synthesis by homogenates of C. albicans, in the presence or absence of the test compounds, was measured over a 5 min incubation period. The addition of DMSO to the homogenate affected measurable RNA synthesis. It was found that without DMSO, 52 fmoles [^{3}H] UMP were incorporated by 50 µl homogenate over a 5 min incubation period at 25° C, compared to 28 fmoles by homogenate when this solvent was added. Thus, the percentage inhibition of the test compound compared to control refers to controls containing the same volume of DMSO. It should be realised that only those compounds which showed inhibitory activity against RNA synthesis are included in this table. The structure of the nucleoside analogues showing inhibitory activity

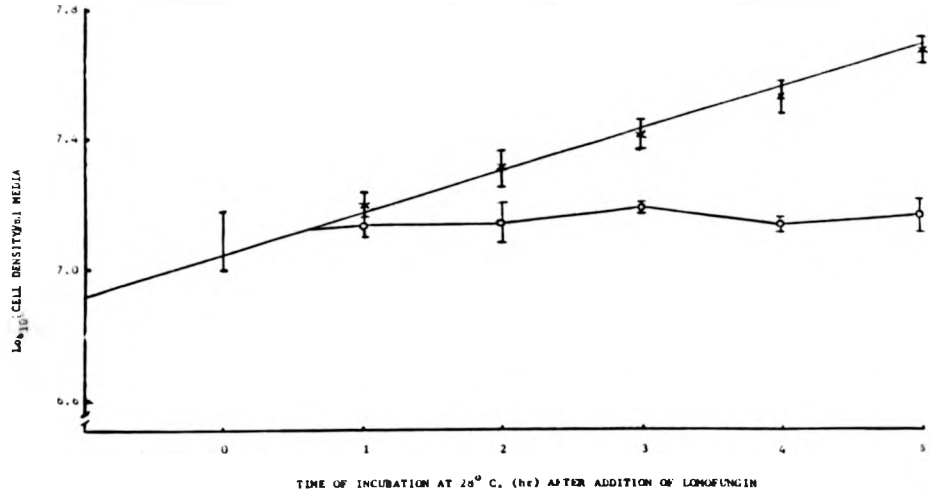


Fig. 5.4 Effect of lomofungin at a concentration of 20 µg/ml on growth of *Saccharomyces*

Inocula of *Saccharomyces* CD-3 strain was grown in 5 ml Y.E.P. broth, as described in 2.4.1. At the cell density indicated (↓), lomofungin, as a solution in .5 M NaOH was added to a final concentration of 20 µg/ml media (o—o µM o—o). The cell density of duplicate aliquots at various times, both before and after the addition of the drug, was estimated using an improved Neubauer haemocytometer. The error bars refer to the standard deviations of the duplicate aliquots. The figure also shows the increase in cell density of control cell cultures, with no lomofungin added (×—×).

	Test compound (ICI compound number in brackets)	RNA polymerase activity in homogenate (fmoles [^{3}H] UTP incorporated/ 5 min/50 µl homogenate)		Percentage inhibition compared to control (%)
Test compound from ICI collection	(69619)	15 ± 6	11 ± 1	55 ± 10
	(78053)	10 ± 6	12 ± 3	62 ± 5
	(78192)	13 ± 2	15 ± 3	52 ± 5
	(82939)	2 ± 1	3 ± 3	91 ± 2
	(111786)	5 ± 3	7 ± 5	79 ± 5
	(111787)	2 ± 4	9 ± 4	81 ± 17
	(154391)	12 ± 2	6 ± 4	89 ± 14
Some known inhibitors of transcription	aphidicolin (69653)	9 ± 2	13 ± 2	74 ± 12
	cycliziding derivatives (95154)	0 ± 3	0 ± 3	100 ± 2
	cycliziding derivatives (95464)	7 ± 1	5 ± 3	79 ± 5
	zincophorin (144255)	9 ± 1	10 ± 4	69 ± 5
	lomofungin	24 ± 6	19 ± 3	26 ± 12
	rifamycin AF/013	26 ± 6	25 ± 3	12 ± 2
	rapamycin	10 ± 7	7 ± 3	71 ± 7
	control	29 ± 2		

Table 5.1 Effect of some known transcription inhibitors [illegible] analogues on RNA synthesis in vitro

Homogenates were prepared from *C. albicans* NCPF 3153 yeast cells growing in exponential phase, as described in 2.2.1. 200 µl aliquots of homogenate were incubated in 200 µl "cocktail", the components of which were present at the final concentrations described in 2.2.5. Test compounds, in DMSO, were added to a final concentration of 1 mM. Duplicate 100 µl aliquots were removed after 0 and 5 min incubation at 25° C. The radioactivity incorporated into TCA insoluble material was measured, as described in 2.5.1. The results of 2 separate experiments are shown (± standard deviations) complete with the average percentage inhibition compared to control (DMSO only added).

	Test compound (ICI compound number in brackets)	Radioactivity incorporated into RNA (pmoles ^{3}H uridine incorporated 0.5 ml aliquots/40 min)	Percentage inhibition compared to control (%)
Test compound from ICI collection	(69619)	0.15 ± 0.08	87
	(78053)	0.09 ± 0.01	92
	(78191)	0.31 ± 0.01	74
	(78192)	0.84 ± 0.10	29
	(113787)	0.17 ± 0.01	86
	(154391)	0.21 ± 0.03	82
Some known inhibitors of transcription	aphidicolin (69653)	0.92 ± 0.04	22
	cyclicidine derivative (95464)	0.60 ± 0.04	49
	zincophorin (144255)	0.12 ± 0.03	90
	lomofungin	0.02 ± 0	99
	rifamycin AF/013	0.11 ± 0	91
	rapamycin	0.82 ± 0.08	30
	control	1.18 ± 0.03	

Table 5.2 Effect of some known transcription inhibitors and nucleoside analogues on RNA synthesis in vivo

Yeast cells of C. albicans NCPF3153 were grown to mid exponential phase of growth in S.D.B. (5×10^{7} cells/ml media), harvested and washed as described in 2.3.3 . The yeast cells were then resuspended, to the same cell density, in 2 ml protoplast buffer and pre-incubated for 10 min at 37° C. The test compound was added in a solution of DMSO to a final concentration of 1 mM prior to the addition of 10 µCi [^{3}H]uridine (specific activity 52 Ci/mmol). Duplicate 0.5 ml aliquots were removed after 0 and 40 min incubation at 37° C., Radioactivity incorporated was measured as described in 2.5.1. The error bars refer to the standard deviation of two separate experiments. The table shows the average percentage inhibition compared to control cultures (DMSO only added).

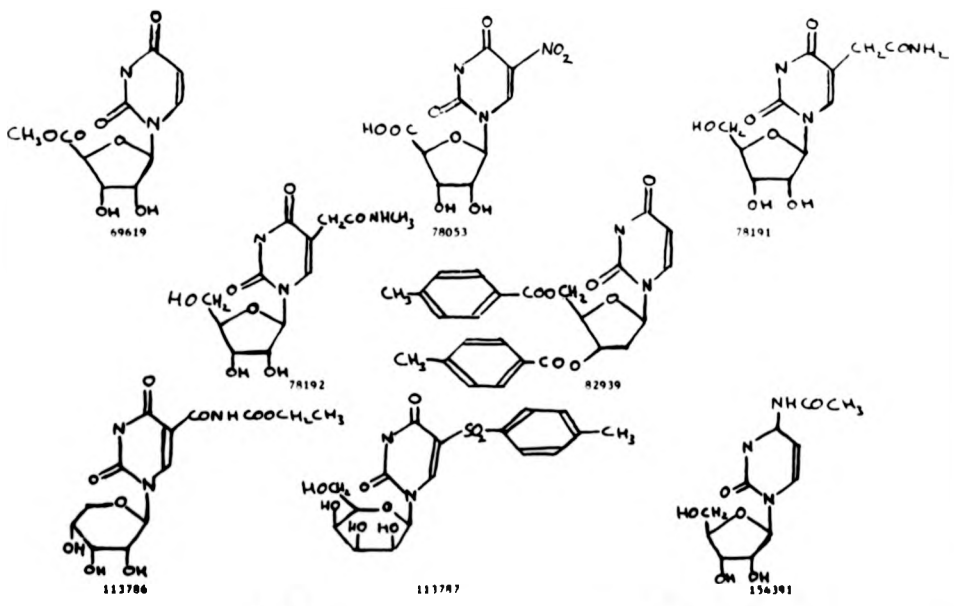
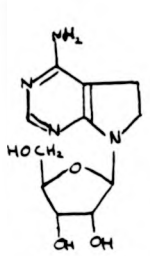
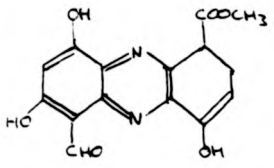


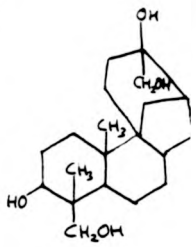
Fig. 5.3 Structures of nucleoside analogues showing inhibitory activity against in vitro or in vivo RNA synthesis



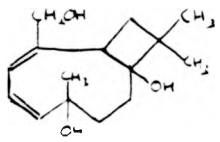
TUBERICIDIN



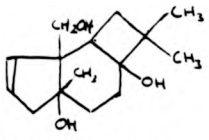
LOMOFUNGIN



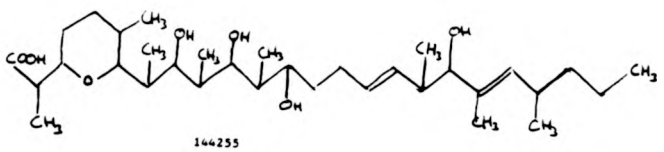
APHIDICOLIN 69653



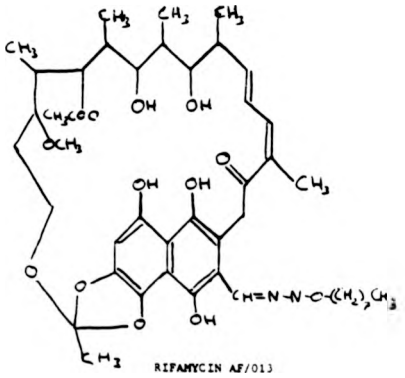
CYCLIZIDINE DERIVATIVE
95154



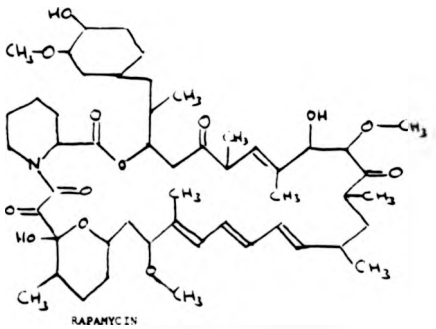
CYCLIZIDINE DERIVATIVE
95464



144255



RIFAMYCIN AF/013



RAPAMYCIN

Fig. 5.6 Some known inhibitors of RNA synthesis

against *in vitro* RNA synthesis by *C. albicans* and also the structure of the known inhibitors of transcription are as shown in Figs. 5.5 and 5.6.

5.3.6 Effect of some known inhibitors of transcription and nucleoside analogues on RNA synthesis by *C. albicans in vivo*

The effect of some nucleoside analogues from the ICI collection and some known inhibitors of transcription on *in vivo* RNA synthesis by *C. albicans* is shown in Table 5.2. The test compound was added in a solution of DMSO to a suspension of yeast cells (harvested and washed whilst growing in the exponential phase of growth) in protoplast buffer . The Table shows the effect of some of the test compounds on RNA synthesis - as measured by incorporation of [^{3}H] uridine into RNA over a 40 min incubation period at 25° C. - compared to controls (DMSO only added). DMSO, at concentrations of 10%, did not inhibit incorporation of [^{3}H] uridine into RNA by *C. albicans* yeast cells *in vivo*. The structure of the nucleoside analogues showing inhibitory activity against RNA synthesis *in vivo* by *C. albicans* is shown in Fig. 5.5. The structure of the known inhibitors of transcription used in this research is shown in Fig. 5.6.

5.4 Discussion

5.4.1 Studies using the inhibitor lomofungin

Lomofungin has a broad antifungal and antibacterial spectrum, active against yeasts and other fungi and both gram positive and gram negative bacteria (184). Kuo *et al* reported that, at a concentration of 5 µg/ml, lomofungin markedly and rapidly inhibited RNA synthesis by *S. cerevisiae* protoplasts (205). In a later paper the same group reported that the antibiotic prevented RNA synthesis by direct interaction with the RNA polymerase and not with the template or substrate (55). (It has been postulated that lomofungin inhibits this enzyme by coordination with a divalent metal ion to the zinc at the RNA polymerases active site (282)). Thus, it did not seem

unreasonable to expect that this drug would have affected growth of C. albicans by inhibition of RNA synthesis. However, as shown in Figs. 5.1, 5.2 and 5.3, lomofungin at concentrations as high as 100 µg/ml did not affect either cellular growth by the six strains of C. albicans used or RNA synthesis by protoplasts. In contrast, growth of S. cerevisiae was completly inhibited by 20 µg lomofungin/ml YEP media (Fig. 5.4). Thus, lomofungin was active against S. cerevisiae but not C. albicans. This is in agreement with Johnson and Dietz (184), who reported that growth of C. albicans A and B serotypes were only inhibited by concentrations of lomofungin of 1 mg/ml.

It is possible that different species of fungi will show differential sensitivities to a drug. However, when examining experimental approaches to fungal chemotherapy and drug discovery and evaluation, a few pertinent points should be borne in mind.

Any method for testing the efficacy of a compound requires a method for measuring its inhibition against the test organism. Attempts to find the lowest concentration of the test drug - in this case lomofungin - which resulted in inhibition were made initially by adding increasing concentrations of the drug to growing cultures of C. albicans. Cell growth was estimated by direct observations of aliquots of cells under the light microscope using an improved Neubauer haemocytometer. Obviously a method for large scale screening of test drugs would require a less time consuming approach. Such an end is usually achieved by a program such as broth or agar dilution (i.e. dilution of the test compound in liquid or solid growth) media or agar diffusion (i.e. diffusion of the test compound from a reservoir). Inhibition of growth is measured by the absence of colonies of the organism growing in/on the media.

An important consideration when investigating potential inhibitors and/or their mode of action is that the test compound will be soluble in the media chosen for growth. The initial studies on the inhibitor lomofungin used a stock solution of lomofungin in 5 mM NaOH. It was reputed that the drug was soluble under such conditions (30). Many antifungal agents are soluble in organic solvents. Indeed, when examining the effects of nucleoside

analogues, and other known inhibitors of transcription on *in vitro* and *in vivo* RNA synthesis, the test compounds were dissolved in DMSO. Other solvents commonly employed are dimethylformamide, acetone, ethanol and polyethyleneglycol (318). The growth media for cultivating the organism should allow free growth, but should not possess constituents which could antagonise the antifungal activity of the compound (see 1.2.5). It has been reported that fungicidal action of lomofungin may be affected by Cu^{++} and Zn^{++} (282). Thus, the content of these metal ions in an undefined media, such as peptone broth, could have an effect on the inhibitory action of lomofungin. However, as growth by *S. cerevisiae* in a similarly ill-defined peptone broth was completely inhibited by lomofungin at 20 µg/ml, another mechanism must account for the differential sensitivity of these two yeast species.

When lomofungin was added, to a concentration of 3.2 mM, to *C. albicans* protoplasts, the rate of *in vitro* RNA synthesis was inhibited 36%, compared to controls (see 5.3.3). In contrast, RNA synthesis *in vivo* was inhibited 99% by lomofungin at a lower concentration of 1 mM (Table 5.2). One factor that may account for this differential sensitivity may be the solvent the drug was dissolved in. This can have an important bearing on the inhibitory action of the drug. DMSO does enhance the susceptibility of some organisms to antifungal drugs by increasing the permeability of the cell membrane to certain molecules (322). Other factors, such as the size of fungal inoculum and also the morphological form are important when trying to evaluate the effects of potential inhibitors. In addition, the incubation conditions can affect the susceptibility of an organism to inhibition. As fungi are aerobes, the conditions for cultivation should allow adequate aeration. This was provided by the use of an orbital shaker.

5.4.2 Inhibitors of *in vivo* and *in vitro* RNA synthesis

Investigations into the efficacy of several known inhibitors of RNA synthesis and nucleoside analogues from the ICI collection against *in vivo* and *in vitro* RNA synthesis are contained in this chapter. The test compounds were

added, in DMSO, to the assay mixture to a final concentration of 1 mM. Such a high concentration was chosen as this would establish whether the test compound did inhibit RNA synthesis and warrant further investigation.

One of the problems in using DMSO as a solvent for the test compounds was that it was found to inhibit in vitro RNA synthesis by C. albicans homogenates. Other groups have commented on this phenomenon (268), believing that the solvent decreases the RNA polymerase - DNA interaction. However, contradictory findings have been reported which suggest that DMSO has no effect, or even stimulates RNA polymerase activity (228). In contrast to in vitro RNA synthesis, the addition of DMSO had no effect on RNA synthesis in vivo by yeast C. albicans in the exponential phase of growth (see 5.3.6).

The results of the efficacy of test compounds against in vitro and in vivo RNA synthesis was as shown in Tables 5.1 and 5.2. In many cases there was a discrepancy between the effectiveness of the test inhibitor in vitro and in vivo. For example, nucleoside analogues 78192, 82939 and 113786 and aphidicolin, the cyclizidine derivatives 95154 and 95464 and rapamycin all had a greater inhibitory effect on in vitro RNA synthesis than in vivo. One reason for this may have been that these inhibitors had difficulty crossing the cell wall and membrane. Nucleoside analogues 113787 and 154391 had similar efficacies against in vitro and in vivo RNA synthesis by C. albicans. This indicates that these two compounds were not affected by the cell wall and membrane in their ability to inhibit RNA synthesis. It was found that some nucleoside analogues had greater inhibitory activity against in vivo RNA synthesis compared to in vitro (i.e. 69619, 78053 and 78191). In addition, lomofungin, rifamycin AF/013 and 144255 showed greater efficacy against RNA synthesis in vivo than in vitro. These results show that, for greater percentage inhibitory effect against RNA synthesis in C. albicans, the undamaged whole organism was required. There could be several explanations to account for these findings. Firstly, particularly in the case of the nucleoside analogues, the compound could be metabolised to more potent inhibitors such as direct substrate analogues (c.f. 5-fluorocytosine, see 1.2.5). Secondly, if the

inhibitor affected a particular stage of RNA synthesis, such as initiation, then *in vitro* RNA synthesis - often cited as mostly elongation of *in vivo* initiated chains (see 3.4.3) - would not be affected. This would explain the preferential activity of rifamycin AF/013 against *in vivo* RNA synthesis compared to *in vitro*. Thirdly, the test compound may affect RNA synthesis as a consequence of inhibition of other metabolic process. The action of a compound such as 144255, an ionophore, may account for such a phenomenon. It should be noted that this compound also affects *in vitro* RNA synthesis. Thus, other mechanisms must account for the ability of 144255 to inhibit RNA synthesis. The finding that lomofungin was more effective against *in vivo* RNA synthesis by *C. albicans* than *in vitro*, seems somewhat surprising. This compound, as noted in 5.4.1, is purported to inhibit RNA synthesis by direct action with the RNA polymerase. This finding could possibly be explained by the action of the drug against other proteins or components involved in transcription as well as the RNA polymerase.

One of the problems encountered, when "screening" such compounds as uridine analogues for their efficacies against *in vitro* RNA synthesis, was that there was low measurable radioactivity incorporated into TCA insoluble material. Unfortunately, such a phenomenon was an integral part of the assay as DMSO has an affect on RNA polymerase activity. (The DMSO was necessary as a solvent for the test compound). In addition, the crude homogenate does contain enzymes such as proteases and RNases, which have a destructive effect on measurement of RNA polymerases (see 4.4.1). Thus, a more accurate assessment of test compounds *in vitro* efficacy could be gauged using partially purified RNA polymerases. This would indicate whether the test drug had any effect against RNA polymerase action. However, inhibition of RNA synthesis *in vitro* is no guarantee of inhibition of this process *in vivo*. This ought to be borne in mind when attempting to design drugs that inhibit this biochemical process.

CHAPTER 6 THE INVOLVEMENT OF RNA SYNTHESIS IN THE YEAST - MYCELIAL TRANSFORMATION OF C. albicans

6.1 Introduction

Investigations in the 19th century revealed that, microscopically, C. albicans sometimes appeared as ovoid budding yeast cells, sometimes as mould-like hyphae. Audrey established that these forms arose from the same organism and the predominant form observed was dependent on the growth medium (12). Rarely has such a simple observation caused such a confused and contradictory literature as dimorphism in C. albicans. Dimorphism may be defined as the reversible interconversion between the yeast-like and filamentous forms.

