Partial Characterization of the Cloned Dihydrofolate Reductase Gene of *Saccharomyces cerevisiae*.

Michael G. Nagel, B.Sc. (Honours)

Department of Biological Sciences.

(Submitted in partial fulfillment of the requirements
for the degree of Master of Science)

Brock University,
St. Catharines, Ontario

July 1985

To my family, for their continual patience and support.

Abstract

The cloned dihydrofolate reductase gene of Saccharomyces cerevisiae (DFR 1) is expressed in Escherichia coli. Bacterial strain JF1754 transformed with plasmids containing DFR 1 is at least 5X more resistant to inhibition by the folate antagonist trimethoprim. Expression of yeast DFR 1 in E. coli suggests it is likely that the gene lacks intervening sequences. The 1.8 kbp DNA fragment encoding yeast dhfr activity probably has its own promotor, as the gene is expressed in both orientations in E. coli.

Expression of the yeast dhfr gene cloned into M13 viral vectors allowed positive selection of DFR 1 - M13 bacterial transfectants in medium supplemented with trimethoprim. A series of nested deletions generated by nuclease Bal 31 digestion and by restriction endonuclease cleavage of plasmids containing DFR 1 physically mapped the gene to a 930 bp region between the Pst 1 and Sal 1 cut sites. This is consistent with the 21,000 molecular weight attributed to yeast dhfr in previous reports.

From preliminary DNA sequence analysis of the dhfr DNA fragment the 3' terminus of DFR 1 was assigned to a position 27 nucleotides from the Eco R1 cut site on the Bam H1 - Eco R1 DNA segment. Several putative yeast transcription termination consensus sequences were identified 3' to the opal stop codon.

DFR 1 is expressed in yeast and it confers resistance to the antifolate methotrexate when the gene is present in 2 - 10 copies per cell. Plasmid-dependent resistance to methotrexate is also observed in a rad 6 background although the effect is somewhat less than that

conferred to wild-type or rad 18 cells. Integration of DFR 1 into the yeast genome showed an intermediate sensitivity to folate antagonists. This may suggest a gene dosage effect. No change in petite induction in these yeast strains was observed in transformed cells containing yeast dhfr plasmids.

The sensitivity of rad 6 , rad 18 and wild-type cell populations to trimethoprim were unaffected by the presence of DFR 1 in transformants. Moreover, trimethoprim did not induce petites in any strain tested, which normally results if dhfr is inhibited by other antifolates such as methotrexate. This may suggest that the dhfr enzyme is not the only possible target of trimethoprim in yeast.

rad 6 mutants showed a very low level of spontaneous petite formation. Methotrexate failed to induce respiratory deficient mutants in this strain which suggested that rad 6 might be an obligate grande. However, ethidium bromide induced petites to a level approximately 50% of that exhibited by wild-type and rad 18 strains.

Acknowledgements.

First and foremost I would like to thank my supervisor, Dr. B. J. Barclay, for his financial support during the initial portion of this study and for his assistance in the preparation of this manuscript. A special thanks goes to my lab colleagues who in years gone by are too numerous to list.

To my typist, Vickie Backus, I extend my appreciation for the professional manner in which she prepared this thesis. I would like to extend my deepest gratitude to Dr. W. H. Cade for his advice and helpful discussion throughout this process.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

Ap	ampicillin
Cm	chloramphenicol
EtBr	ethidium bromide
G418	geneticin
MTX	methotrexate
Neo	neomycin sulfate
Sulf	sulfanilamide
Tc	tetracycline
TRM	trimethoprim
gpt	xanthine-guanine phosphoribosyl transferase
dhfr	dihydrofolate reductase
gal	galactose
his	histidine
lac	lactose
leu	leucine
met	methionine
pro	proline
thi	thiamine
trp	tryptophan
ura	uracil
ori	DNA replication origin
OD	optical density

EDTA	ethylene-diaminetetraacetic acid
TRIS	2-amino-2-hydroxymethyl-1, 3-propane diol
TTC	2,3,5-triphenyltetrazolium chloride
A	adenine
C	cytosine
G	guanine
T	thymine
dATP	2'-deoxyadenosine-5'-triphosphate
dCTP	2'-deoxycytosine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dTTP	2'-deoxythymine-5'-triphosphate
ddNTP	2',3'-dideoxynucleoside triphosphate
FU	5-fluorouracil
FdUMP	5-fluoro-2'-deoxyuridine-5'-monophosphate
DNA	deoxyribonucleic acid
ssDNA	single-stranded deoxyribonucleic acid
hr	hour
min	minute
sec	second
uCi	microcurie
g	gram
mg	milligram
ug	microgram

mg	milligram
ug	microgram
kbp	kilobase pair
bp	base pair
l	litre
ml	millilitre
ul	microlitre
rpm	revolutions per minute
K	1,000 rpm
v/v	volume to volume
rad 6 rad 18	radiation sensitive mutants of S. cerevisiae