The phenomenon of dimorphism is of interest for two reasons. Firstly, there is the view that mycelium formation may be correlated with the pathogenicity of C. albicans. However, conflicting evidence implicating yeast (301), mycelial (289) or indeed both forms (372), as the virulent entity has tended to confuse rather than clarify the situation. Secondly, the regulation of cell divergence remains a basic and poorly understood problem in development biology. As a dimorphic fungus, C. albicans presents a suitable model to study this problem.

Extensive research has been undertaken by many groups over the past 100 years to achieve a greater understanding of dimorphism. Research into the environmental factors that have been considered to affect C. albicans morphogenesis has resulted in many stimulants being advocated. These may promote either yeast-like or mycelial growth (e.g. see 278). The importance of temperature (66), inoculum density (84), media composition (10) and strain variation (248) have been widely reported. Also, several specific stimulants have been reported as being capable of inducing the formation of mycelia. These include low Zn^{++} concentrations (406), Co^{++} (275) and Fe^{++} (207) salts, amino acids such as proline (84) and the hexosamine N-acetyl glucosamine (NAG) (349). However, attempts to define a single, universal environmental stimulus have foundered as many of the results have often been confused and contradictory.

In contrast to those reports on environmental factors, biochemical studies of the differences between the yeast and mycelial forms have been more fruitful. These have contributed much to the understanding of dimorphism. Initial biochemical studies were carried out by Nickerson's group (274). These workers believed that the morphology arose from an interplay between cellular growth and cellular division and how this affected the cell wall - the principal determinant of cell shape . Biochemical comparisons have generally revealed quantitative differences between the two forms (e.g. in content of membrane (20) and cell wall (23)). In addition, some enzymes concerned with the relative levels of these components have been found to differ markedly in the two forms (e.g. Chitin synthetase (72)).

More recent studies on C. albicans dimorphism have been focussed upon germ tube formation. Germ tubes are the initial morphological form observed during the yeast-mycelial transformation and may be identified as cylindrical outgrowths of new cellular material from any point on the yeast's surface (see 1. 2.2). A variety of complex media may induce germ tube formation. Germ tubes may also be induced from starved or stationary phase cells when incubated in buffer containing NAG (349) or glucose plus glutamine (glu plus gln) (345) at temperatures above 35° C.. Under these conditions it has been reported that there is extensive RNA and protein synthesis (345).

Strain variation is an important parameter in the ability of C. albicans to form germ tubes. It was with this point in mind that studies on germ tube formation were made, using a wild type strain, i.e. NCPF3153. This strain was obtained from a clinical isolate. As such, it is probable that this strain is similar, in many respects, to wild type strains used by other groups working in this field. How valid such a prediction was in the light of the results presented in this chapter will be discussed.

Initial studies examined the effects of a buffered solution containing "complex" (i.e. serum) or "simple" (i.e. NAG) stimulating agents on germ tube formation by C. albicans NCPF3153. The effect of other "simple" stimulating agents, such as glucose plus glutamine (glu plus gln), on germ tube formation

by this strain of C. albicans was also examined. A range of concentrations of these different stimulating agents were examined for their effects on germ tube formation and cell growth by buffered yeast suspensions.

The total RNA and protein content of the yeast, incubating in germ tube inducing conditions, was monitored. This was done to investigate whether the levels of these macromolecules changed during germ tube formation. De novo RNA synthesis was monitored by incorporation of labelled uridine into TCA insoluble material. The effect of altering the concentration of the different stimulating agents on RNA synthesis was investigated. This would indicate whether there was any correlation between the level of stimulating agent, in the buffer, and RNA synthesis by the yeast. The effects of several RNA and protein synthesis inhibitors on both germ tube formation and RNA synthesis were investigated. This would confirm that RNA synthesis was necessary for germ tube formation and not merely accompanying this phenomenon. The RNA species synthesised could give an insight into the RNA polymerase isozymes involved in germ tube formation. It was with this in mind that RNA synthesised during this process, either [^{32}P] or [^{14}C] labelled, was analysed by standard electrophoretic techniques.

The conclusions that were drawn from the results shown in this chapter will be compared with those found by other workers. Also, this chapter will discuss how valid such a comparison is.

6.2 Materials and Methods

6.2.1 Organism and growth conditions used

C. Albicans A serotype (strain NCPF3153) was used in all experiments reported in this chapter. The yeast cells were cultivated in S. and S medium and stored in phosphate buffer, as described in 2.3.3. The thick cell suspension was kept at 0 - 4° C., for at least 24 hr, in order to starve the cells prior to induction of germ tubes.

6.2.2 Germ tube formation by C. albicans

The method used for inducing germ tubes from the strain of C. albicans used in this chapter was based on that described by Shepherd and Sullivan (346). The thick cell suspension, prepared as described in 2.3.3, was diluted in 20 mM imidazole HCl buffer pH 6.6 containing 0.2 mM $MnCl_2$ (imidazole buffer) to give a cell suspension with a final cell density of 1×10^5 - 5×10^5 cells/ml buffer. When required, human serum, N-acetyl glucosamine (NAG), glucose (glu) or glucose plus glutamine (glu plus gln) were present in the cell suspension at final concentrations indicated in the legends of the figures in the results section of this chapter. The cell suspensions were then incubated at 37° C. for the lengths of time indicated in the legends. The formation of germ tubes was assessed by light microscopy (x 400 magnification) of samples of the cell suspension removed at various times during the incubation. Cell growth during germ tube formation was monitored by changes in O.D. of cell suspension. Measurements were made of the O.D. using a Pye-Unicam SP500 spectrophotometer.

Protein and RNA content of the cells were estimated using methods based on those described by Lowry et al (224) and Schnieder (330) as cited in 2.6.3 and 2.6.2 respectively.

6.2.3 Measurement of RNA synthesis during germ tube formation

RNA synthesis by C. albicans during the yeast-mycelial transformation was measured by incorporation of [^{3}H] or [^{14}C] uridine into RNA. The precursor was added to cell suspensions of C. albicans incubating at 37° C., as subsequently described in the legends of the figures. Incorporation of label into TCA insoluble material by duplicate aliquots of cell suspension was measured as described in 2.5.1.

6.2.4 Extraction and analysis of RNA synthesised during germ tube formation

The RNA synthesised by C. albicans during germ tube formation

was radioactively labelled with either [^{14}C] uridine or [^{32}P] (as sodium orthophosphate). Typically 10 µCi of [4^{14} - C] uridine or 5 - 25 µCi of sodium [^{32}P] orthophosphate were added per ml of C. albicans yeast cell suspension incubating as described in 6.2.3. The labelled RNA was extracted as described in 2.7.1. The extraction buffer contained 0.5 mg C. utilis RNA/ml solution in order to obtain a visible nucleic acid precipitate after the ethanol cooling step. RNA containing [^{14}C] uridine was analysed by polyacrylamide gel electrophoresis without a DNase treatment step. The RNA was analysed on 2.5% polyacrylamide/0.5% agarose composite tube gels, as described in 2.8.1. [^{32}P] labelled RNA was extracted as described for the [^{14}C] labelled RNA, with the exception that the nucleic acid was DNAse treated, as described in 2.5.4. The RNA was then precipitated as described in 2.7.1 and analysed by agarose gel electrophoresis, using 1.3% agarose slab gels as described in 2.8.1. The position of the labelled migrating RNA was visualised by autoradiography using γ-sensitive photographic plates. These were developed as described in 2.8.9.

6.3 Results

6.3.1 Pretreatment of C. albicans for germ tube formation

Table 6.1 shows the effect of the growth medium used, and the cell density of inocula, on the ability of yeast to form germ tubes. In order to obtain reproducibly high percentages of cells with germ tubes, it was necessary to grow the cells for at least 50 hr in S. and S. medium. The yeast cells were then starved for at least 24 hr at 0 - 4° C.. In addition, it was necessary to use small inocula of cells - i.e. 1×10^5 - 5×10^5 cells per ml buffer. It was found that imidazole buffer containing 2% serum enabled all the cells to possess germ tubes after 6 hr incubation at 37° C. when these preconditions were met. (If the C. albicans strain used in this research was grown in S. and medium for only 20 - 30 hr - followed by starvation at 0 - 4°C. - then the cells did not form germ tubes).

Media used to cultivate cells	Percentage of cells with germ tubes		
	Cell density of inocula on dilution in buffer containing serum		
	5×10^7 cells/ml	5×10^6 cells/ml	5×10^5 cells/ml
S.D.B.	-	-	-
S. and S. medium	10%	16%	100%

Table 6.1 Growth media and inoculum density on ability of C. albicans to form germ tubes

Yeast cells of C. albicans were grown in either S.D.B. or S. and S. medium and stored as described in 2.3.3. Aliquots of the cell suspension were diluted in imidazole buffer containing 2% serum, to the final cell densities indicated in the table. After 6 hr incubation at 37° C., the percentage of cells with germ tubes was estimated by light microscopy (x 400 magnification).

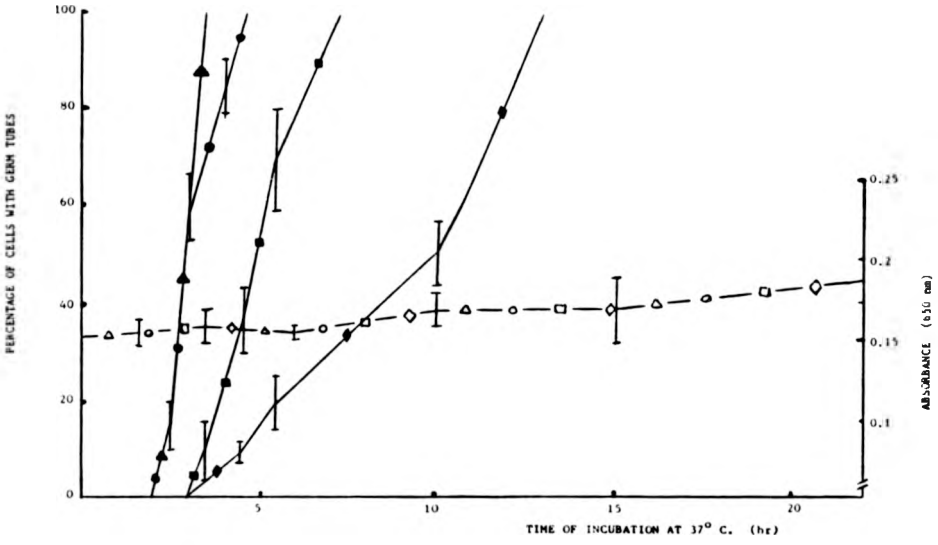


Fig. 6.1 Effect of varying serum content of imidazole buffer on germ tube formation by C. albicans

Yeast cells of C. albicans were grown in S. and S. medium as described in 2.3.3. After starvation for 24 hr, samples of the cells were diluted, in imidazole buffer, to final cell densities of 2×10^5 yeast cells/ml. Serum was present in the buffer at final concentrations of 5% (—●—), 2% (-●—), 0.5% (—■—) or 0.25% (—◆—). The percentage of cells with germ tubes, in these buffered media at the times indicated in the figure, was estimated by light microscopy (x 400 magnification). The error bars refer to the standard deviations of estimates of percentage numbers of cells with germ tubes, when duplicate aliquots were removed from the suspension incubating at 37°C.. The absorbance at 650 nm, of yeast cells incubating in buffer containing 5% (—▲—), 2% (—●—), 0.5% (—■—) or 0.25% (—◇—) serum, was monitored over a 22 hr incubation at 37°C.. The error bars refer to the standard deviations of duplicate measurements of the O.D., after the times of incubation indicated in the figure.

6.3.2 Germ tube formation by C. albicans in various buffered systems and the effect of varying the concentration of the stimulant

Pre treatment of C. albicans NCPF3153, as described in 6.3.1, allowed this strain to reproducibly form germ tubes when incubated in imidazole buffer containing a suitable stimulating agent. These were human serum, NAG, glu plus gln and glu only. (It was found that cells incubating in imidazole buffer only, at 37° C., did not, at any time, form germ tubes). The effect of varying the concentration of these four inducing agents in the imidazole buffer is shown in Figs. 6.1 - 6.4. The figures also show the absorbance, at 650 nm, of the cell suspensions over a 22 hr incubation period at 37° C.. This parameter was used as a measure of cell growth.

Fig. 6.1 shows the effect of varying the concentration of human serum in the buffer on the yeast's ability to form germ tubes. Increasing the concentration of serum in the buffer decreased the time taken for all the yeast cells to possess germ tubes. In buffer containing 5% serum all yeast cells possessed germ tubes within 3½ hr from the start of incubation. In buffer containing 2%, 0.5% or 0.25% serum, the time taken for all yeasts to produce germ tubes was longer (i.e. 4½, 7½ and 12½ hr respectively). The absorbance of the cell suspensions in buffer containing 5%, 2%, 0.5% or 0.25% serum increased only slightly over the 22 hr incubation period (i.e. by 0.038 O.D. units). This indicated that there was only a small amount of cellular growth by C. albicans in these buffered systems over the incubation period.

Fig. 6.2 shows the time course for the yeast cells to possess germ tubes in NAG-containing buffer. All the cells possessed germ tubes after 10 hr incubation at 37° C. when viewed under the light microscope. Varying the concentration of NAG in the buffer did not have any effect on the time course for germ tube formation. The optical density of the cell suspensions in NAG containing buffer increased only slightly over the 22 hr incubation period, i.e. by 0.028 O.D. units. Thus, under these conditions there was only a small amount of cellular growth shown by C. albicans.

The effect of varying the concentration of both glu plus gln on the ability

Fig. 6.2 Germ tube formation by C. albicans in NAG containing buffer

C. albicans yeast cells were grown in S. and S. medium as described in 2.3.3. After starvation for 24 hr, samples of the cells were diluted, in imidazole buffer containing 1.25 mM NAG, to a final cell density of 2×10^5 yeast cells/ml. The cell suspension was incubated at 37° C., and samples removed at the times indicated in the figure. The percentage of yeast cells with germ tubes was estimated by light microscopy (x 400 magnification) (———). The error bars refer to the standard deviations of duplicate aliquots, removed from the cell suspension at the appropriate times. The absorbance at 650 nm, of yeast cells incubating in buffer containing 1.25 mM NAG, was monitored over a 22 hr incubation (- - -). The error bars refer to the standard deviations of duplicate measurements of the O.D., after the times indicated in the figure.

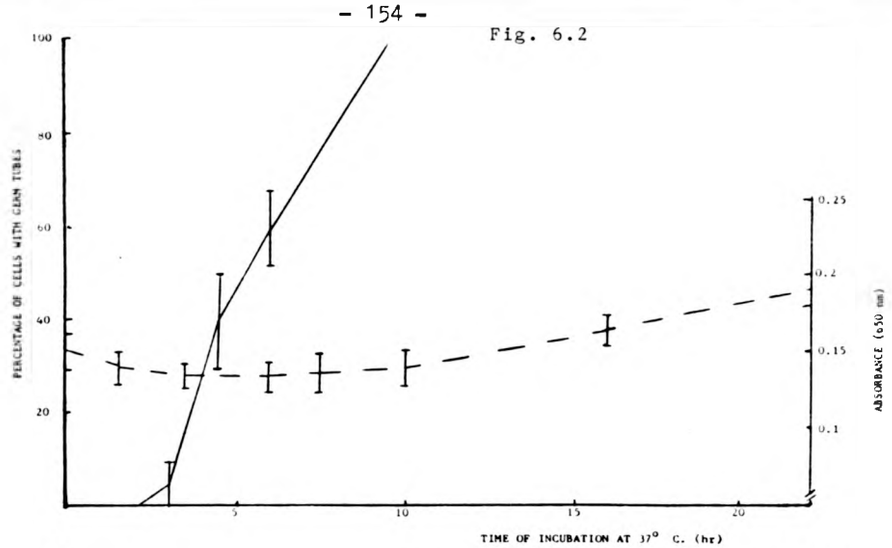


Fig. 6.3

Fig. 6.3 Effect of varying concentration of glu plus gln, in imidazole buffer, on germ tube formation by C. albicans

C. albicans yeast cells were grown in S. and S. medium and stored as described in 2.3.3. After starvation for 24 hr, samples of the cells were diluted, in imidazole buffer to final cell densities of 2×10^5 yeast cells/ml. The buffer contained glu plus gln, both at final concentrations of 25 mM (—▲—), 5 mM (—●—), 1.25 mM (—●—) or 0.25 mM (—◆—). The cell suspensions were incubated at 37° C., and samples removed at the times indicated in the figure. The percentage of yeast cells with germ tubes was estimated by light microscopy (x 400 magnification). The error bars refer to the standard deviations of estimates of percentage numbers of cells with germ tubes at the times shown. The absorbance at 650 nm, of yeast cells incubating in buffer containing 25 mM (—▲—), 5 mM (—○—), 1.25 mM (—□—) or 0.25 mM (—◇—) glu plus gln, was monitored over a 22 hr incubation at 37° C. The error bars refer to the standard deviations of duplicate measurements of the O.D., after the times of incubation indicated in the figure.

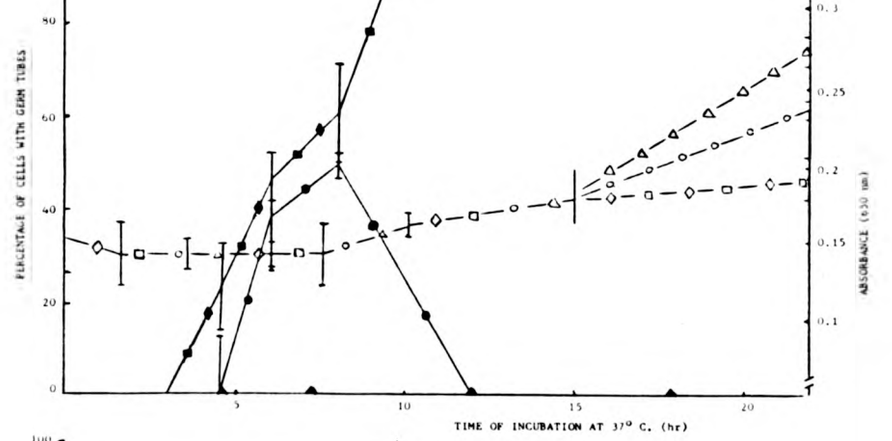
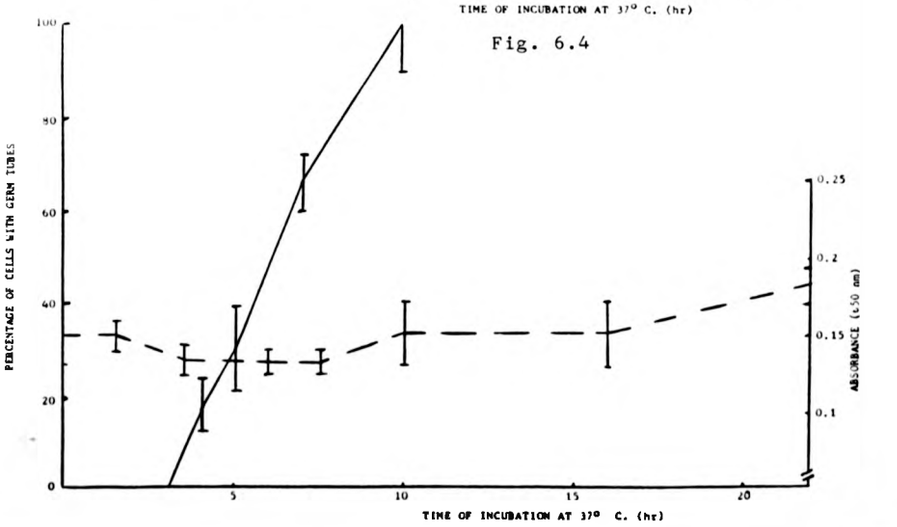


Fig. 6.4

Fig. 6.4 Germ tube formation by C. albicans in glu containing buffer

C. albicans yeast cells were grown in S. and S. medium and stored as described in 2.3.3. After starvation for 24 hr, samples of the cells were diluted, in imidazole buffer containing 1.25 mM glu, to a final cell density of 2×10^5 yeast cells/ml. The cell suspension was incubated at 37° C. and samples removed at the times indicated in the figure. The percentage of yeast cells with germ tubes was estimated by light microscopy (x 400 magnification) (———). The error bars refer to the standard deviations of estimates of percentage numbers of cells with germ tubes at the times shown. The absorbance at 650 nm, of yeast cells incubating in buffer containing 1.25 mM glu, was monitored over a 22 hr incubation at 37° C. The error bars refer to the standard deviations of duplicate measurements of the O.D., after the times indicated in the figure (- - -).



of C. albicans to form germ tubes was shown in Fig. 6.3. Yeast cells incubated in buffer containing equal amounts of glu plus gln, at concentrations of 0.25 mM and 1.25 mM, all produced germ tubes within 10 hr at 37° C.. Cells incubating in buffer containing both compounds at final concentrations of 25 mM did not form germ tubes at any time over the 22 hr incubation period at 37° C.. In buffer containing both glu plus gln at final concentrations of 5 mM 50% of the yeast cells possessed germ tubes after 7½ hr incubation at 37° C.. After 16 hr incubation no cells possessed germ tubes, when viewed by light microscopy.

Over the first 10 hr incubation at 37° C. the absorbance of the cell suspension did not change markedly - whatever the concentration of glu plus gln . Over the next 12 hr the absorbance of the cell suspension containing 25 mM glu plus gln increased (i.e. by 0.136 O.D. units). This indicates almost a twofold increase in cellular material by C. albicans yeast cells in this medium. In buffer containing 5 mM glu plus gln the absorbance increased (i.e. by 0.088 O.D. units) over a similar 12 hr incubation period at 37° C.. This indicates an increase in cellular material by C. albicans yeast cells in this buffer. Cells incubating in buffer containing glu plus gln, at concentrations of 1.25 mM or 0.25 mM, showed a slight increase in absorbance over the same period of 0.04 O.D. units. This indicated that there was only a small increase in cellular material under these conditions.

Fig. 6.4 shows the time course for germ tube formation by yeast cells in buffer containing glu only. All the cells possessed germ tubes after 10 hr incubation at 37° C., when viewed under the light microscope. Varying the concentration of glu in the buffer did not effect the time course of germ tube formation. The optical density of the cell suspension in glu containing buffer increased only slightly over the 22 hr incubation period (i.e. by 0.022 O.D. units). This indicated that there was only a slight increase in cellular material shown by C. albicans over the 22 hr incubation period in glu containing buffer.

In media that promoted germ tube formation by C. albicans yeast cells this

phenomenon was always accompanied by flocculation of cells. Germ tubes were approximately 10 - 30 μm before growth stopped.

6.3.3 RNA and protein content of C. albicans during germ tube formation

Fig. 6.5 shows the RNA content of C. albicans yeast cells before, and during, the formation of germ tubes in buffer containing 2% serum or 1.25 mM NAG. In serum-containing buffer the RNA content per yeast cell increased more than three fold over a 6 hr incubation period at 37° C.. In buffer containing NAG the RNA content per yeast cell increased more than two fold over the same period.

The protein content of yeast cells incubating in buffer containing serum over a 6 hr incubation period at 37° C. was as shown in Fig. 6.6. The protein content of individual yeast cells did not change markedly over the incubation period. It was found that the protein content per unit yeast cell increased by only five percent over 6 hr.

6.3.4 RNA synthesis by C. albicans during germ tube formation

RNA synthesis by C. albicans, during the yeast-mycelial transformation was measured by incorporation of [^{14}C] uridine into TCA insoluble material. In the absence of a suitable stimulating agent in the buffer, there was negligible [^{14}C] incorporation into RNA.

The effect of varying the concentration of serum, in the imidazole buffer, on incorporation of [^{14}C] uridine into RNA by C. albicans is shown in Fig. 6.7. Increasing the concentration of serum in the buffer increased the amount of [^{14}C] uridine incorporated into RNA for the first 16 hr incubation. The highest level of [^{14}C] incorporation by C. albicans was found when the yeast cells were incubated in buffer containing 5% serum. Decreasing the serum content decreased the level of [^{14}C] incorporated into RNA by the yeast. Over the next 6 hr incubation the level of [^{14}C] incorporated into RNA fell.

A time course of incorporation of [^{14}C] into RNA by yeast incubating in NAG-containing buffer is shown in Fig. 6.8. The amount of [^{14}C] incorporated

Fig. 6.5 Changes in RNA content of C. albicans during germ tube formation

C. albicans yeast cells were grown in S. and S. medium and stored as described in 2.3.3. After starvation for 24 hr, samples of the cells were diluted, in imidazole buffer containing either 1.25 mM NAG or 2% serum, to cell density of 2×10^5 ml. The cell suspension was incubated at 37° C. and 100 ml aliquots removed at the times shown in the figure. The cells were collected by filtration using GF/C glass fibre disks. The RNA content of cells incubating in buffer containing either NAG (—●—) or serum (—■—) was estimated as described in 2.6.2. The error bars refer to the standard deviations of duplicate estimates of RNA content. Assay blanks consisted of serum - containing buffer only.

Fig. 6.6 Changes in protein content of C. albicans during germ tube formation

C. albicans yeast cells were grown in S. and S. medium and stored as described in 2.3.3. After starvation for 24 hr, samples of the cells were diluted, in imidazole buffer containing 2% serum to a final cell density of 4×10^5 cells/ml. After the times of incubation at 37° C., indicated in the figure, the cells in 5 ml suspension were collected by centrifugation. The protein content of the cells was estimated as described in 2.6.3. The error bars refer to the standard deviations of duplicate estimates of protein content. Assay blanks consisted of serum containing buffer only. The regression line of "protein content" against "time of incubation" was estimated as described in 2.9.1.

Fig. 6.7 Effect of varying serum content of buffer on incorporation of [^{14}C] uridine into RNA by C. albicans during germ tube formation

Yeast cells of C. albicans were grown in S. and S. medium and stored as described in 2.3.3. After starvation for 24 hr, samples of the cells were diluted, in imidazole buffer containing serum, to final cell densities of 5×10^5 cells/ml. Serum was present in the buffer at concentrations of 5% (—●—), 2% (—●—), 0.5% (—■—) or 0.25% (——). 50 μCi of [5-^{14}C] uridine (specific activity 488 mCi/mmol) was added per ml suspension. Duplicate 0.1 ml aliquots were removed after the times of incubation at 37° C., shown in the figure. The radioactivity incorporated into TCA insoluble material was measured as described in 2.5.1. The error bars refer to the standard deviations of duplicate aliquots removed at the times shown.

Fig. 6.5

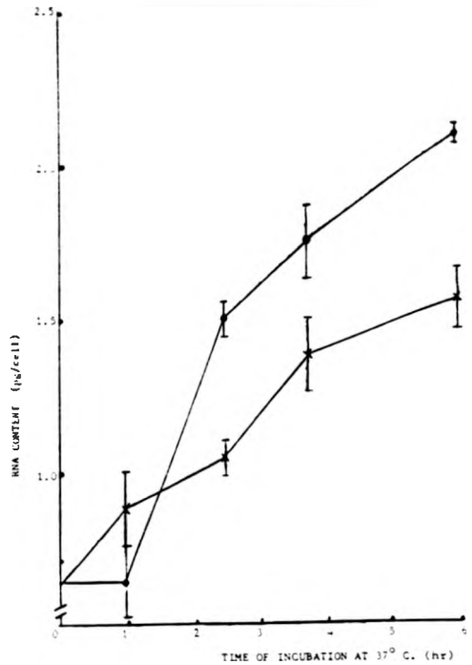


Fig. 6.6

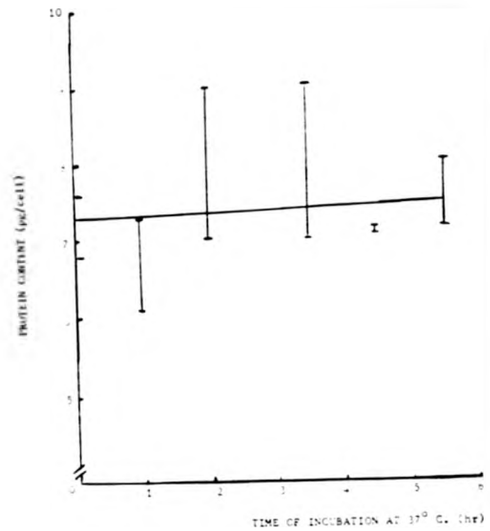
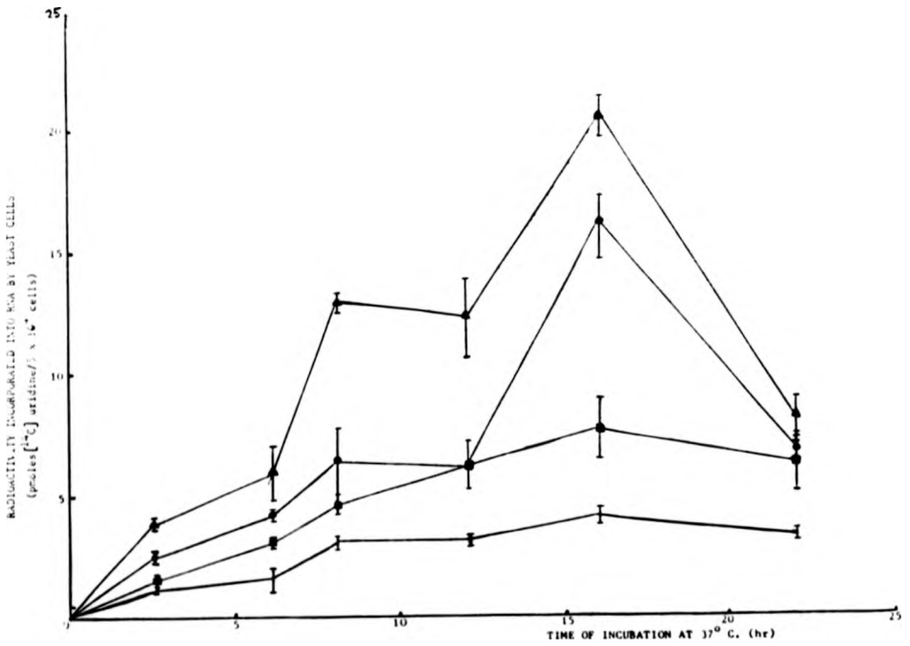


Fig. 6.7



into RNA increased over the first 8½ hr of incubation at 37° C.. Over the next 14 hr the level of [^{14}C] incorporated into RNA did not increase or decrease. Varying the concentration of NAG in the buffer did not affect the rate of incorporation of [^{14}C] uridine into RNA by the yeast.

Fig. 6.9 shows a time course of incorporation of [^{14}C] into RNA by C. albicans over a 22 hr incubation and the effect of altering the glu concentration in the buffer. The level of [^{14}C] incorporated into RNA increased with time over this period. Altering the concentration of glucose did not affect the rate of [^{14}C] incorporation.

The effect of varying the concentration of glu plus gln, in the buffer, on incorporation of [^{14}C] into RNA by C. albicans is shown in Fig. 6.10. The level of [^{14}C] uridine incorporated increased with time when yeast cells were incubated at 37° C.. Over the first 12½ hr incubation, increasing the concentration of glu plus gln in the buffer, increased the rate of incorporation of [^{14}C] into RNA by C. albicans. After this period, the level of [^{14}C] uridine incorporated into RNA by yeast, in buffer containing 25 mM glu plus gln gln, decreased. A similar result was found after 16 hr incubation for yeast incubating in buffer containing 5 mM glu plus gln. The level of [^{14}C] incorporated into RNA by yeast, in buffer containing 1.25 mM or 0.25 mM glu plus gln, increased over the 22 hr incubation period.

The effect of adding the labelled precursor to cell suspensions of C. albicans in 2% serum, 1.25 mM NAG, 1.25 mM glu or 1.25 mM glu plus gln at differing times during germ tube formation is shown in Figs. 6.11 to 6.14. The addition of [^{3}H] uridine to cells incubating in buffer containing 2% serum after 0, 2, 4 and 6 hr incubation at 37° C. is shown in Fig. 6.11. When label was added to the cell suspensions after these times, there was linear incorporation of [^{3}H] into RNA at rates of 0.31, 0.30, 0.33 and 0.27 pmoles [^{3}H] hr^{-1} by 1.5 x 10 yeasts. The rates were calculated from regression lines of "pmoles [^{3}H] incorporated into RNA by 1.5 x 10^{5} cells" against "time of incubation at 37° C.". When label was added after 0, 2, 4 and 6 hr respectively, values for r of 0.80, 0.81, 0.77 and 0.65 were found. These values indicated high

Fig. 6.8

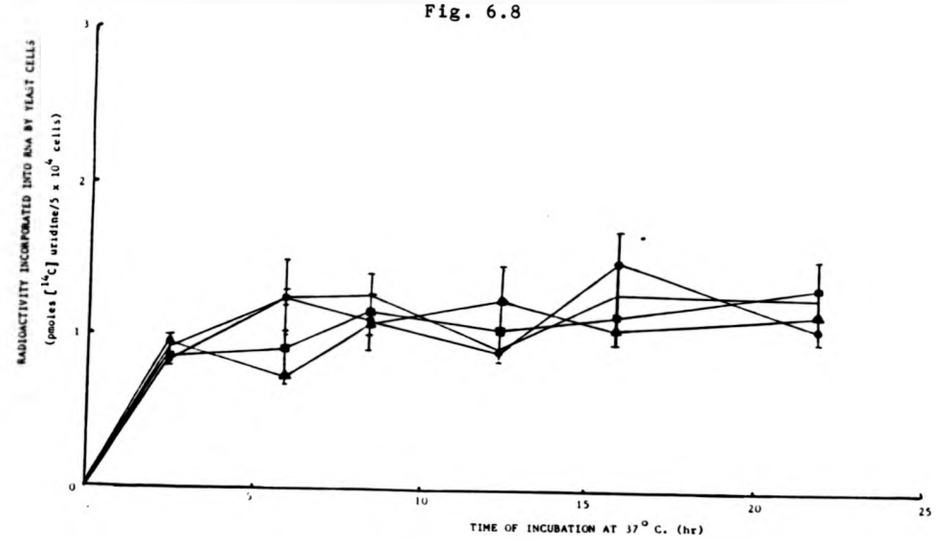


Fig. 6.8 Effect of varying NAG concentration of buffer on incorporation of [^{14}C] uridine into RNA by C. albicans during germ tube formation

Yeast cells of C. albicans were grown in S. and S. medium and stored as described in 2.3.3. After starvation for 24 hr, samples of the cells were diluted, in imidazole buffer containing NAG, to final cell densities of 5 x 10^5 cells/ml. NAG was present in the buffer at concentrations of 25 mM (—●—), 5 mM (—●—), 1.25 mM (—●—) or 0.25 mM (———). 50 μCi of [^{14}C] uridine (specific activity 488 mCi/mmol) was added per ml suspension. Duplicate 0.1 ml aliquots were removed after the times of incubation at 37° C., shown in the figure. The radioactivity incorporated into TCA insoluble material was measured as described in 2.5.1. The error bars refer to the standard deviations of duplicate aliquots removed at the times shown.

Fig. 6.9

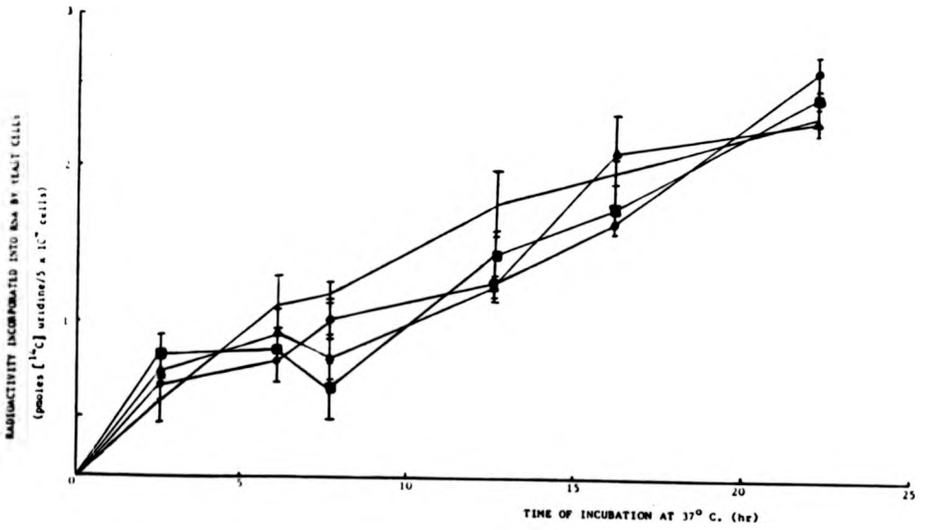


Fig. 6.9 Effect of varying glu concentration of buffer on incorporation of [^{14}C] uridine into RNA by C. albicans during germ tube formation

Yeast cells of C. albicans were grown in S. and S. medium and stored as described in 2.3.3. After starvation for 24 hr, samples of the cells were diluted, in imidazole buffer containing glu, to final cell densities of 5 x 10^5 cells/ml. Glu was present in the buffer at concentrations of 25 mM (—●—), 5 mM (—●—), 1.25 mM (—●—) or 0.25 mM (———). 50 μCi of [^{14}C] uridine (specific activity 488 mCi/mmol) was added per ml suspension. Duplicate 0.1 ml aliquots were removed after the times of incubation at 37° C., shown in the figure. The radioactivity incorporated into TCA insoluble material was measured as described in 2.5.1. The error bars refer to the standard deviations of duplicate aliquots removed at the times shown.

Fig. 6.10

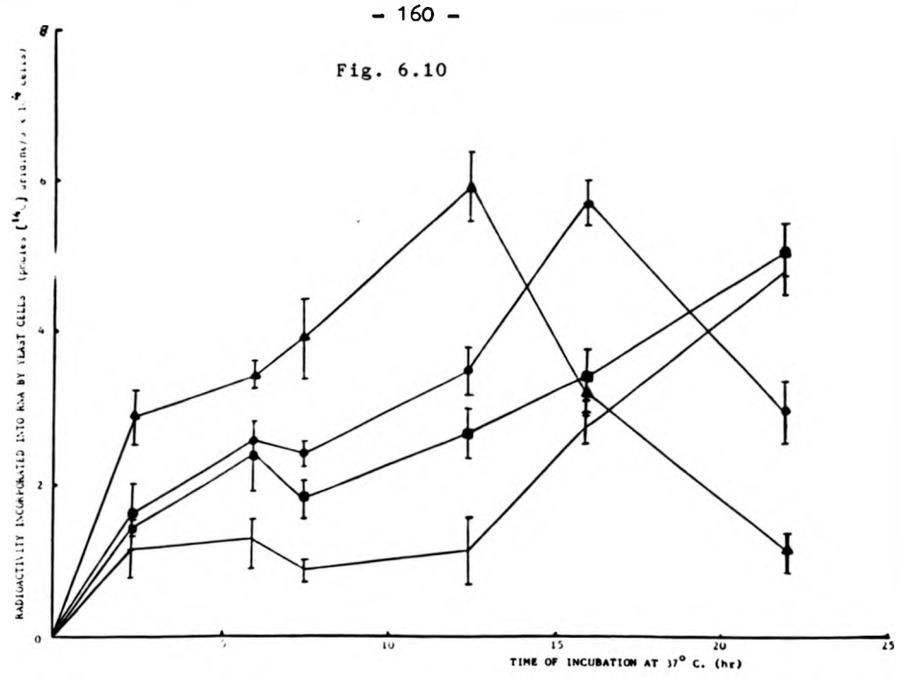


Fig. 6.11

Fig. 6.12

Fig. 6.10 Effect of varying glu plus gln concentration of buffer on incorporation of [^{14}C] uridine into RNA by C. albicans during germ tube formation

Yeast cells of C. albicans were grown in S. and S. medium and stored as described in 2.3.3. After starvation for 24 hr, samples of the cells were diluted, in imidazole buffer containing glu plus gln, to final cell densities of 5 x 10^5 cells/ml. Glu plus gln was present in the buffer at concentrations of 25 mM (—●—), 5 mM (—●—), 1.25 mM (—●—) or 0.25 mM (——). 50 μCi of [4, ^{14}C] uridine (specific activity 488 mCi/mmol) was added per ml suspension. Duplicate 0.1 ml aliquots were removed after the times of incubation at 37° C., shown in the figure. The radioactivity incorporated into TCA insoluble material was measured as described in 2.5.1. The error bars refer to the standard deviations of duplicate aliquots removed at the times shown.

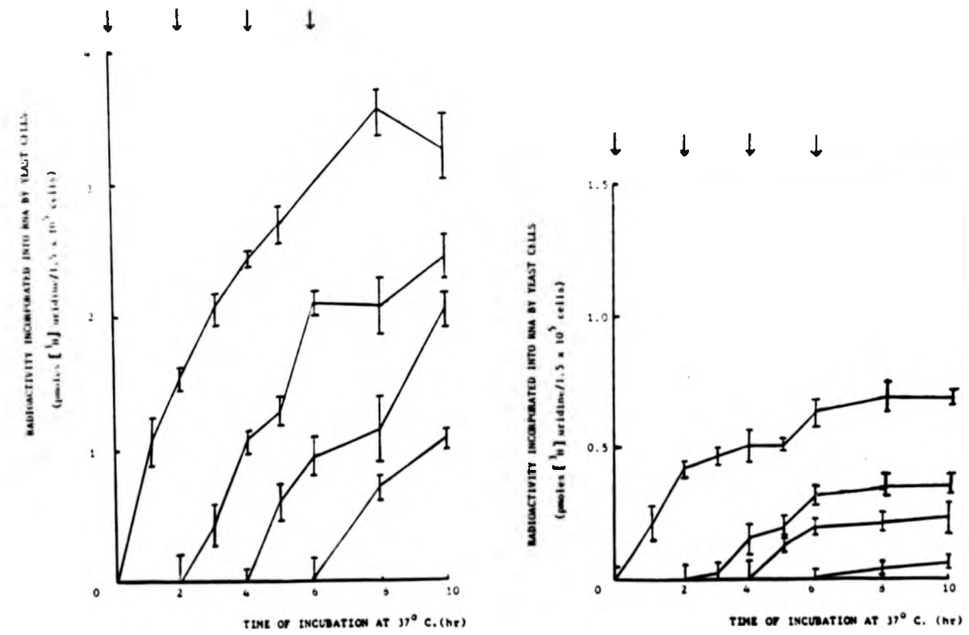


Fig. 6.11 Effect of addition of [^{3}H] uridine at different times on incorporation of label into RNA by C. albicans in buffer containing serum

Yeast cells of C. albicans were grown in S. and S. medium and starved for 24 hr, as described in 2.3.3. Samples of the cells were diluted, in imidazole buffer containing 25% serum, to final cell densities of 3 x 10^5 cells/ml. 10 μCi at [5, ^{3}H] uridine (specific activity 23 Ci/mmol) was added per ml of suspension, incubating at 37° C., at the times indicated in the figure (↓). Duplicate 0.5 ml aliquots were removed at the times shown in the figure. The radioactivity incorporated into TCA insoluble material was measured as described in 2.5.1. The error bars refer to the standard deviations of duplicate aliquots.