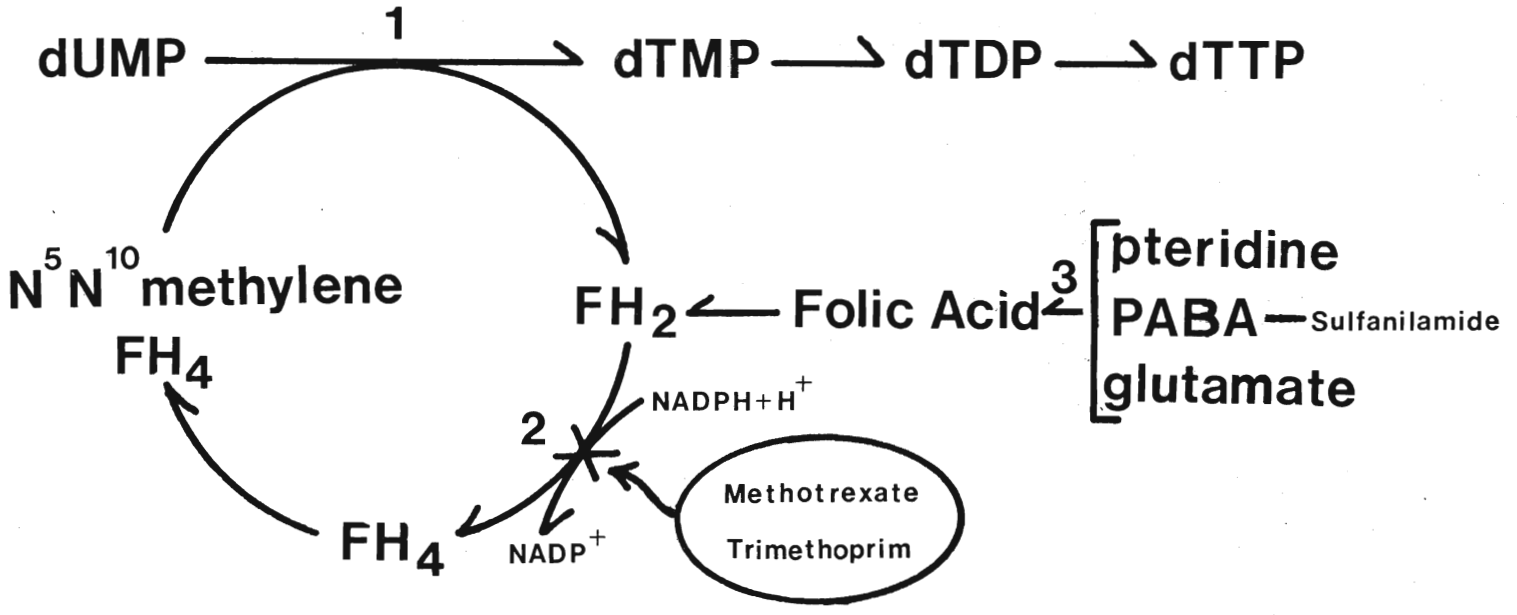
Introduction

Dihydrofolate reductase (dhfr; 5,6,7,8, - tetrahydrofolate: NADP+ oxidoreductase; EC 1.5.1.3.) is an essential enzyme which catalyzes the NADPH dependent reduction of dihydrofolate to tetrahydrofolate (Osborn and Huennekins, 1958). A major function of this protein is to recycle tetrahydrofolate from the dihydrofolate which is formed in the conversion of deoxyuridylic acid to deoxythymidylic acid (see Figure 1). This reaction, catalyzed by thymidylate synthase (5,10 - methylene - tetrahydrofolate: dUMP C-methyltransferase; EC 2.1.1.45), produces stoichiometric amounts of dihydrofolate which is then converted to tetrahydrofolate to maintain the supply of pteroylglutamic acid cofactors (Smith and Calvo, 1982). These reduced folate derivatives are required for one carbon transfer in various biosynthetic reactions. These include: purine formation, thymidylate synthesis and the anabolism of certain amino acids (primarily methionine, serine and glycine) in a variety of organisms.

A derivative of folic acid (N10 - formyltetrahydrofolate) is the formyl donor in the conversion of $_{met}tRNA^{Fmet}$ to $_{fmet}tRNA^{Fmet}$ The latter charged transfer RNA initiates protein synthesis in bacteria as well as in the mitochondria of eukaryotes (for a review see Blakely, 1969). In addition to their role in protein synthesis, folates are involved in an important way with other aspects of macro-molecular synthesis including RNA and DNA metabolism. This finding and the fact that folates are ubiquitous in nature has suggested to some investigators that the vitamin might have some regulatory function in cells (Kirschner and Bisswanger, 1976).