Fig. 6.12 Effect of addition of [^{3}H] uridine at different times on incorporation of label into RNA by C. albicans in buffer containing NAG

Yeast cells of C. albicans were grown in S. and S. medium and starved for 24 hr, as described in 2.3.3. Samples of the cells were diluted, in imidazole buffer containing 1.25 mM NAG, to final cell densities of 3 x 10^5 cells/ml. 10 μCi of [5, ^{3}H] uridine (specific activity 23 Ci/mmol) was added per ml of suspension, incubating at 37° C., at the times indicated in the figure (↓). Duplicate 0.5 ml aliquots were removed at the times shown in the figure. The radioactivity incorporated into TCA insoluble material was measured as described in 2.5.1. The error bars refer to the standard deviations of duplicate aliquots.

correlation between "pmoles [^{3}H] uridine incorporated into RNA by 1.5 x 10^5 cells" and "time of incubation at 37° C.".

Fig. 6.12 shows a time course for incorporation of [^{3}H] uridine into RNA by cells incubating in buffer containing 1.25 mM NAG. The label was added to the suspension after 0, 2, 4 and 6 hr incubation. Addition of label at the start of incubation revealed a non-linear increase in amount of [^{3}H] incorporated into RNA by yeast cells. A non-linear increase in incorporation of [^{3}H] into RNA, for the first 6 hr incubation, was found when the label was added to the suspension after 2 hr. After 8 hr incubation there was no change in the level of [^{3}H] incorporated into RNA. Addition of the label after 4 and 6 hr incubation also revealed that between 8 and 10 hr there was no change in level of [^{3}H] incorporated into RNA by the yeast.

The addition of [^{3}H] uridine to yeast cells, in buffer containing 1.25 mM glu only, after 0, 2, 4 and 6 hr incubation at 37° C., is shown in Fig. 6.13. When label was added to the cell suspensions after these times of incubation, incorporation of [^{3}H] uridine into RNA was found at rates of 0.07, 0.08, 0.06 and 0.06 pmoles hr^{-1} by 1.5 x 10^5 yeast cells. These values were calculated by regression lines of "pmoles of [^{3}H] incorporated into RNA by 1.5 x10^5 cells" against "time of incubation of cell suspension at 37° C.". Values for r of 0.80, 0.85, 0.79 and 0.63 for the two variables were found, when label was added to the cell suspensions after 0, 2, 4 and 6 hr incubation respectively. These values indicate high positive correlation between "pmoles of [^{3}H] incorporated into RNA by 1.5 x 10^5 cells" and "time of incubation of the cell suspension at 37° C.".

The effect of the addition of labelled uridine at different times of incubation at 37° C. of cells suspended in buffer containing both 1.25 mM glu plus gln is shown in Fig. 6.14. Regression lines were calculated for "incorporation of [^{3}H] into RNA by 1.5 x 10^5 cells" against "time of incubation" from time courses when the label was added after 0, 2, 4 and 6 hr incubation. These revealed rates of incorporation of label into RNA of 0.18, 0.17, 0.16 and 0.12 pmoles hr^{-1} by 1.5 x 10^5 cells respectively. Values for r of 0.86, 0.85, 0.79 and 0.61, when label was added after 0, 2, 4 and 6 hr incubation, indicated high

Fig. 6.13 Effect of addition of [^{3}H] uridine at different times on incorporation of label into RNA by C. albicans in buffer containing glu

Yeast cells of C. albicans were grown in S. and S. medium and starved for 24 hr, as described in 2.3.3. Samples of the cells were diluted, in imidazole buffer containing 1.25 mM glu, to final cell densities of 3 x 10^5 cells/ml. 10 µCi of [5,^{3}H] uridine (specific activity 23 Ci/mmol) was added per ml of suspension, incubating at 37° C., at the times indicated in the figure (↓). Duplicate 0.5 ml aliquots were removed at the times shown in the figure. The radioactivity incorporated into TCA insoluble material was measured as described in 2.5.1. The error bars refer to the standard deviations of duplicate aliquots.

Fig. 6.14 Effect of addition of [^{3}H] uridine at different times on incorporation of label into RNA by C. albicans in buffer containing glu plus gln

Yeast cells of C. albicans were grown in S. and S. medium and starved for 24 hr, as described in 2.3.3. Samples of the cells were diluted, in imidazole buffer containing 1.25 mM glu plus gln, to final cell densities of 3 x 10^5 cells/ml. 10 µCi [5,^{3}H] uridine (specific activity 23 Ci/mmol) was added per ml of suspension, incubating at 37° C., at the times indicated in the figure (↓). Duplicate 0.5 ml aliquots were removed at the times shown in the figure. The radioactivity incorporated into TCA insoluble material was measured as described in 2.5.1. The error bars refer to the standard deviations of duplicate aliquots.

Fig. 6.15 Effects of addition of inhibitors of RNA and protein synthesis on germ tube formation by C. albicans

C. albicans yeast cells were grown in S. and S. medium, and starved for 24 hr, as described in 2.3.3. Samples of the cells were diluted to final cell densities of 3 x 10^5 cells/ml in imidazole buffer containing 1.25 mM NAG. Actinomycin D (—●—), rifamycin AF/013 (—◆—), lomefungin (—◆—) and rapamycin (—▲—) were added, as a solution in DMSO to final concentrations of 0.2 mM, to cell suspensions. Cycloheximide (—■—) and chloramphenicol (—▲—) were added, as a solution in H_2O, to final concentrations of 0.1 mM, to cell suspensions. Control cultures had DMSO only added to a final concentration of 1%. The cell suspensions were incubated at 37° C. and samples removed at the times indicated in the figure. The percentage of cells with germ tubes was estimated by light microscopy (x 400 magnification). The error bars refer to the standard deviations of duplicate samples removed from the suspension at the appropriate times.

Fig. 6.13

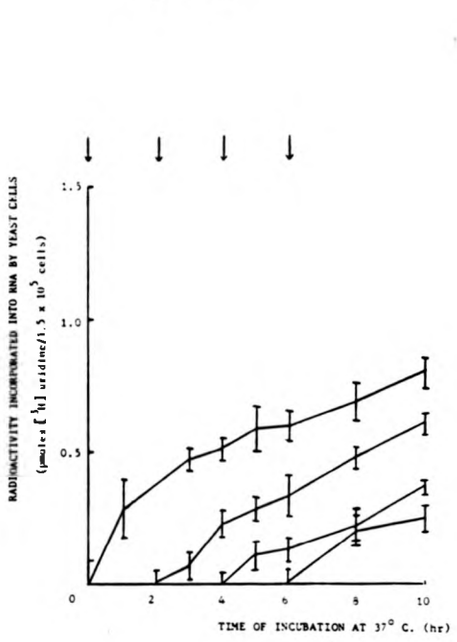


Fig. 6.14

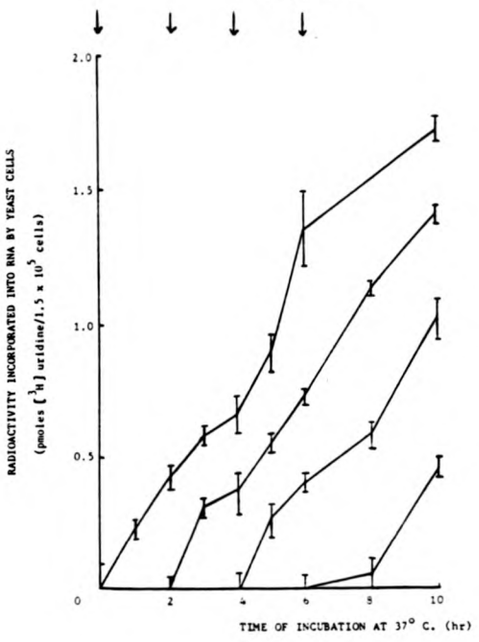
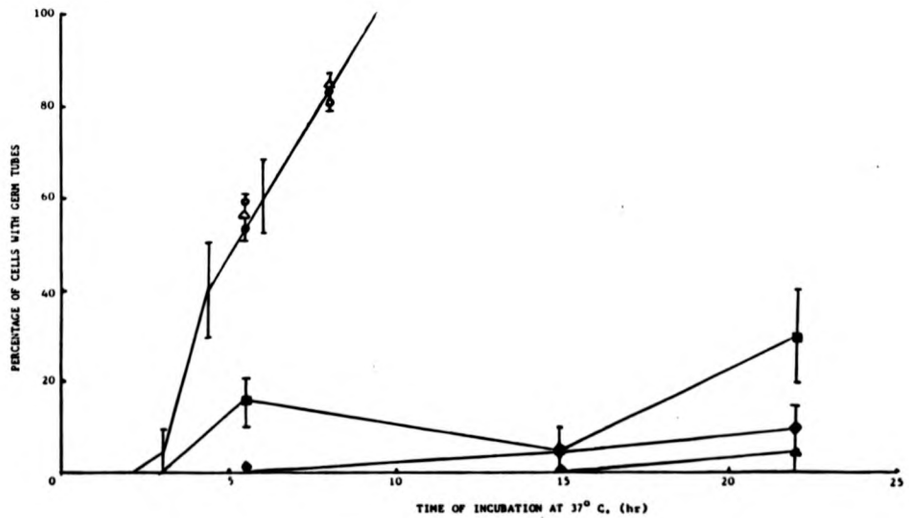


Fig. 6.15



correlation between the two variables.

6.3.5 Inhibitors of transcription and translation on germ tube formation by C. albicans

The effect of some inhibitors of RNA and protein synthesis on germ tube formation by C. albicans is shown in Fig. 6.15. The drugs were added in H_2O or DMSO to the final concentrations indicated in the legend of the figure. The figure shows the effect of the drugs on germ tube formation by C. albicans in buffer containing 1.25 mM NAG. In this buffer all cells possessed germ tubes after 10 hr incubation. Actinomycin D, chloramphenicol or cycloheximide, at the concentrations tested, did not have an affect on the time course for germ tube formation by C. albicans. In the presence of lomofungin, rifamycin AF/013 and rapamycin only a small percentage (approximately 10%) of cells had produced germ tubes after 16 hr incubation. After 22 hr incubation in buffer containing 1.25 mM NAG and 0.2mM rifamycin AF/013 the percentage of cells with germ tubes increased to 30%. Similar results to these found by C. albicans incubating in 1.25 mM NAG containing buffer were found when cells were incubated in buffer containing 1.25 mM glu, 1.25 mM glu plus gln and 2% serum. However, cells incubating in buffer containing 2% serum formed germ tubes after 4½ hr incubation at 37° C..

The effect of inhibitors of transcription RNA synthesis, over a 6 hr period, by C. albicans in suitable buffer for germ tube formation is shown in Table 6.2. Actinomycin D, rifamycin AF/013 and lomofungin all inhibited RNA synthesis by 47% - 99%. These inhibitors had an affect on RNA synthesis by yeast incubating in buffer containing serum, NAG or glu plus gln. It was found that incorporation of [^{3}H] into RNA was inhibited when cells were incubated in buffer containing 2% serum and 0.2 mM rapamycin. Cells incubating in buffer containing 1.25 mM glu plus gln and 1.25 mM NAG showed an increase in incorporation of [^{3}H] uridine into RNA when rapamycin was added to the suspensions. All the drugs, added from stock solutions in DMSO, were added to the suspensions, so the final concentration of the solvent was 1%. Increasing

Table 6.2 Effect of inhibitors of RNA synthesis on incorporation of [^{3}H] uridine into RNA by C. albicans during germ tube formation

Yeast cells of C. albicans were grown in S. and S. medium and stored as described in 2.3.3. After 24 hr, aliquots were diluted, in imidazole buffer, to final cell densities of 1 x 10^5 yeasts/ml. The cells were incubated at 37° C., in buffer containing either 2% serum, 1.25 mM NAG, 1.25 mM glu or 1.25 mM glu plus gln. The inhibitors were added to a final concentration of 0.2 mM in DMSO, to the suspensions. 10 μCi of [5,^{3}H] uridine (specific activity 23 Ci/mmol) was added per ml yeast suspension. After 6 hr at 37°C., the radioactivity incorporated into duplicate 0.5 ml aliquots was measured as described in 2.5.1. The table includes the standard deviations of radioactivity incorporated by the duplicate aliquots. The figures in brackets refer to the percentage inhibition compared to controls (DMSO only added to the suspension).

Figs. 6.16 - 6.20 PAGE of [^{14}C] labelled RNA extracted from C. albicans during germ tube formation in buffer containing serum

C. albicans yeast cells were grown in S. and S. medium and starved for 24 hr as described in 2.3.3. Samples of the cells were diluted, to final cell densities of 3 x 10^5 yeast cells/ml, in imidazole buffer containing 2% serum. 10 μCi of [4, ^{14}C] uridine (specific activity 488 mCi/mmol) was added per ml suspension. The RNA from yeast cells in a volume of 5 ml of suspension, was extracted as described in 2.7.1. The RNA extracted after 1 (Fig. 6.16), 2 (Fig. 6.17), 3 (Fig. 6.18), 4 (Fig. 6.19) and 5 (Fig. 6.20) hr incubation was analysed by PAGE as described in 2.8.1. The distance migrated by the bromophenol blue (4 S), T. pyriformis 25 S, 17 S, 5.8 S and 5 S rRNA and E. coli 23 S and 16 S rRNA species are shown.

Figs. 6.21 - 6.25 PAGE of [^{14}C] labelled RNA extracted from C. albicans during germ tube formation in buffer containing NAG

C. albicans yeast cells were grown in S. and S. medium and starved for 24 hr, as described in 2.3.3. Samples of the cells were diluted, to final cell densities of 3 x 10^5 yeast cells/ml, in imidazole buffer containing 2% NAG. 10 μCi of [4, ^{14}C] uridine (specific activity 488 mCi/mmol) was added per ml suspension. The RNA from yeast cells in a volume of 5 ml of suspension was extracted as described in 2.7.1. The RNA extracted after 1 (Fig. 6.21), 2 (Fig. 6.22) 3 (Fig. 6.23), 4 (Fig. 6.24),and 5 (Fig. 6.25) hr incubation was analysed by PAGE as described in 2.8.1. The distance migrated by the bromophenol blue (4 S), T. pyriformis 25 S, 17 S, 5.8 S and 5 S rRNA and E. coli 23 S and 16 S rRNA species are shown.

Table 6.2

Germ tube inducing component of buffer	Radioactivity incorporated into yeast cells (pmoles[^{3}H]uridine incorporated into RNA/5 x 10^4 cells/6 hr)				
	control (DMSO only)	+ 0.2 mM actinomycin D	+ 0.2 mM rifamycin AF/013	+ 0.2 mM lomofungin	+ 0.2 mM rapamycin
2% serum	0.985 ± 0.011	0.266 ± 0.056 (73%)	0.175 ± 0.016 (82%)	0.121 ± 0.011 (88%)	0.574 ± 0.106 (42%)
1.25 mM glu plus gln	0.294 ± 0.024	0.085 ± 0.011 (71%)	0.014 ± 0.001 (95%)	0.005 ± 0.001 (98%)	0.349 ± 0.049 (-19%)
1.25 mM NAG	0.127 ± 0.021	0.067 ± 0.008 (47%)	0.020 ± 0.001 (84%)	0.014 ± 0.001 (89%)	0.193 ± 0.032 (-53%)

Fig. 6.16

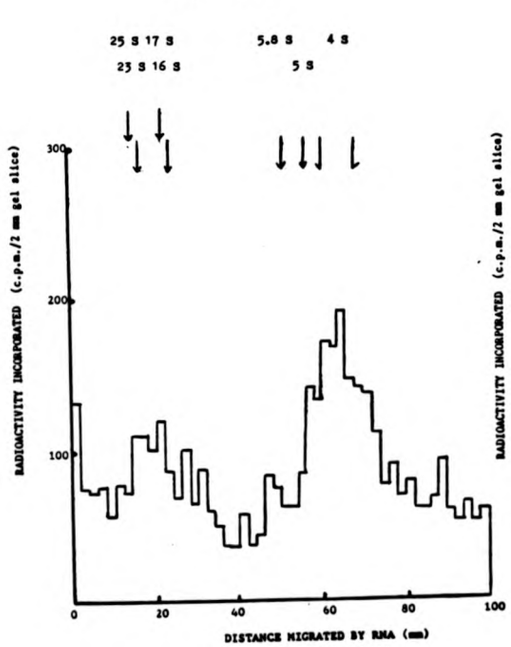
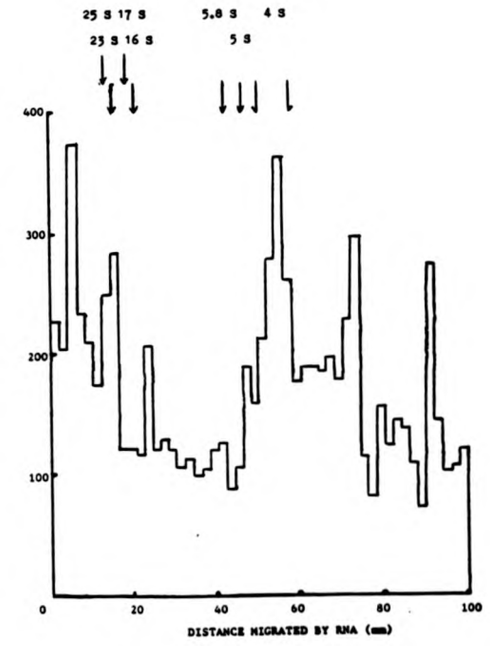


Fig. 6.17



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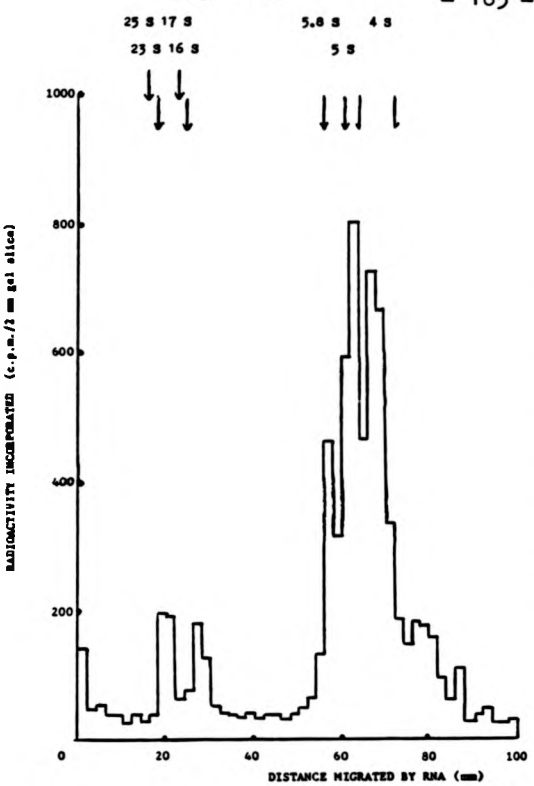


Fig. 6.19

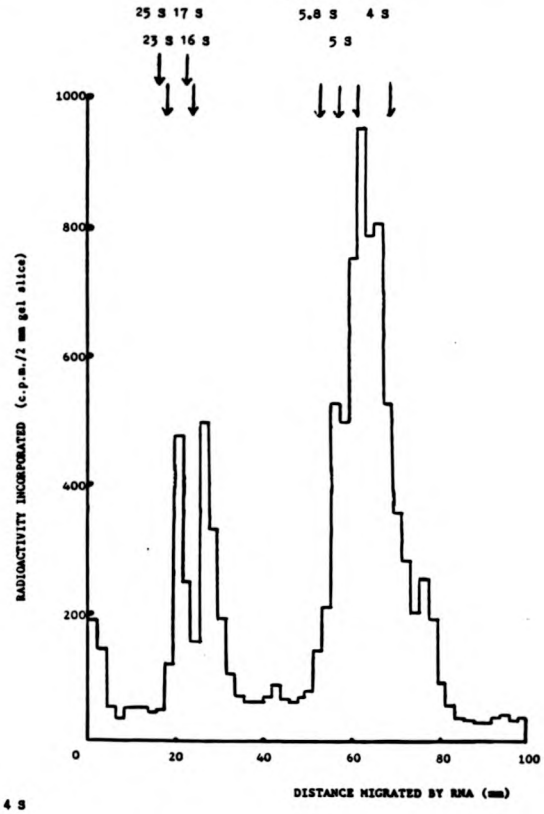


Fig. 6.20

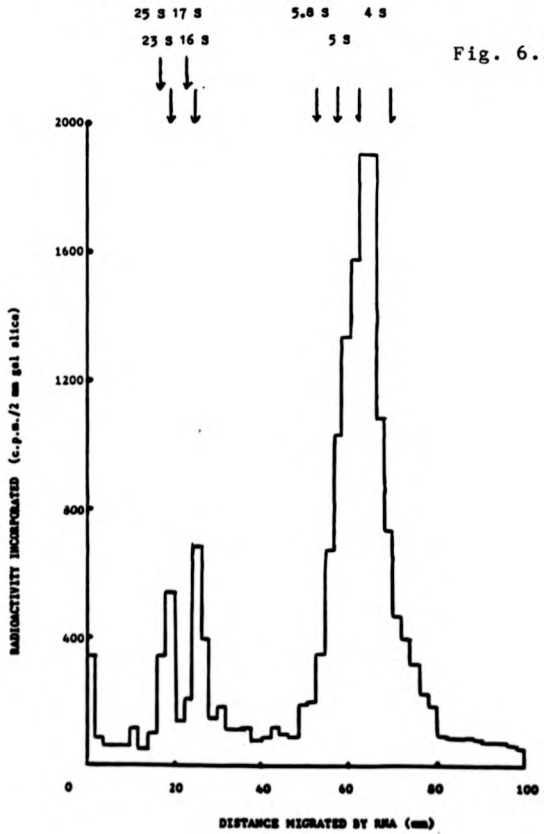


Fig. 6.21

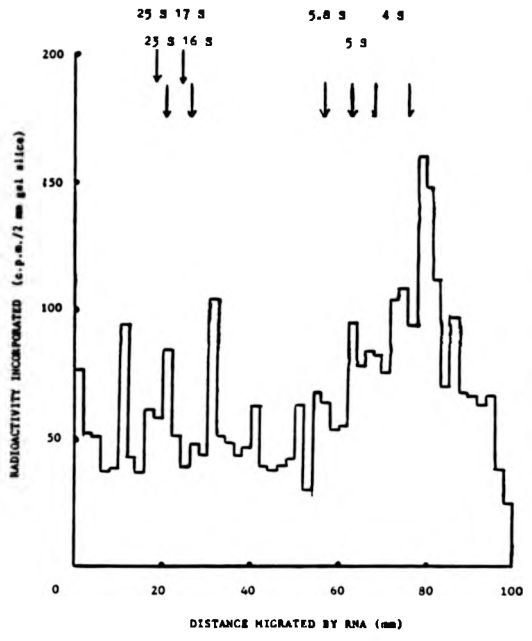


Fig. 6.22

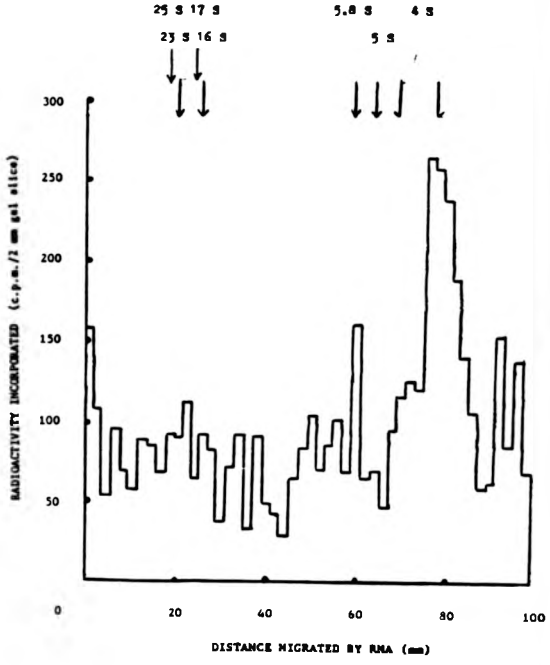


Fig. 6.23

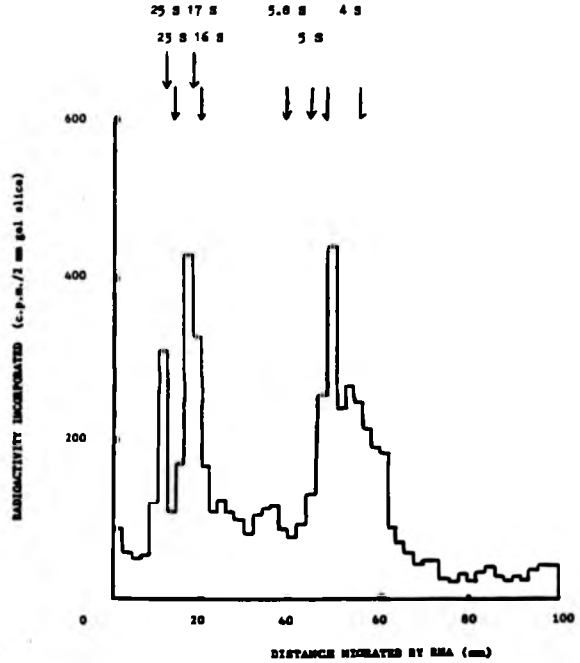


Fig. 6.24

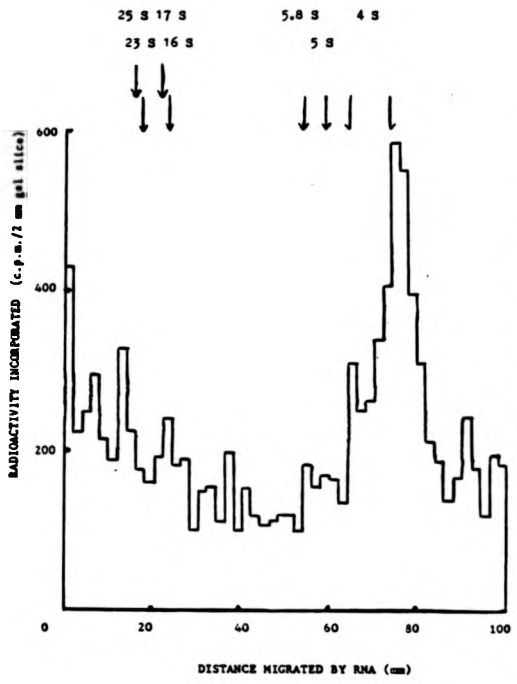
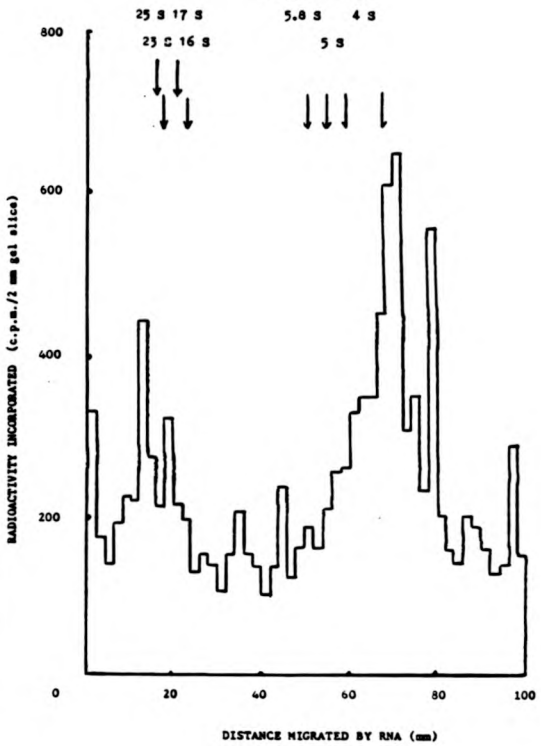


Fig. 6.25



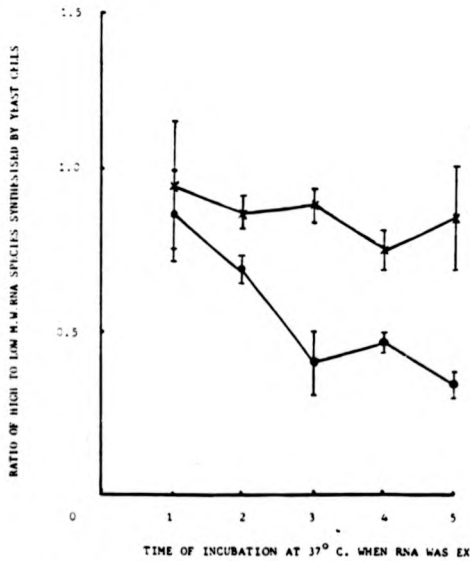


Fig. 6.26 Changes in the ratio of high M.W. to low M.W. RNA species during germ tube formation by C. albicans

RNA synthesised by C. albicans during germ formation in buffer containing either NAG or serum, was labelled, extracted and analysed as described in the legends of Figs. 6.16 - 6.25. The proportion of high M.W. species (larger than 5 S) to low M.W. species (smaller than 5 S) was determined for RNA extracted from C. albicans in buffer containing 1.25 mM NAG (—✕—) and 2% serum (—●—). This ratio was determined by measurement of the c.p.m. in gel slices containing RNA larger and smaller than the 5 S RNA marker species. The position of the 5 S RNA species was estimated from the mobility T. pyriformis 5 S rRNA migrated under the same conditions. The error bars refer to the standard deviations of separate determinations.

the DMSO content of the suspension to 5% or 10% resulted in an inhibition in the ability of cells to form germ tubes in suitable media.

6.3.6 Analysis of RNA synthesised by C. albicans during germ tube formation

RNA synthesised by C. albicans yeast cells incubating in buffer containing 2% serum or 1.25 mM NAG was extracted at various times during the formation of germ tubes. Figs. 6.15 - 6.25 show analysis by PAGE, RNA extracted after 1, 2, 3, 4 and 5 hr incubation at 37° C. of yeast in buffer containing 1.25 mM NAG or 2% serum. The Figs. show peaks of [^{14}C] corresponding to the high M.W., 25 S and 17 S rRNA species and also the lower M.W. (5.8 S and 5 S rRNA and 4 S tRNA) species. The position of these species was estimated from the position marker RNA species migrated (i.e. T. pyriformis 25 S, 17 S, 5.8 S and 5 S rRNA, E. coli 23 and 16 S rRNA and bromophenol blue 4 S). A high proportion of [^{14}C] was associated with the small 5 S and 4 S RNA species. This qualitative observation was confirmed by autoradiography of agarose slab gels containing [^{32}P] labelled RNA. The RNA was extracted during the yeast-mycelial transformation of C. albicans in buffer containing 2% serum or 1.25 mM NAG. Most [^{32}P] labelled RNA migrated as low M.W. species (results not shown).

The proportions of RNA greater or smaller than 5 S rRNA species during the yeast-mycelial transformation of cells in buffer containing 2% serum or 1.25 mM NAG was estimated as described in the legend of Fig. 6.26. The ratio of [^{14}C] in RNA larger than 5 S to that smaller than 5 S fell from 0.95 in that extracted from cells in buffer containing 2% serum after 1 hr incubation to 0.3 after 5 hr. The ratio of [^{14}C] in RNA larger than 5 S to that smaller than 5 S did not change markedly over the 5 hr period the yeast cells were incubating in buffer containing 1.25 mM NAG (i.e. approximately 0.85).

6.4 Discussion

6.4.1 Pretreatment of C. albicans for germ tube formation

In order to achieve reproducible germ tube formation by C. albicans NCPF3153, pretreatment of the yeast inoculum was necessary. The cells had to be grown in nutritionally impoverished media, starved for at least 24 hr and inoculated in suitable germ tube inducing media. The methodology used was based on that described by Shepherd et al (345). This group reported reproducible germ tube formation, by C. albicans ATCC10261, when grown for 20 hr, in glucose-salts-biotin medium, and starved for 24 hr, prior to inoculation in suitable media. The choice of growth medium for C. albicans NCPF3153 was an important parameter for reproducible germ tube formation. It was found that if the yeast was grown in peptone broth (S.D.B.), the cells were unable to form germ tubes, even when starved for 24 hr prior to inoculation in suitable media (Table 6.1).

It was necessary to grow C. albicans NCPF3153 for 50 - 70 hr in glucose-salts-biotin medium. Lower yields, and slower growth rates are found, when C. albicans is grown in defined media compared to complex media. A greater yield of yeast, per unit volume growth media, was obtained when C. albicans NCPF3153 was grown for 50 - 70 hr, compared to 20 - 40 hr growth. The yeast cells were then presumably in the stationary phase of growth as the yield of cells did not change between 50 and 70 hr incubation. It was found that if this strain of C. albicans was grown for 20 - 40 hr, the yeast did not form germ tubes. These results indicate that the growth phase is an important parameter for germ tube formation by this strain of C. albicans. It has been suggested that C. albicans must accumulate in stationary phase - of the G_1 phase of the cell cycle - before germ tube formation, in suitable media, can occur (248,345). However, by no means all groups working on the yeast-mycelial transformation of C. albicans are in agreement. Ahrens et al reported that the strain of C. albicans used in their experiments was able to form germ tubes in the exponential phase of growth. Synchronous germ tube formation occurred when the yeast cells were diluted into suitable media at 37° C. (4). Thus,

no hard and fast rule can be made concerning the growth phase of C. albicans, as a species, for germ tube formation to occur.

A further pre-requisite for reproducible germ tube formation by C. albicans NCPF3153 was that the yeast had to be starved, for at least 24 hr, after harvesting. This was achieved by storing the yeast in 20 mM phosphate buffer pH 5.6. Shepherd et al (345) stressed that starvation was a necessary precondition for reproducible germ tube formation by C. albicans ATCC10261. Starvation of the yeast may be reflected by changes in cellular metabolism that are requirements for C. albicans to form germ tubes. An analysis of the levels of metabolites in C. albicans ATCC10261, during the exponential and stationary growth phases and during starvation has recently been made (368). It was found that, during starvation, the levels of hexose phosphates decreased, whilst the levels of storage polysaccharide (e.g. glycogen) and the size of the free amino acid pools increased. Such changes should be found whenever a strain of C. albicans undergoes germ tube formation for these criteria to be applicable to the species as a whole.

It has long been recognised that inoculum density plays an important role in the ability of C. albicans to form germ tubes. In order to obtain a high percentage of cells with germ tubes a small inoculum was required (see Table 6.1). These results are in agreement with the general findings reported in the literature. Typically, a low inoculum (1×10^5 - 5×10^5 yeast/ml media) is required for C. albicans to form germ tubes (110). It is thus somewhat unusual that Shepherd et al (345) found that high inocula (0.8×10^8 to 1.2×10^8 yeast/ml media) were able to form germ tubes. It has recently been found that yeast C. albicans, at high cell densities, produced a substance Hazen and Cutler termed "morphogenic auto-regulatory substance" ("MARS") (159). This compound was found in the growth media of yeast at high cell densities (1×10^8/ml). MARS was capable of suppressing germ tube formation by yeast in media that normally promoted this phenomenon. Hazen and Cutler later reported that this compound is probably a proline analogue (160).

The important preconditions required for reproducible germ tube formation

by C. albicans NCPF3153 may thus be summarised: Firstly, the yeast must be grown to stationary phase in nutritionally impoverished media. Secondly, the yeast must be starved before a small inoculum may be added to suitable buffered media.

6.4.2 Media used to induce germ tube formation by C. albicans

Several stimulating agents could be added to C. albicans NCPF3153 which resulted in the buffered suspensions forming germ tubes. Two important requirements for formation of germ tubes were the presence of a suitable inducing agent in the buffer and an incubation temperature of 37° C..

It is now firmly established that the temperature for incubation of the yeast is a very important parameter in the formation of mycelia. However, this is not the single environmental stimulus that triggers morphogenetic conversion as it does for other dimorphic fungi, (e.g. H. capsulatum (203)). The existence of strain variation argues against such a single, universal stimulus . The requirement for high temperatures (typically greater than 35° C. (66)) may reflect a need for membrane fluidity by yeast C. albicans before germ tubes may be initiated - the first visible sign of the yeast-mycelial transformation. It was found that if C. albicans NCPF3153 was incubated at temperatures below 35° C. (i.e. 30° C.) the yeast did not form germ tubes.

Germ tube formation could be induced by incubation of C. albicans NCPF3153 in imidazole HCl buffer pH 6.6 containing 0.2 mM $MnCl_2$ and either serum, NAG, glu plus gln or glu only. Shepherd et al also reported that the same buffer containing NAG or glu plus gln could stimulate starved C. albicans yeast to produce germ tubes. However, this group reported that C. albicans was unable to form germ tubes in the presence of glu only (345). Hrmova et al (167) recently reported that low concentrations of glu - in the presence of monorden (an antibiotic) - stimulated germ tube formation by C. albicans. This same group reported that higher (100 mM) concentrations favoured unicellular yeast growth. The result that low (i.e.less than 25 mM) glu concentrations stimulated germ tube formation by C. albicans was similar to the results reported in this

thesis (see Fig. 6.3). The addition of other hexoses, such as fructose or galactose, to the buffered yeast suspension did not induce germ tubes by C. albicans NCPF3153.

Observation under the light microscope revealed that floculation of the cells occurred at the same time as germ tube initiation. This was found whatever system was used to induce germ tubes by C. albicans. This made estimation of cell numbers difficult - thus growth had to be monitored by other means. Other groups have commented on this phenomenon during germ tube formation and have tried various methods to disrupt the clumps of cells. These have included the addition of inositol, bovine serum albumin, Tween 80 or Triton X-100 to yeast suspensions (345). Unfortunately, these attempts proved unsuccessful. Sonication did successfully disrupt the cell clumps. However, it also resulted in the shearing of the germ tube from the cell. Clumps of cells are seen during mycelial formation by growth of daughter blastospores along the hyphal shoot (see 1.2.1 and 2.3.4). The aggregation of yeasts, reported in this thesis, probably reflected flocculation of single blastospores rather than growth of daughter cells. An apparent decrease in cell numbers, per unit volume, was found when cells were observed by light microscopy, using a haemocytometer, during germ tube formation. In addition, measurement of the absorbance of the cell suspensions during germ tube formation did not show any marked increase in the optical density. This contradiction, i.e. an increase in cellular material - germ tubes - not reflected in an increase in absorbance of the cell suspension - will be discussed below.

Methods for measuring increases in cellular growth include dry weight estimations, cell counts, DNA estimations and optical density. Measurement of dry weight and DNA content of the yeasts would have proved difficult. Assuming a dry weight of 40 pg for a yeast cell (see results in 4.3.2) it was necessary to obtain 2.5×10^8 cells for measurable (10 mg) quantities. This represents all the cells in a total volume of 500 ml (assuming a cell density of 5×10^5 cells/ml - as low cell densities are required to successfully obtain a high proportion of cells with germ tubes). Practical problems arise

when attempting to harvest such low cell densities from such large volumes of media. These practical problems are applicable to DNA estimates; in order to obtain measurable - i.e. more than 10 µg DNA - it was necessary to extract the DNA from 10^8 - 10^9 cells (see 2.6.1). Cell aggregation makes estimates of cell numbers by haemocytometry difficult - as described in the preceeding paragraph. Thus, measurement of the optical density appeared to offer a quick and reliable estimation of cell growth.

A variety of stimulating agents could induce germ tube formation by C. albicans. Yeast incubated in imidazole buffer containing 5% serum showed the most rapid ability to produce germ tubes. In this buffer all yeast cells had germ tubes within 3½ hr incubation at 37° C.. As a comparison, a small inoculum of yeast, incubated in 100% serum, all produced germ tubes within 3 hr incubation at 37° C. (see 2.3.4). Fig. 6.1 showed that a decrease in the serum content of the buffer resulted in an increase in the time taken for all the cells to produce germ tubes. These results indicated that serum possessed component(s) which stimulated the production of germ tubes by starved yeast cells in a dose-response manner. Serum - from both human and animal sources - has long been recognised as a medium capable of inducing germ tube formation (227). Barlow et al (21) reported a protein isolated from seminal plasma and serum of low molecular weight (2,000 - 3,000) which would induce germination in low concentrations (67). It is this component of serum which may be responsible for inducing germ tubes by the yeast cells.

The results of Fig. 6.1 showed that the absorbance of the cell suspension increased only slightly over the 22 hr incubation period. Varying the concentration of serum in the medium did not affect the slight increase in optical density over the 22 hr incubation period. The absorbance of the cell suspension did not change markedly over the period the cells were forming germ tubes. This seems to be a contradictory finding to that expected, i.e. an increase in cellular material - germ tubes - not reflected by an increase in absorbance.