The parent compound, folic acid, was first isolated in pure form

Figure 1 Site of action of various antifolate drugs in the metabolic pathway leading to the synthesis of tetrahydrofolic acid.



1 Thymidylate Synthase
2 Dihydrofolate Reductase
3 Pteroate Synthase

from spinach (Mitchell et al., 1944). It soon became apparent that folates (pteroylglutamic acids) consisted of a group of over 100 closely related metabolites (Hoffbrand, 1975). More recent studies have shown that these folates differ from the parent compound in that they are reduced to dihydro- or more commonly tetrahydro- forms at positions 5,6,7,8, in the pyrazine ring. Also, many folate moieties have one of 5 single carbon units attached at N_5 or N_{10} (see Figure 2; Mackenzie and Baugh, 1980). In addition, in both prokaryotic and eukaryotic systems, intracellular folates have been found to be conjugates of pteroylglutamate consisting of chains up to 7 glutamic acid residues (Baugh and Krumdieck, 1971).

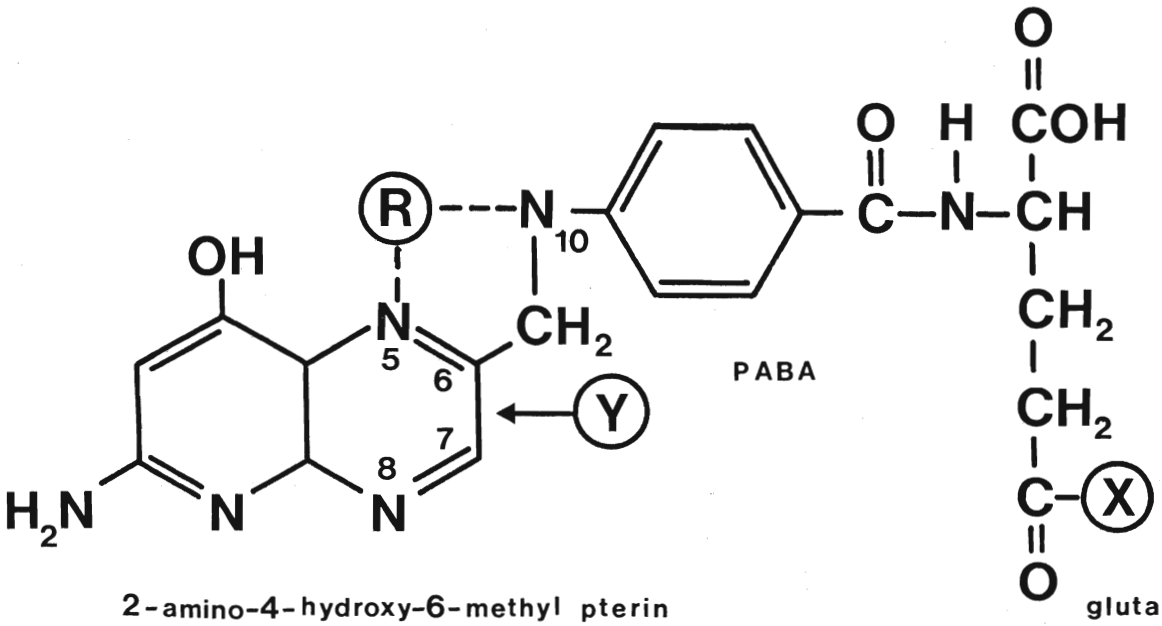
As early as the 1930's folate metabolism became an important subject of clinical investigation when it was discovered that sulfanilamide, a structural analogue of p-Aminobenzoic acid (pABA), protected laboratory mice against streptococcal infections (Buttle et al., 1936). Subsequently, it was found that another antibiotic trimethoprim inhibited folate metabolism, although its mode of action was quite different from that of the sulfonamide compounds. Further investigation revealed that sulfanilamides act as competitive inhibitors of the pteroate synthase catalyzed condensation of PABA and pteridine. Trimethoprim was found to be a species specific inhibitor of dihydrofolate reductase (for a review see Wormser and Keusch, 1983).

Folate antagonists such as trimethoprim (in bacteria) and methotrexate (in mammals), which inhibit dihydrofolate reductase activity cause a depletion of the intracellular tetrahydrofolate pools. This may ultimately result in "thymineless" death (Cohen and

Figure 2 Biologically significant derivatives of folic acid.