Formation of a germ tube from a yeast cell will only reflect a small percentage increase in total cellular material of the yeast. A small increase

of cellular material would be compounded by the low absorbance of the cell suspension at the start of the incubation. This may be illustrated by the observation that a cell suspension of 2×10^5 cells/ml had an absorbance of 0.15 O.D. units at 650 nm. As the absorbance of the cell suspension only increased slightly over the 22 hr period of incubation, this indicates that there was no full development to the mycelial state. This was confirmed by observation of the cells after 22 hr incubation by light microscopy. This revealed the yeast produced germ tubes, with a length of 15 - 30 μm. Altering the concentration of serum in the buffer did not affect the length of the germ tubes. These results seem to be in contrast to those of Chiew et al (70). This group reported that development of C. albicans in complex media, such as serum, continues to the multicellular mycelial state. However, it should be borne in mind this group used 100% serum as the medium used to induce germ tubes. In this research fairly low concentrations (less than 5%) of serum were added to buffer to induce germ tube formation. It is possible that development to the multicellular hyphal form requires a medium containing components for growth, in addition to stimulating agents.

Possibly the simplest media to date that has been described for the successful induction of germ tubes is a buffered solution containing the hexosamine NAG. More than 90% of the cells had formed germ tubes when an inoculum was incubated for 10 hr, at 37° C., in NAG-containing buffer (Fig. 6.2). Altering the concentration of NAG in the buffer did not have any effect on the time course for germ tube formation. It has been hypothesised that the capability of NAG to trigger germ tube formation is related to an activation of the synthesis of chitin (39, 349). Chitin is a biopolymer consisting of (1 - 4) linked NAG subunits. In addition to glucans and mannans, the cell wall of C. albicans has a small amount of chitin. About 1% in the yeast and 5% in the mycelia form of total cell wall polysaccharide is chitin (23). This polymer appears to be important to the growth process as the bud ring and septum are predominantly chitin (54) and it is an important component in the growth of hyphal tips (22).

The formation of germ tubes has been accompanied by an increase in activity of such enzymes as chitin synthetase (22) and other enzymes of NAG metabolism, e.g. glutamine fructose-6-phosphate amino transferase (71), N-acetylglucosamine-6-phosphate deacetylase (296). Recent studies have shown that other N-acetyl hexosamines, such as N-acetyl mannosamine, may gratuitously induce germ tube formation with glucose (369). Indeed, it has been found that NAG need not enter a cell to induce germ tube formation (347) and the subsequent increase in the activities of the enzymes associated with NAG metabolism. However, these enzymes are probably not control points for germ tube formation as the increases in activity may be induced by NAG whether or not C. albicans yeast undergoes dimorphic change (126). Also, the activities of enzymes concerned with cell wall "re-modelling" (chitin synthetase and chitinase) have been found to increase during the exponential phase of growth (22).

The time taken for all the yeast cells of C. albicans strain NCPF3153 to produce germ tubes was much longer than that found by Shepherd et al (345). This group used a different strain of C. albicans to that used here. In addition, this group reported that the time course for germ tube formation was the same whether the yeast was incubated in buffer containing NAG, serum or glu plus gln. This group reported buffer containing gln and other hexoses (such as D fructose, D galactose and D mannose) was also capable of inducing germ tubes by C. albicans (345). However, a lower percentage of yeast had germ tubes when incubated in these buffers compared to the NAG or glu plus gln systems.

Low concentrations of glu plus gln (both at 0.25 or 1.25 mM) could induce germ tube formation by C. albicans NCPF3153 (Fig. 6.3). The time course for all cells to form germ tubes was similar to that buffer containing NAG. However, at higher (25 mM) glucose and glutamine concentrations no germ tube formation was observed by the yeast. The reversible nature of the yeast mycelial transformation was illustrated by incubating cells in buffer containing 5 mM glucose and glutamine. In this buffer approximately 50% of cells had developed germ tubes after 7½ hr incubation at 37° C.. 8½ hr later no yeast

cells were visible which had germ tubes.

A two fold increase in optical density at 650 nm over a 22 hr incubation period of cells suspended in buffer containing 25 mM glu plus gln was found (Fig. 6.3). This indicated an increase in the amount of cellular material of yeasts in this buffer. The absorbance of cells suspended in buffer containing 5 mM glucose and glutamine also increased over the same period. However, the increase in O.D. was not as great as for cells in buffer containing the higher (i.e. 25 mM) concentrations of glu plus gln. The absorbance of yeast incubating in buffer containing serum, NAG, glu or glu plus gln (at a concentration of 0.25 mM and 1.25 mM) did not increase over 22 hr. This indicated that there was no marked increase in cellular material in these buffers. Hence, germ tube formation by C. albicans, in these systems, was not accompanied by development to a multicellular state. Shepherd et al (345) also reported that incubation of cells in buffer containing NAG or glu plus gln was not accompanied to full mycelial development. However, this group did report that development to the multicellular hyphal state did occur when yeast cells were incubated in buffer containing serum.

The time course for all yeast to form germ tubes was similar in buffer containing NAG, low concentrations of both glu plus gln and glu only. All cells had germ tubes within 10 hr of inoculation. This suggests that similar mechanisms were responsible for germ tube formation by C. albicans NCPF3153 when incubated in these "simple" media.

It is apparent that some of the results reported here, in this thesis, are different from those found by other groups. Johnson et al (183) have found that the formation of germ tubes in suitable buffer (i.e. glucose plus glutamine) was strain dependent. This group used small inocula to achieve germination of the susceptible strains at 37° C.. As noted in 6.1, almost as many strains of C. albicans. and conditions described "suitable" for the induction of germ tubes, have been reported as there are groups working in the field. Thus, it is not surprising that many apparently conflicting results have been found. As the formation of germ tubes is the first indication of

morphogenesis many studies on the phenomenon have investigated the cell wall and membrane and any changes therein. Unfortunately, there does seem to be a paucity of information regarding the involvement of RNA synthesis. The next section discusses the results obtained when this process was investigated during germ tube formation.

6.4.3 Protein and RNA Content during the Yeast-Mycelial Transformation of C. albicans

It was hoped that studying RNA synthesis before, during and after the formation of germ tubes would add to the knowledge of this phenomenon. In order to confirm that RNA content was increasing - as found by Shepherd et al (345) - the levels of total RNA in the cell were measured before and during germ tube formation (Fig. 6.5). The orcinol method was used to estimate the levels of RNA in yeast cells in buffer containing 2% serum and 1.25 mM NAG. It should be noted that serum contained components that would react with the orcinol reagent. This problem was easily overcome by the use of "blanks" in the assay for the RNA content, containing buffer and 2% serum treated as the samples as cited in the legend of Fig. 6.5.

The RNA content of the yeast cell was estimated as 0.64 pg at the start of incubation. In buffer containing 2% serum all cells possessed germ tubes after 4½ hr incubation at 37° C.. After 6 hr incubation the RNA content had increased over three fold to 2.10 pg/cell. The RNA content of yeast cells in 1.25 mM NAG containing buffer increased to 1.56 pg/cell over the same period. Yeast cells in this buffer all produced germ tubes after 10 hr incubation at 37° C.. Thus, two firm conclusions are that RNA content/cell increased during the formation of germ tubes and, in buffer containing serum, the RNA content still increased after all the cells possessed germ tubes.

It is worth commenting on the qualitative and quantitative levels of RNA per yeast cell during the yeast mycelial transformation. Qualitatively, the results shown in Fig. 6.5 for yeast in serum containing buffer are similar to those found by Shepherd et al (345), i.e. a trebling in the RNA content per cell over a 6 hr incubation period at 37° C.. However, quantitatively the

RNA content per yeast cell reported by this group of 50 fg was much lower than that reported in this thesis. In complete contrast the RNA content of C. albicans has been estimated elsewhere at 8 pg/yeast cell (389). Such high values for total cellular RNA seem typical for Candida spp as a whole, with RNA accounting for approximately 15% of the dry weight of the cell (123, 185, 246) (Assuming a dry weight of 40 pg per cell, the average RNA content of Candida spp, in these reports, was approximately 6 pg/cell). The value of 0.64 pg/yeast reported here is rather lower than that found in the reports cited. It has been found that the RNA content of yeast cells has been found to decrease on "ageing" from 15% to 5% of the dry weight (7). Thus, starvation may have accounted for the apparently "low" RNA content of C. albicans yeast reported here. As a further comparison, the RNA content of S. cerevisiae - an organism often compared with C. albicans (e.g. 345) - was estimated as 3.9 pg/cell (32).

In contrast to the finding that RNA content increased markedly during germ tube formation by C. albicans, the protein content per cell only increased slightly (Fig. 6.6). Thus, it would appear that RNA synthesis was not followed by an increase in protein content. The method used for estimation of protein content - i.e. that of Lowry et al (see 2.6.3) - was based on the quantitative colorimetric measurement of tyrosine and tryptophan and the peptide bonds in a protein. This would only measure the total protein content of the cell; no increase in protein content would be apparent if there was also an increase in protein degradation. In addition, this technique may only be sufficiently sensitive to monitor marked changes in protein content, i.e. a greater than 30% increase in newly synthesised protein.

Previous data on protein content on yeast form cells, germ tubes and hyphae of C. albicans reported by other groups has been somewhat conflicting and contradictory. Some workers have reported that there is a decrease in protein content of yeat during germ tube formation (64), whilst others maintain there is an increase (345). It should be emphasised that the data cannot be readily compared due to the different conditions and times by which

germ tubes of different strains of C. albicans are formed.

Undoubtedly, de novo synthesis of proteins does occur in C. albicans during germ tube formation. It has been found by several groups of workers that high levels of labelled precursor amino acids are rapidly incorporated into protein over this period (4, 42, 43, 234). In addition, germ tube formation by C. albicans, in suitable media, may be prevented by the addition of protein synthesis inhibitors, such as trichodermin and cycloheximide (345).

Alterations that occur during morphogenesis in protein synthesis have prompted several workers to try and identify and quantitate any phase specific proteins. Manning and Mitchell (234) used two dimensional (2 D) gel electrophoresis to analyse labelled proteins, synthesised during germ tube formation. The proteins were extracted from two different strains of C. albicans, growing at two different temperatures, which resulted in germ tube formation in the susceptible strain. This group found that there did not appear to be any major differences between the major protein components of the cell. However, they did report the existence of 33 yeast and 10 mycelial specific proteins. The mycelial proteins were found to have similar antigenic determinants to those in the yeast form (235). This indicated that the 10 proteins apparently specific to the mycelial form may have been modified from proteins also found in the yeast form. Brown and Chaffin (42) also reported the existence of yeast form specific proteins, on analysis of approximately 230 proteins, by 2 D gel electrophoresis. This group used a temperature shift to induce germ tube formation by C. albicans from which only 5 proteins, specific to the yeast, were observed. The high number of apparent specific yeast form proteins found by Manning and Mitchell (234) almost certainly includes some proteins specific to the different strains.

The existence of yeast phase specific proteins is in contrast to the results of Brummel and Soll (43). These workers reported there appeared to be no major differences between proteins synthesised during "germ tube formation" and blastospore development. However, examination of this report reveals that these workers may have been studying pseudo-mycelial development, rather than germ tube formation. Work recently published has complicated rather than

clarified the position over form-specific proteins. Ahrens et al reported both yeast and mycelial specific proteins (4). This group used a pulse/chase method of protein labelling and temperature induced germ tube formation of yeast cells growing in exponential phase. This group also analysed proteins precipitated by a low speed centrifugation, in addition to those in cell free extract which the other groups (42, 43, 234) discarded. These groups had labelled proteins with either [^{35}S] or [^{3}H] precursors and induced germ tube formation from cells in the stationary phase of growth. The major problem with the attempts to identify phase specific proteins is that differences in environmental conditions and strain variation have been used as a basis for the induction of germ tubes. Thus, one should be aware of this problem when trying to draw any firm conclusions from these workers research.

It seems reasonable to suggest that proteins newly synthesised during the formation of germ tubes will include those associated with NAG metabolism. As noted in 6.4.2 polymerised NAG subunits form the important cell wall component chitin. It was mentioned on page 179 that the increase in activity of these enzymes was sensitive to protein synthesis inhibitors. In addition, the development of suitable systems for the uptake of inducing agents, like NAG, has also been found to be sensitive to protein synthesis inhibitors (126). One may expect, as germ tube formation is a cell wall morphogenesis, the synthesis of cell wall and membrane associated proteins.(e.g. chitin synthetase (73) and β(1 - 3)glucan synthetase (125)). Recent work (60) has indicated that these cell wall proteins are the same in both phases of C. albicans.

As the majority of proteins from both phases of the fungus are similar, it is possible that any different less abundant proteins may serve a regulatory function. Brown and Chaffin (42) noted that the total proteins observed in their analysis represented a small proportion of the total genetic coding capacity of C. albicans - only those relatively abundant were visualised. Thus, the dimorphism of C. albicans may be regulated by hyphal or yeast phase specific proteins or proteins that modify proteins already present.

Knowledge that protein synthesis may be regulated by cyclic nucleotide

levels, has led several groups to investigate possible roles for these compounds in dimorphic transformations. Larsen and Sypherd (208) first remarked on a correlation between intracellular cAMP levels and the morphological form of the fungus Mucor racemus. (Later research by the same group indicated that cAMP levels were not the sole factor for controlling mycelial to yeast development (283)). The dimorphic fungus H. capsulatum is reported to have five times the cAMP concentration in the mycelial compared to the yeast form (237).

It has recently been found (68, 276) that cAMP levels increased to at least twice those of yeast C. albicans prior to germ tube formation. In addition, cAMP may induce the germination of 60% yeast cells under conditions that normally did not result in germ tube formation. However, evidence implicating cAMP in the phenomenon of germ tube formation is by no means unequivocal. Sullivan and Shepherd (369) reported that the cAMP levels did not change over the period of germ tube formation in buffer containing serum. This group believed that the conflicting results were due to the cellular metabolism adjusting to the effect of the nitrogen rich sources, used by Nimii et al (276) and Chattaway et al (68), for inducing the yeast-mycelial transformation. Thus, the precise aetiological relationship between cAMP levels and the yeast-mycelial transformation remains unclear.

6.4.4 RNA synthesis by C. albicans during the yeast-mycelial transformation

De novo RNA synthesis by C. albicans during and after germ tube formation was measured by the incorporation of labelled uridine into TCA insoluble material. Essentially similar conditions to those described in 3.3.2 were used to measure this process in yeast cells and protoplasts.

An interesting result was found when exponential phase yeast cells were incubated in protoplast buffer (containing 50 mM fructose) at 37° C. and 1 nmole of [^{14}C] uridine. It was found that 29 pmoles of [^{14}C] uridine were incorporated by 2.5×10^7 yeast cells over 40 min incubation at 37° C.. If one assumed a linear rate of incorporation, over the 40 min, 2.9×10^{-10} moles

of [^{14}C] were incorporated min^{-1} per exponential phase yeast cell. As a comparison, the level of [^{14}C] incorporated into C. albicans in germ tube inducing media was measured. It was found 71, 23 and 12 pmoles of [^{14}C] were incorporated by 2.5 x 10^5 cells in buffer containing 2% serum, 1.25 mM glu plus gln and 1.25 mM NAG respectively. Assuming linear incorporation of label into RNA over the assay period, the rate of incorporation may be found. 1.6 x 10^{-18}, 5.1 x 10^{-19} and 3.2 x 10^{-19} pmoles of [^{14}C] were incorporated min^{-1} by yeast in buffer containing serum, glu plus gln and NAG. These values represent levels of incorporation of [^{14}C] into RNA 55, 17 and 11 times that of cells harvested in the exponential phase of growth. It should be borne in mind that any attempt at comparing relative rates of RNA synthesis, based on incorporation of label into RNA, should take into account any effects due to dilution of the label in the precursor pools. Thus, the higher rates of [^{14}C] incoporation into RNA by yeast during germ tube formation, compared to exponential phase, may reflect less dilution of the label in the pools rather than greater levels of RNA synthesis.

The effect of altering the serum content in the imidazole buffer and the concomitant effect on labelled uridine incorporation by yeast cells, during the yeast-mycelial transformation, was as shown in Fig. 6.7. Increasing the concentration of serum in the buffer increased the rate for all the cells to possess germ tubes (see Fig. 6.1). In addition, increasing the serum content increased the rate, and maximum amount, of [^{14}C] uridine incorporated into RNA. Thus, higher levels of RNA synthesis were associated with conditions that promoted the most rapid germ tube formation. RNA synthesis continued after all the cells had formed germ tubes. This may reflect RNA synthesis required for elongation of the hyphal shoot until development ceased.

It was found that the level of [^{14}C] incorporated by the yeast into RNA decreased after 16 hr incubation in buffer containing any of the concentrations of serum tested. Two explanations could account for this decrease in the level of [^{14}C] incorporation. Firstly, degradation of the labelled RNA (synthesised by the yeast cell since the start of incubation) could occur as

part of the natural turnover of RNA. If unlabelled uridine, synthesised by the yeast itself was used as the precursor for new RNA the level of [^{14}C] incorporated would decrease. Alternatively, RNA synthesis by the yeast cell may have slowed, or terminated, with no replacement of RNA that had been degraded by RNases. The former point would imply that exogenous label was diluted to a greater and greater extent as the incubation progressed. (This ought to be reflected by a non-linear increase in incorporation of label into RNA).

A pertinent question concerns the extent the label was diluted by the pool of "cold" precursor uridine. Labelled uridine was added, at different times during germ tube formation, to investigate if the precursor was diluted to the same extent over this period. When [^{3}H] uridine was added to the suspension, after 0, 2, 4 and 6 hr incubation, the label was immediately incorporated into RNA at similar linear rates (Fig. 6.11). One may tentatively conclude that there was little change in the rate of incorporation of label into the intracellular pool and thence into RNA.

The effect of different concentrations of NAG, in the imidazole HCl buffer, on RNA synthesis by C. albicans incubating at 37° C. is shown in Fig. 6.8. It was found that altering the concentration of NAG did not markedly alter the time course for incorporation of labelled uridine into RNA. Incorporation of [^{14}C] uridine into RNA of cells incubating in buffer containing NAG increased with time for 6 hr incubation at 37° C.. After this period the level of [^{14}C] incorporated into RNA remained at a similar level for the next 14 hr. A time course for germ tube formation, in NAG containing buffer, showed all yeast had produced germ tubes after 10 hr incubation (Fig. 6.2). Thus, RNA synthesis, by yeast in NAG-containing buffer, was associated with germ tube formation by the cells.

Addition of [^{3}H] uridine to yeast in buffer containing NAG, after 0 and 2 hr incubation at 37° C., revealed non-linear incorporation of [^{3}H] uridine for 6 hr (Fig. 6.12). After 6 hr there was no change in the level of [^{3}H] uridine incorporated into RNA. When labelled uridine was added to the cell

suspensions, after 4 and 6 hr incubation, little or no incorporation of label into RNA after 6 hr, was found. These findings could be explained by the level of RNA synthesis, by yeast, decreasing as the incubation - and germ tube formation - progressed. These findings suggest RNA synthesis, by C. albicans during germ tube formation in NAG-containing buffer, was linear for the first 6 hr. Label was incorporated from the medium into RNA, without dilution by the precursor pools. However, after this period there was a decreasing rate of incorporation of label into RNA and an increasing dilution effect of the pyrimidine pools.

The effect of varying the concentration of glu in the imidazole buffer on RNA synthesis by yeast cells is shown in Fig. 6.9. A time course showed that [^{14}C] incorporation into RNA increased with time over the 22 hr period of incubation at 37° C.. Varying the concentration of glucose, in the buffer, did not alter the rate of incorporation of [^{14}C] uridine into RNA by yeast. Uridine incorporation into RNA proceeded at a similar rate to that found by cells in NAG containing buffer for the first 8 hr incubation. However, over the next 12 hr incubation, the amount of [^{14}C] incorporated into RNA by cells in glu-containing buffer increased. In this buffer, yeast C. albicans formed germ tubes after 10 hr incubation at 37° C. (Fig. 6.4). Thus, RNA synthesis was associated with germ tube formation and continued after all the cells had produced germ tubes. If [^{3}H] uridine was added to yeast at different times of incubation in glu-containing buffer, the label was immediately incorporated at a similar linear rate (see Fig. 6.13). Thus, incorporation of the radioactively labelled precursor into the pyridine pool, and then into RNA, proceeded at a linear rate for the first 10 hr incubation.

Yeast incubating in buffer containing NAG or glu all produced germ tubes with a length of 15 - 30 μm. Incubation in these buffered media was not accompanied by development of the yeast to the multicellular hyphal state. Hence, the initial stages of the yeast-mycelial transformation by C. albicans in buffer containing NAG or glucose were similar in these respects. However, in the latter buffer C. albicans continued the synthesis of RNA long

after this process had slowed/stopped on incubation in NAG containing buffer.

The effect on incorporation of [^{14}C] uridine into RNA by C. albicans in buffer containing different concentrations of glu plus gln is shown in Fig. 6.10. Increasing the concentration of glu plus gln in the buffer increased the rate of incorporation of label into RNA for the first 12½ hr of incubation. After this period, the level of [^{14}C] incorporated into RNA by yeast in buffer containing 25 mM glu plus gln decreased over the following 10 hr. The level of [^{14}C] incorporated into RNA by yeast in buffer containing 5 mM glu plus gln decreased after 16 hr incubation at 37° C.. It was found cellular growth (as measured by absorbance - see Fig. 6.3) increased after 16 - 22 hr incubation of C. albicans in buffer containing 25 mM and 5 mM glu plus gln. Thus, the decrease in [^{14}C] incorporation into RNA appeared to be associated with a period of growth by the yeast. This contradiction could possibly be explained by natural turnover of the RNA synthesised (and hence labelled) since the start of the incubation. If this RNA was replaced with RNA containing unlabelled nucleotides, synthesised by the yeast using unlabelled precursors, the level of [^{14}C] incorporation would decrease. The level of [^{14}C] uridine incorporated into yeast, in buffer containing either 1.25 mM or 0.25 mM glu plus gln, increased over the 22 hr incubation period. Qualitatively, this result is similar to that found for yeast incubating in buffer containing low concentrations of glu. RNA synthesis by C. albicans in buffer containing low concentrations of glu plus gln was associated with germ tube formation. Incorporation continued after all the cells possessed germ tubes. Quantitatively, higher rates of [^{14}C] incorporation were found, when yeast cells were incubated in buffer containing 1.25 mM glu plus gln compared to yeast in buffer containing glucose only. When labelled uridine was added to yeast, after various times of incubation in buffer containing 1.25 mM glu plus gln, it was incorporated immediately into RNA at similar rates (see Fig. 6.14). Thus, the [^{3}H] uridine was incorporated into the precursor pool and then into the RNA at a similar rate when added to the suspension.

Perhaps the overall conclusions from the results shown in this section, is that RNA synthesis is associated with germ tube formation by C. albicans NCPF3153.

In contrast to the findings of Shepherd et al (345), it was found that the presence of different inducing agents, at various concentrations, resulted in different rates of incorporation of labelled uridine into RNA. Shepherd et al (345) reported that incorporation of label into RNA by cells incubating in "simple" (i.e. NAG or glu plus gln) buffered media ceased when 60% of the cells possessed germ tubes. It was reported here that only cells in buffer containing NAG showed a similar qualitative result. In more "complex" tissue culture media (e.g. serum), Shepherd et al reported a continuation of RNA synthesis until the yeast developed to the hyphal state. RNA synthesis continued after germ tube formation, when C. albicans NCPF3153 was incubated in buffer containing serum, glu plus gln and glu only. However, full development to the multicellular state did not occur after the cells formed germ tubes. Thus, the level, and rate, of labelled uridine incorporation into RNA was dependent upon the buffered medium, in which the yeast cells were incubating, to a greater extent than the cells ability (or otherwise) to form germ tubes.

6.4.5 The effect of inhibitors of transcription and translation on germ tube formation by C. albicans

In order to further examine the involvement of RNA synthesis in the yeast-mycelial transformation, the effect of several inhibitors of transcription on germ tube formation was investigated. The results obtained in Fig. 6.15 show the effect of some inhibitors of transcription and translation on germ tube formation by yeast cells incubating in buffer containing 1.25 mM NAG. A similar result for time course and sensitivity of germ tube formation to the drugs was found for cells in 1.25 mM glu plus gln and 1.25 mM glu only. Cells in buffer containing 2% serum exhibited similar sensitivities to the drugs, although the time course for germ tube formation was more rapid. It should be noted that the figure shows the effects of the drugs on germ tube formation and not on elongation.

Inhibitors of RNA synthesis were added to the cell suspension, at the commencement of incubation, to final concentrations of 0.2 mM. The drugs were added

in DMSO, so the final concentration of the solvent was not greater than 1%. It was found that DMSO concentrations higher than this had a deleterious effect on the ability of cells to form germ tubes. This was reflected in the lower percentage numbers of cells (i.e. 50%) having germ tubes, after 10 hr incubation in buffer containing higher (i.e. 5%) concentrations of DMSO compared to control cultures. It is well known that DMSO is a solvent that can alter the integrity of the cell membrane (see 5.4.2 and also 322). It is this property that is presumably responsible for the effect on germ tube formation, when concentrations of DMSO greater than 1%, were added to cell suspensions. The yeast-mycelial transformation manifests itself visually as an outgrowth from the membrane. In addition, RNA synthesis by cells was inhibited by concentrations of DMSO as low as 10% when incubated in buffer containing components which promoted germ tube formation.

It has been noted in the literature that inhibitors of RNA synthesis can inhibit germ tube formation (126,280,345). Indeed, it was found (Fig. 6.15) that lomofungin, rifamycin AF/013 and rapamycin all had an effect on germ tube formation by C. albicans. Both lomofungin and rifamycin AF/013 inhibited RNA synthesis by yeast, in suitable germ tube inducing media, by approximately 90 - 100% and 80 - 95% respectively (see Table 6.2). These results suggest RNA synthesis is necessary for germ tube formation.

The addition of rapamycin to yeast in serum-containing buffer inhibited RNA synthesis. Rapamycin is a strong inhibitor of growth of C. albicans (16), with concentrations as low as 0.005 μg/ml (5 nM) showing some effect on growth (351). Concentrations of 0.02 μg/ml (0.02μM) inhibited incorporation of H_3 [^{32}P] O_4 into RNA by C. albicans by 49%. Thus, it seems surprising that 0.1 mM (100 μg/ml). rapamycin actually stimulated incorporation of [^{3}H] uridine into RNA during germ tube formation in imidazole buffer containing NAG or glu plus gln. Singh et al commented that rapid growth, in a "complete" medium, seemed to be essential for the inhibitory effect of rapamycin. It was found when C. albicans was suspended in Tris HCl buffer containing glucose, rapamycin did not have any effect on RNA synthesis (351). The increase in incorporation of [^{3}H] uridine into RNA, found

when C. albicans was incubated in buffer containing NAG or glu, may have arisen as a consequence of the drug affecting other metabolic processes.

It was established, in the previous section, that the level of RNA synthesis by C. albicans NCPF3153 depended upon the medium in which the cells were incubated. Thus, the activity of those many enzymes and components which control RNA synthesis may vary according to the incubation conditions. Any compound which affected the control of gene expression would exhibit an effect on RNA synthesis which was apparently dependent upon the media. Thus, rapamycin may have had a direct effect on expression of DNA. Whatever method accounted for the effects of rapamycin against C. albicans, it was clear that this compound inhibited germ tube formation.

Actinomycin D did not inhibit germ tube formation. This was a surprising result in view of the result found using other RNA synthesis inhibitors. Neither the time course nor the percentage of cells possessing germ tubes was affected by the presence of actinomycin D. It was found (Table 6.2) that, at the same concentration, RNA synthesis by yeast cells in suitable buffer was 26 - 54% that of controls. This presumably represents synthesis of A-U rich RNA by C. albicans in media that promoted germ tube formation. (Actinomycin D inhibits RNA synthesis by intercalation with G-C rich regions of the DNA (31). This germ tube formation may be associated with the synthesis of A-U rich RNA. It is possible that translation of such an RNA transcript would result in a protein containing hydrophobic amino acids as part of the sequence (394). Such a protein would be an ideal candidate for a membrane associated component. Since germ tube formation is initially distinguishable as a membrane associated event, it is possible the synthesis of such a protein may be required for this process.

It was noted earlier, in this section, that the addition of lomofungin and rifamycin AF/013 to the yeast suspension inhibited germ tube formation. These compounds greatly inhibited RNA synthesis, by inhibition of initiation by RNA polymerases (see 5.1). The germ tubes formed by yeasts in suitable buffer containing actinomycin D were 5 - 10 μm in length. In contrast, the germ tubes of yeast from control cultures were longer than 15 μm. It is possible that the

synthesis of G-C containing RNA molecules was required for this step. Alternatively, the mRNA species that coded for proteins necessary for elongation may have been present, prior to the induction of the germ tube and the addition of actinomycin D.

The effects of rifamycin AF/021, lomofungin and rapamycin on germ tube formation were reversible. It was found that inocula, kept for 12 hr in buffer containing the drugs, were able to form germ tubes when resuspended in suitable media. In contrast to the effects of inhibitors of RNA synthesis, it was found that cycloheximide and chloramphenicol did not have any effect on germ tube formation. This would appear to indicate that protein synthesis was not required for successful germ tube formation by the yeast. Cycloheximide concentrations of 10^{-6} - 10^{5} M strongly inhibit eukaryotic protein synthesis in systems as diverse as Chlorella and rats (392). However, it is probable that the concentrations of cycloheximide used in the buffer (i.e. 10^{-4} M) were insufficient to inhibit protein synthesis by C. albicans. It has been found by other workers (e.g. 345, 409) that other protein synthesis inhibitors such as trichodermin or puromycin prevented germ tube formation by the yeast. Mitochondrial protein synthesis was not required for germ tube formation as chloramphenicol - an inhibitor of prokaryotic and mitochondrial protein synthesis - was not effective.

It was reported that RNA polymerase III from C. albicans yeast was sensitive to 200 µg α-amanitin/ml (see 4.4.2). It was found that concentrations of 200 µg/ml completely inhibited germ tube formation by C. albicans in serum-containing buffer. At a concentration of 20 µg/ml the amatoxin did not have any effect on germ tube formation by yeast in conditions that promoted this phenomenon (results not shown). This result indicated that at least RNA polymerase III directed synthesis was required for successful germ tube formation.

6.4.6 RNA species synthesised by C. albicans during germ tube formation

It is now firmly established that the ultimate control of cell differentiation resides in gene activation and repression. Regulation at the transcriptional level received its original impetus from the studies of Monod, Jacob

et al on inducible and repressible enzymes in bacteria (175,265). Since then, attempts to unravel the mechanisms involved in the regulation of this process, in both prokaryotes and eukaryotes, have continued unabated. Many investigations into the regulation of specific genes have been conducted in organisms that produce abundant quantities of the desired gene product (e.g. the vitellogenin gene from X. laevis oocytes (114)). Attempts have also been made to correlate the levels of abundant RNA species (i.e. rRNA and tRNA) with changes in cellular growth or differentiation. This section will investigate whether the levels of these RNA species changed over the initial 5 hr period of yeast in media that promoted germ tube formation.

RNA synthesised by C. albicans, incubating in buffer containing either 1.25 mM NAG or 2% serum, was labelled with either [^{14}C] uridine or [^{32}P] sodium orthophosphate. Qualitative information was provided by electrophoresis of [^{32}P] labelled RNA on 1.3% agarose slab gels. These revealed that the [^{32}P] labelled RNA was associated with the more abundant rRNA and tRNA species. The nature of slab gel electrophoresis is such that it is difficult to discern quantitative levels of the higher and lower M.W. RNA species. As a consequence, the changes in the relative levels of these RNA molecules was monitored using methods that allowed a quantitative interpretation to be made.

The [^{14}C] labelled RNA was extracted from yeast in the two buffered media used to promote germ tube formation. The RNA was extracted after 1, 2, 3, 4 and 5 hr incubation in buffer containing NAG or serum. This RNA was analysed, by PAGE, on 2.5% polyacrylamide/0.5% agarose tube gels. Figs. 6.16 - 6.25 show the position the [^{14}C] labelled RNA migrated under electrophoresis in denaturing conditions. These figures show the peaks of [^{14}C] label incorporated into the 25 S, 17 S and 5 S rRNA and 4 S tRNA. The position of the 5.8 S rRNA was infered from the mobility of T. pyriformis RNA under the same conditions (see 3.3.3).

Several eukaryotic organisms have been studied to obtain information on the quantitative and qualitative activies of the RNA polymerase isozymes. However, the results have not led to any definitive conclusions in correlating the synthetic rates, or the quality and quantity of RNA present in the cells, with the level of

in vitro RNA polymerase activity. It was reported in 6.4.3 and 6.4.4 that the quantitative levels of RNA synthesised (measured by incorporation of labelled uridine into RNA and by the orcinol method for levels of total RNA) depended upon the media in which the yeast cells were suspended. It was found that during germ tube formation by *C. albicans*, the products of RNA polymerase I and III directed synthesis increased. Quantitative information on the levels of these RNA species would give an approximate indication of the *in vivo* activities of the RNA polymerases.

The ratio of [^{14}C] incorporated into RNA larger than 5 S to that smaller than 5 S approximates to the *in vivo* activities of RNA polymerases I and II to RNA polymerase III. Evidence that yeast *C. albicans* RNA polymerase III was responsible for the synthesis of 5 S rRNA and 4 S tRNA was provided in 4.3.12. However, it was not possible to positively correlate the synthesis of the large rRNA precursor and hnRNA with RNA polymerases I and II by the same method. This was because RNA polymerase II exhibited unusual (for higher eukaryotes at least) sensitivity to α-amanitin. It was assumed that RNA polymerases I and II from *C. albicans* yeast were responsible for the synthesis of rRNA and hnRNA respectively. This is a characteristic attributable to all eukaryotes possessing three isozymes, which are resolvable by DEAE-sephadex chromatography (see 1.3.3).

Fig. 6.26 showed how the ratios, for RNA extracted from yeast in NAG or serum-containing buffer, changed over a 5 hr incubation. The ratio of RNA polymerase I and II directed synthesis to that of RNA polymerase III, of yeast in NAG-containing buffer, did not change markedly over this period. At the end of this period only 50% of yeast had germ tubes. In contrast, the ratio of isozyme activities decreased, over the same period, when yeast cells were incubated in serum-containing buffer. In this media, all yeast produced germ tubes after 4½ hr incubation at 37° C.. These results indicate that the period before the appearance of germ tubes by yeast was associated with relatively high levels of RNA polymerase I and II directed synthesis. As soon as all the yeast had germ tubes most of the [^{14}C] was associated with RNA synthesised by RNA polymerase III. Hence, the levels of RNA species synthesised by the three RNA polymerases changed

over the period of germ tube formation by yeast in serum containing buffer.

Ribosomes are required for protein synthesis. Continuous formation of ribosomes does take place throughout the life cycle of eukaryotic cells (222). The stimulation of rRNA synthesis has been reported in rapdily growing fibroblast cells (315). It has been reported that there is a dramatic increase in rRNA synthesis in rapidly growing S. cerevisiae (390). This presumably represents the requirement for a higher level of protein synthesis during such growth periods as these. (In addition, there is amplification of the rDNA genes in cells undergoing rapid growth - such as Xenopus oocytes (222)). Regulation of rRNA synthesis may operate by changing the number of active rDNA genes or the rate of transcription of these genes. It is evident (Figs. 6.16 - 20) that, in serum-containing buffer, there is a decrease in the relative amount of RNA as rRNA in ribosomes, as all the yeast produced germ tubes. In contrast, there was no marked change in the proportion of RNA as rRNA over the 5 hr period the yeast were incubated in NAG containing buffer. These results suggest that there was a high level of protein synthesis associated with germ tube formation. Subsequent growth - i.e. germ tube elongation - was associated with lower relative levels of protein synthesis - at least when incubated in serum-containing buffer.

The synthesis of RNA polymerase III directed transcription is independent of the synthesis of the rRNA precursor in both higher (139) and lower eukaryotes (225). This is especially surprising in the case of 5 S rRNA synthesis. This molecule is an integral part of the large (60 S) subunit, always present in a 1:1 molar ratio to the 25 - 28 S and 5.8 S rRNA species. In addition, the number of gene copies per genome is about the same for 5 S rDNA and rDNA (379). The rate of 5 S rRNA synthesis in sea urchin has been found to fluctuate with the growth stage of the organism (225) which suggests the synthesis of this gene product responds to the stimulus of growth. The levels of tRNA - also required for protein synthesis during periods of growth - have been found to increase during growth in S. cerevisiae (390).

As protein synthesis requires mRNA for translation, one might expect an

increase in the levels of these species during a period of growth. However, extraction and analysis of RNA, as described in the methodologies of 2.7.1 and 2.8.1, is unlikely to yield information on mRNA levels during germ tube formation. This is because much lower levels of mRNA are found compared to the other more abundant RNA species. It is probable that the mRNA synthesised during the yeast-mycelial transformation contained sequences coding for the synthesis of such proteins as the large and small ribosomal subunit proteins and enzymes such as chitin synthetase pro-enzyme and other proteins concerned with changes in the cell wall. These proteins would respectively be required for new ribosome synthesis and also for wall morphogenesis - i.e. germ tube formation (see 6.4.2).

CHAPTER 7 GENERAL DISCUSSION

7.1 Introduction

This chapter will assess the suitability of RNA synthesis as a target site for novel inhibitors of C. albicans in relation to other sites for chemotherapy. The results from the thesis will be reviewed and discussed in relation to possible lines of future research.

7.2 RNA synthesis in C. albicans

The overall conclusion from results presented in this thesis is that RNA synthesis in C. albicans, whilst showing a few similarities to the same process in S. cerevisiae, is more typical of fungi imperfectii. This may be illustrated by the α-amanitin insensitivity of RNA polymerase II from yeast form C. albicans. It was found this enzyme was insensitive to concentrations of 1 mg α-amanitin/ml (see 4.3.12). The α-amanitin sensitivity of this isozyme was completely unexpected. RNA polymerase II is usually sensitive to very low (less than 1 µg/ml) concentrations of α-amanitin. It is only RNA polymerase II from fungi, especially fungi imperfectii, that show unusual sensitivity to this octapeptide (see 4.4.2). However, the monovalent and divalent cation sensitivities were similar to the results found for S. cerevisiae RNA polymerases I, II and III. The differences in α-amanitin sensitivities between C. albicans and mammalian RNA polymerases suggest a potentially exploitable difference between the pathogenic fungus and it's host.

A number of nucleoside analogues (78192, 82939 and 113786) were discovered to be very effective inhibitors when tested in an in vitro assay for RNA polymerase, but were less effective in an in vivo assay. This pattern of inhibition was the same for the two cyclizidine derivatives (95154 and 95464) and for aphidicolin and rapamycin. On the other hand nucleoside analogues 69619, 78053 and 78191 showed greater inhibitory activity against in vivo compared to in vitro RNA synthesis. This suggests these compounds may be metabolised to more potent inhibitors of RNA synthesis. Fairly high

concentrations (1 mM) were used to establish whether the test compound showed any inhibitory activity. It would be necessary to show these nucleoside analogues were effective inhibitors of RNA synthesis at lower (1 μM) concentrations in order to warrant further investigation.

It was shown in chapter 6 that radiolabelled uridine was rapidly incorporated into RNA by yeast cells during germ tube formation. In fact the rate of incorporation of [^{14}C] uridine into RNA by yeast during this process was more than seventeen-fold greater than that of exponential phase yeast cells (see 6.4.4). These results suggest that the yeast-mycelial transformation might be an excellent target for chemotherapy by inhibitors of RNA synthesis. It was found that the rate of incorporation of radiolabelled uridine into RNA was affected by the media used to promote germ tube formation (see 6.4.4). In addition, over the period all yeast produced germ tubes, the proportion of RNA synthesised by RNA polymerase III increased. This suggests that *in vivo*, RNA polymerase activity of *C. albicans* responds to environmental stimulii.

7.3 Other potential sites for chemotherapy

Any drug that is used against *C. albicans*, as a pharmaceutical preparation, must show differential sensitivity between fungus and host. Present ethical and commercial demands dictate that any antifungal agent produced by a pharmaceutical company is demonstrably safe - whatever the mode of action.

At the present time, the sites which best hold the promise of new *C. albicans*-specific agents are the cell wall and membrane. Differences between *C. albicans* and mammalian cell membranes are well documented. Mammalian cells, of course, have no cell wall. Inhibitors specifically against *C. albicans* cell wall or membrane would, hopefully, show no activity against the mammalian cell. As the cell wall and membrane are the boundaries between cell and environment, any inhibitor of this site need not be transported into the cell. However, the enzymes responsible for the synthesis

concentrations (1 mM) were used to establish whether the test compound showed any inhibitory activity. It would be necessary to show these nucleoside analogues were effective inhibitors of RNA synthesis at lower (1 μM) concentrations in order to warrant further investigation.

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of cell wall and membrane components are located on the inside of the membrane. Thus, only inhibitors of the relatively inert outer membrane or cell wall would avoid problems of membrane impermeability. Some inhibitors of these sites currently available, such as the polyene antibiotics, have the disadvantage of toxicity to mammalian cells (see 1.2.6).

Attempts could be made to produce antifungal agents that inhibit C. albicans-specific enzymes on the inner membrane, such as chitin synthetase or β (1-3) glucan synthetase. Indeed, there are some inhibitors of chitin synthetase which have been tested for their efficacy against C. albicans. These compounds - the polyoxins - inhibit chitin synthetase activity in vitro (22). However, they have no effect on growth processes which require an increase in the amount of chitin, such as germ tube formation. It has been suggested that the polyoxins are not getting to the site of chitin synthetase - possibly as a consequence of membrane impermeability (345).

7.4. Further research on RNA synthesis in C. albicans

It would be extremely satisfying, intellectually, to design an antifungal agent that did not inhibit mammalian cells, based on the peculiarities of RNA synthesis in C. albicans. Subsequent research should concentrate on further exploring differences in RNA synthesis to those reported here. There are various techniques that could be used to achieve this aim.

7.4.1 Further research on RNA polymerases of C. albicans

Three RNA polymerase isozymes were obtained from yeast, and one from mycelial form C. albicans after ion exchange chromatography on DEAE-Sephadex (see 4.4.1). This is a similar result to that reported by Boguslawski et al for RNA polymerases from mycelial and yeast forms of the fungus H. capsulatum (35). (However, three classes of RNA polymerases were recovered from the mycelial form and one from the yeast form of this dimorphic fungus). This group later reported that treatment of the cell

free extract from yeast, with polymin P, resulted in three peaks of enzyme activity after ion exchange chromatography (203). It is possible that such a procedure might result in the resolution of three peaks of RNA polymerase activity when the cell free extract from mycelial C. albicans is chromatographed on DEAE-Sephadex. Purification of these isozymes from mycelia would still be hampered by the need for large amounts of cellular material. As mycelial formation is only induced at low cell densities, large volumes of media (i.e. 100L) would thus be required.

Further research into RNA polymerases from C. albicans will require elucidation of the subunit composition of the three enzymes from both morphological forms. In addition, the divalent and monovalent ion optima, temperature and α-amanitin sensitivities would show how comparable each RNA polymerase isozyme was. This would show if these enzymes from C. albicans had similar properties to those from other dimorphic fungi, such as H. capsulatum (203).

Another approach to characterising RNA polymerases from C. albicans is on the basis of antibody specificity. It has been reported that antibodies, raised against RNA polymerase III from yeast form H. capsulatum showed only slight cross-reactivity against the same isozyme from the mycelial form (204). This indicated there were structural differences between yeast and mycelial RNA polymerase III. However, other workers in this field (e.g. 138) believe that any subunit differences are a consequence of artefacts arising during purification. It would be interesting to investigate whether RNA polymerases from both morphological forms of C. albicans showed similar cross-reactivity to those reported for H. capsulatum. This would necessitate the purification to homogeneity of RNA polymerase enzymes from both morphological forms of C. albicans.

Production of antibodies, by conventional means, requires large (200 - 300μg) quantities of highly purified enzyme. However, the introduction of hybridoma cell lines to produce monoclonal antibodies has resulted in important advantages over production of antibodies by conventional means.

Unpurified molecules, that constitute only a small part of a complex mixture, may be injected into a test laboratory animal. The antibody-secreting B lymphocytes may be fused with tumour B lymphocyte cell lines and the hybridoma grown on selective media. The cells secreting antibodies against the enzyme may be selected from other clones. Such antibodies would constitute powerful tools for further investigations into RNA polymerases from C. albicans.

Factors which stimulate transcription by RNA polymerases have been purified from the nuclei of plants (218), animals (359) and lower eukaryotes (108). Purification of such a factor from C. albicans would potentially be very useful as a full understanding of the mechanism for stimulating enzyme activity is required for the design of specific inhibitors. Sawadogo et al have identified a protein from S. cerevisiae that stimulates RNA polymerase I and II on ds DNA templates (327). This basic protein, termed P37, (M.W. 37,000) does not stimulate wheatgerm or calf thymus RNA polymerase II (326).

Permeabilised cells present an excellent opportunity to study RNA polymerases, and the other components that direct accurate transcription in an almost in vivo state. This overcomes problems arising as a consequence of cellular membrane impermeability to large or charged molecules that may be used to probe RNA synthesis. Methods have been described for the preparation of permeabilised cells (298). These would require some modifications as the exact conditions to permeabilise the yeast cell depend upon the enzyme to be studied.

Isolated chromatin preparations present an opportunity to study transcription of DNA in its natural state. However, the components that direct accurate transcription initiation are lost during isolation (see 3.4.3). Thus, efforts should be made to isolate these components before any useful studies can be made on this in vitro system.

7.4.2 Further research on RNA biosynthesis by C. albicans

Investigations were made into rRNA and tRNA synthesis by C. albicans during germ tube formation (see 6.4.6). Further research on the biosynthesis of these relatively abundant RNA species could be conducted during other growth periods, e.g. the regeneration of protoplast cell walls. It was found that a high proportion of RNA extracted from protoplast had high M.W. values (see 3.4.1). It would be interesting to see how the levels of these RNA species changed over the period of cell wall repair. In addition, rRNA and tRNA synthesis during exponential phase growth or mycelial development could also be monitored. Results from these investigations would show if there were any similarities in biosynthesis of rRNA and tRNA by C. albicans in such widely differing situations.

Different approaches to those used for investigations into rRNA and tRNA synthesis are required for studies on mRNA biosynthesis. Most RNA extracted, as described in 2.8.1, is rRNA and tRNA (see 3.3.4). However, mRNA (and hnRNA from which it is derived) may be purified by the selective annealing of the poly A at the 3' end to a column containing covalently bound synthetic polynucleotides of T or U residues. The mRNA synthesised during various periods of growth could be radioactively labelled and extracted. Complementary DNA (cDNA) copies could be made of these RNA molecules using reverse transcriptase. These could be hybridised with mRNA synthesised during other periods of growth. This would show the extent of homology between mRNA synthesised during such periods.

Most studies on proteins synthesised during the yeast-mycelial transformation have indicated the majority of such proteins are synthesised by both morphological forms (see 6.4.3). It is possible that any protein(s) responsible for regulating the dimorphic transformation may be present in minute quantities. Thus, previous attempts at radioactively labelling, extracting and analysing these proteins may not have been sufficiently sensitive for their identification. Amplification of the mRNA sequences that direct the synthesis of protein(s) responsible for regulation is possible

using cDNA copies. These cDNA copies could be cloned in a suitable vector. Thus, large amounts of DNA would be obtained that had sequences which could direct the synthesis of mRNA found in C. albicans during the yeast-mycelial transformation. This DNA could be sequenced and the amino acid sequence of proteins synthesised during germ tube formation elucidated.

Any comparative studies on RNA biosynthesis during different growth transitions should take into account the effects of changes in the purine and pyrimidine precursor pool sizes. It is possible these would change over the life cycle of C. albicans. These could be measured by techniques such as thin layer chromatography, as described by other workers (300).

7.4.3 Studies on the transport of drugs into C. albicans

Nucleoside analogue inhibitors were found which were very powerful when tested in an in vitro assay for RNA polymerase, but were not so effective in an in vivo assay (see 5.4.2). This may have arisen as a consequence of cellular membrane impermeability - one of the major problems of drug design. One method that has been suggested for overcoming this problem is to covalently link the potential drug to a protein as a conjugate. Steinfeld et al chemically synthesised a 5FC-peptide conjugate using 1-(ethoxycarbonyl)-2-ethoxy-1,2-dihydroxyquinoline as the coupling agent and a variety of di and tripeptides (361). These were effective inhibitors of C. albicans growth at similar concentrations to 5FC (374). Tripeptides such as trimethionine have been suggested as "carriers" for inhibitors that were usually impermeable to the cell membrane (271). Hence, conjugates of drugs and peptides could enter C. albicans through the peptide transport system. Further research is required to obtain sufficient knowledge to suggest tripeptides that would be transported specifically into C. albicans. Thus, peptides covalently linked to some nucleoside analogues suggested in 5.4.2 may be suitable carriers for these drugs.

It has been suggested that cellular membrane impermeability may account for some mammalian cell culture lines showing lower sensitivity to

∝-amanitin than isolated RNA polymerase II. Hencin and Preston suggested the use of ∝-amanitin-B.S.A. conjugates to transport the amatoxin into susceptible cells. The covalent link would then be hydrolysed to result in free ∝-amanitin (161). This finding seems in contrast to the results shown in 4.3.12. These revealed that, at a concentration of 200 µg/ml, the amatoxin was able to enter C. albicans and inhibit RNA polymerase III directed synthesis. This suggests membrane impermeability to ∝-amanitin may only be significant at very low concentrations of the amatoxin.

There are other oligo-peptides that are able to inhibit RNA synthesis in C. albicans. A protein has already been purified, from bull semen that inhibits this process in vivo. This protein, termed seminal plasmin (M.W. 6,300), inhibits RNA synthesis in other micro-organisms (373). It would be interesting to study how this protein was transported into the cell and it's effect on the various components that control transcription.

7.4.4 Combination chemotherapy and alternative approaches to candidosis

Synergystic combinations of two or more antifungal agents has been suggested as a possible technique for reducing the effective cidal concentrations of these drugs when treating candidosis. The presence of sub-lethal concentrations of amphotericin B reduces the concentration of 5FC required to inhibit growth of C. albicans - presumably by disrupting membrane integrity (see 1.2.5). It is possible the efficacy of the nucleoside analogues that inhibited RNA synthesis in C. albicans might be increased by such an approach. This might provide an alternative method for overcoming problems of cellular membrane impermeability shown by some nucleoside analogues to that suggested in 7.4.4 (i.e. by nucleoside analogue-peptide conjugates).

Other approaches have been made to antifungal chemotherapy over recent years. It has been reported that some micro-organisms may be inhibited when grown in the presence of furanoquinolines and UV light (377). If such UV mediated antibiotics had no side effects against mammalian dermis

cells, there is a possible application for them as topical remedies against candidosis (376). However, as these compounds were mediated by UV radiation, they would only be applicable against superficial candidosis of cutaneous sites. Some workers have suggested vaccination against C. albicans as a possible treatment for candidal vaginitis (252). Later research has revealed that components which stimulate the granulocytic response include muramyl dipeptides (117, 243). These components could be used prophylactically to stimulate the host's immune response system against C. albicans. Some workers have even suggested a use for mannans from C. albicans as antiviral agents (201). However, such compounds that stimulate an immune response would not be useful against the most serious cases of candidosis, i.e. those in the immuno-compromised host (see 1.2.4).

7.5 Final conclusion

A start has been made on exploring RNA synthesis in C. albicans as a potential target site for antifungal agents. There are some results in this thesis which suggest RNA synthesis in higher eukaryotes might be sufficiently different to that in C. albicans. It is possible that these differences are worth pursuing to achieve a C. albicans-specific antifungal agent.

Attempts to prepare suitable systems to study RNA synthesis in C. albicans were described in chapter 3. Protoplasts were successfully prepared from yeast cells in the exponential phase of growth. These were capable of incorporating radiolabelled uridine into RNA - most of which had a higher M.W. than 4 S. Protoplasts incorporated radiolabelled uridine into RNA at a slower rate than the exponential phase blastospores from which they were prepared. Thus, studies on in vivo RNA synthesis usually involved the use of exponential phase cells. The development of an in vivo assay to measure RNA synthesis in yeast cells was useful, as it allowed studies to be made on the effect of inhibitors on this process.

Nuclei were successfully prepared by the lysis of protoplasts. In view

of the delicate nature of the nuclear membrane, and high degree of chromosomal organisation (see 1.3.1), it is perhaps not surprising that low yields of nuclei were obtained. The "high" DNA content of C. albicans nuclei is further indication that these organelles were disrupted during preparation. The assay conditions that supported RNA synthesis by C. albicans nuclei were similar to those described by workers using nuclei from other eukaryotes. There was evidence for disengagement of the RNA polymerases after transcription termination, which is a characteristic often reported for nuclei from a wide variety of sources (e.g. 381). C. albicans nuclei showed higher levels of incorporation of radiolabelled UMP into RNA at higher salt concentrations. It was found that a higher level of radiolabelled UMP was incorporated into RNA when the nuclei were incubated in buffer containing 100 mM compared to 10 mM $MgCl_2$. This is an unusual result compared to that found for nuclei from other organisms, where optima of 100 mM $MgCl_2$ are reported (251).

It is evident from the results discussed in 3.4.3 that RNase activity was a considerable problem in the preparation of nuclei from C. albicans protoplasts. The deleterious effect of these enzymes on the RNA species synthesised in vitro is evident (see 3.4.4). A greater proportion of [^{14}C] UMP was associated with the high M.W. RNA species, when nuclei were incubated in buffer containing 100 mM compared to 10 mM $MgCl_2$. The probable reason for this result was that high Mg^{++} concentrations affect the secondary structure of the RNA. At higher concentrations, there is an increase in the extent of ds regions of RNA. The effect of RNases acting on ss regions is therefore curtailed. Thus, the finding that C. albicans nuclei showed higher levels of incorporation of [^{14}C] UMP into RNA in media containing higher $MgCl_2$ concentrations, probably arose as an artefact of this particular system.

The purification of RNA polymerases from yeast form C. albicans, and the part-purification of those from the mycelial form was reported in chapter 4. Some characteristics of these enzymes were also reported in

this chapter. The first step in the purification of the RNA polymerases was disruption of cells. The best method for this was homogenisation of the cells, with balotini, for 3 min at 0 - 4° C.. The following steps in the purification, needed to be carried out as quickly as possible. This was due to the degradative effects of proteases and RNases in the homogenate, which were not removed until the gel filtration step. RNA polymerase activiy was monitored after each step in the purification. Initially this was done using the assay conditions that gave maximal incorporation of [^{14}C] UMP into RNA by nuclei (see 3.4.3). However, it was found that the $MgCl_2$ concentration, of the assay "cocktail", had to be reduced to 10 mM to give optimal RNA polymerase activity in the homogenate. RNA polymerase activity appeared to decrease during the initial stages of purification, i.e. until the gel filtration step. It is probable that the methodologies used for these initial stages, i.e. high salt sonication and nucleic acid precipitation interfered with enzyme activity.

Ion exchange chromatography resolved the three RNA polymerases from yeast form C. albicans. In contrast, only one peak, of low activiy, was found for mycelial form RNA polymerases. RNA polymerases I and II from the yeast form were further purified by DNA cellulose chromatography. The KCl concentrations, of eluting buffer, required to desorb these enzymes from DNA cellulose were similar to those reported for S. cerevisiae. However, much higher salt concentrations were required to desorb C. albicans RNA polymerase III than those reported for the same isozyme from S. cerevisiae. RNA polymerase III from C. albicans yeast was not chromatographed on DNA cellulose. The high salt concentrations required to elute this isozyme from the DNA would also have eluted the DNA from the cellulose.

Glycerol gradient centrifugation was the final stage in the purification of the three RNA polymerase isozymes from yeast form C. albicans. This step resulted in a drastic loss in the total activity of the three RNA polymerases. The specific activity of RNA polymerases I and II also decreased markedly. This indicates that the purified RNA polymerases are

highly labile. Glycerol gradient centrifugation resulted in the purification, to electrophoretic homogeneity of the three isozymes. Only one band of protein could be visualised using the extremely sensitive staining technique devised by Merril et al (258). Purification to homogeneity of the RNA polymerases from the mycelial form was hampered by the low RNA polymerase activity after ion exchange chromatography. Also, the low yields of mycelia obtained required very large volumes of growth media to cultivate the cells.

Some characteristics of partially purified RNA polymerases were discussed in 4.4.2. The divalent cation optima for all three isozymes were 10 mM and 0.5 m - 1.0 mM for Mg^{++} and Mn^{++} respectively. The salt optima for RNA polymerases I and II were 40 mM and 100 mM and biphasic optima at 100 mM and 240 mM for RNA polymerase III. These values are similar to those reported in the literature for S. cerevisiae. In addition, the Km for CTP of the three isozymes were estimated at 0.01, 0.02 and 0.06 mM respectively. These values are marginally higher than those reported for other eukaryotic RNA polymerases.

Possibly the most unusual finding of chapter 4 was that the three RNA polymerases from yeast C. albicans showed unusual sensitivity to amanitin. The α-amanitin concentrations required to inhibit RNA polymerases I and III were similar to those reported for S. cerevisiae (138). Much higher levels of the amatoxin were necessary to inhibit RNA polymerase II from C. albicans than found for this isozyme from most other eukaryotes. It is worth remembering that RNA polymerase II from fungi in general, and fungi imperfectii in particular, show unusual sensitivity to α-amanitin. Thus, the results shown in 4.3.12 may be a confirmation of this trend.

Chapter 5 dealt with some aspects of inhibitors of RNA synthesis in C. albicans. Initial investigations were conducted on the efficacy of lomofungin against growth and RNA synthesis of C. albicans. These studies revealed that lomofungin, at a concentration of 320 μM, did not affect growth of C. albicans. This was in agreement with the findings of other groups (184), who reported that concentrations of 1 mg/ml (3.2 mM) were

necessary to inhibit growth of C. albicans. RNA synthesis, in protoplasts, was inhibited by the same concentration. In contrast, later studies revealed that concentrations of 1 mM, and even 0.2 mM, inhibited RNA synthesis in vivo. The most probable reason for this discrepancy lies in the relative insolubility of lomofungin in water. Later studies used lomofungin, from a stock solution in DMSO. In this solvent lomofungin could be more easily mixed with the cell sample than when suspended in 5 mM NaOH. This illustrates the need for a suitable solvent, for dissolving a test drug, when studying the mode of action of inhibitors. Unpublished results (J.F. Ryley, personal communication) showed that lomofungin, at concentrations of 25 µg/ml (80 µM), inhibited growth of both morphological forms of C. albicans.

DMSO was the solvent used to prepare stock solutions of the uridine analogues tested against in vivo and in vitro RNA synthesis in C. albicans. These compounds were chosen from the collection of ICI Pharmaceuticals Division, on the basis of a similarity to uridine, a precursor nucleoside for RNA. The efficacy of these compounds was compared to other, well known inhibitors of transcription. It was found that 69619, 78053, 78192, 82939, 113786, 113787 and 154391 inhibited RNA synthesis in vitro in cell free extracts. ICI compounds 69619, 78053, 78191, 78192, 113787 and 154391 inhibited RNA synthesis in vivo in yeast cells. Potentially the most useful are those showing inhibitory activity against in vivo RNA synthesis.

RNA synthesis during the yeast-mycelial transformation was discussed in chapter 6. In order to achieve reproducible germ tube formation from C. albicans NCPF3153, the yeast had to be grown in nutritionally impoverished media and starved for at least 24 hr. Germ tube formation by C. albicans was induced when a small inoculum was incubated in buffer containing suitable inducing agents at temperatures above 37^{o} C..

The time taken for all the yeast cells to have germ tubes depended upon the buffer in which the cells were incubated. Yeast cells incubating in buffer containing 5% serum showed the shortest time to form germ tubes. Lowering the concentration of serum increased the time taken for all the

yeast cells to form germ tubes and decreased the rate of incorporation of radiolabelled uridine into RNA. Yeast cells in buffer containing NAG, glu or glu plus gln were all able to form germ tubes. Varying the concentration of NAG or glu in the buffer did not affect either the time taken for all the cells to form germ tubes or the rate of incorporation of radiolabelled uridine into RNA.

The rate of incorporation of [^{3}H] uridine into RNA by yeast in buffer containing NAG, serum, glu or glu plus gln was measured over a 10 hr period. A linear rate was obtained when yeast were incubated in buffer containing serum, glu or glu plus gln. When yeast was incubated in NAG-containing buffer, there was a non-linear increase in the rate of incorporation of label into RNA over 6 hr. After this period, there was no increase in the amount of label into RNA. These results indicated that [^{3}H] uridine was incorporated into the intracellular uridine pool and into the RNA at a similar linear rate, whenever it was added to the cell suspension.

Chapter 6 also showed the results of studies on the effects of some inhibitors of RNA synthesis on germ tube formation. In agreement with other groups, these studies found that RNA synthesis was necessary for germ tube formation.

The ratio of high to low M.W. species was measured during germ tube formation by C. albicans. It was found that this ratio decreased during germ tube formation in serum-containing buffer. The ratio of high to low M.W. RNA, from yeast cells in NAG-containing buffer, did not change markedly over the same 5 hr period. All yeast, in buffer containing serum, had germ tubes after 4½ hr incubation. Only 50% of yeast cells, in NAG-containing buffer, had germ tubes after 5 hr incubation. These results indicated that the ratios of RNA polymerase I and II to RNA polymerase III directed RNA synthesis changed as the cells formed germ tubes.

The levels of RNA polymerase I directed synthesis were highest during the initial stages of germ tube formation - assuming, in C. albicans, RNA polymerase I was responsible for the synthesis of rRNA. It follows that

there was a high level of ribosome synthesis, implying a high level of protein synthesis. Although the total RNA content of the cells increased during germ tube formation, there was no marked increase in total protein content. This apparent paradox may be explained by the synthesis of new proteins using amino acids from the pools of precursors found in the yeast cells.

In conclusion, the results presented in this thesis have indicated that RNA polymerases from C. albicans are different to those from higher eukaryotes. This suggests RNA synthesis is a possible target site for novel inhibitors. Some novel inhibitors were suggested in this thesis and these could be useful against growth transitions of C, albicans which are accompanied by high rates of RNA synthesis, such as germ tube formation.

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