One carbon groups carried by tetrahydrofolate

Oxidation state	Group	
most reduced	$-CH_3$	Methyl
intermediate	$-CH_2-$	Methylene
most oxidized	-CHO	Formyl
	-CHNH	Formimino
	-CH=	Methenyl

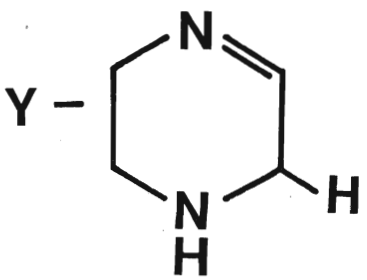


2-amino-4-hydroxy-6-methyl pterin

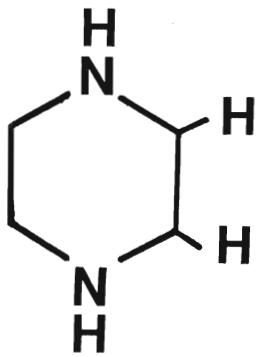
glutamic acid

R
- CH_3 at N_5
- CHO at N_5 or N_{10}
- CHNH at N_5
- CH at N_5, N_{10}
- CH_2 at N_5, N_{10}
- H at N_5 or N_{10}

X
- poly (γ-glutamyl)$_n$ glutamic acids
- OH



7,8 dihydro



5,6,7,8 tetrahydro

Barner, 1954). The loss of viablity due to thymidylate depletion occurs in a variety of organisms from bacteria to humans (Pritikin and Romig, 1969; Brendel and Fath, 1974; Barclay and Little, 1978; Hyrnick and Bertino, 1973).

In addition to cell killing, thymine starvation has been shown to be extremely mutagenic in thy- Bacillus subtilis and Salmonella typhimurium (Bresler et al., 1970; Holmes and Eisenstark, 1968). Thymineless mutagenesis has been shown to cause specific AT - CG transitions in Escherichia coli (Kunz and Glickman, 1985) and bacteriophage T4 (Smith et al., 1973). In contrast, the nuclear genomes of yeast and cultured human cells are relatively insensitive to mutagenesis by thymine starvation (Barclay and Little, 1978; Dimnik and Hoar, 1983). However, high levels of mitochondrial mutation occur in both S. cerevisiae and HeLa cell lines when grown under "thymineless" conditions, provoked by either chemical treatment or in mutants defective in dTMP biosynthesis, grown in the absence of the nucleotide. The induction of respiratory deficient mutants or "cytoplasmic petites" (ρ^-) in yeast has also been observed after exposure to a variety of chemical mutagens (for a review see Oliver, 1977). Thymine deprivation has also been shown to induce recombination in E. coli (Gallant and Spottswood, 1964), in yeast (Barclay and Little, 1978) and in mouse cells (Hori et al., 1984).

The toxicity associated with folate depletion has been exploited clinically for many years in the treatment of various human pathogens. Dihydrofolate reductases from bacterial and mammalian sources exhibit different binding affinities to various folate analogues (Baccanari et al., 1975). Thus, evidence has been presented by Burchall (1983)

that bacterial dhfr binds trimethoprim more readily than does its mammalian counterpart. It is this differential binding affinity which results in a high therapeutic index for trimethoprim even though its target is common to host and parasite.

In addition to the use of inhibitors of folate biosynthesis as antibacterial agents, these compounds are important clinically in cancer therapy. It appears that folic acid derivatives play some important role in the maintenance of rapid growth and division of neoplastic cells. The high demand of cancer cells for dietary folate has been well documented (for a review see Hitchings, 1983). As a consequence, the inhibition of folate metabolism by drugs such as methotrexate and aminopterin have for many years proven to be a clinically useful approach to the treatment of certain cancers (Conduit, 1971). Protocols which use antifolates have evolved either as a treatment of choice for various cancers or as an adjuvant to surgery or radiotherapy (Bertino, 1979).

There are several physiological properties of the target cell, which influence the relative ability of methotrexate to inhibit cell growth (Gallivan, 1979). These include the capacity of the cell to transport methotrexate across the cell membrane and the maintenance of an intracellular drug concentration which is sufficient to inhibit dihydrofolate reductase activity (Sirotnak and Donsbach, 1974). A complication in the clinical pharmacology of the drug arises from the finding that methotrexate, like naturally occuring folates, can be converted to polyglutamate derivatives both in normal and tumour cells (Nair and Baugh, 1973). Thus, it is unclear whether methotrexate itself or a polyglutamated derivative of the drug is the actual dhfr

inhibitor *in vivo*. Because folate depletion affects preferentially rapidly dividing cell types, methotrexate toxicity is not limited to neoplastic cells, but also effects those of the gastric mucosa, hair follicule and haematopoetic system. This non-specific inhibition is the probable cause of the side effects associated with the use of the drug in cancer chemotherapy (ie. nausea, hairloss and anemia).

In spite of these side effects the use of methotrexate as a cancer therapy has long been shown to be efficacious in slowing the rate of progress of the disease and in some cases malignant neoplasms have gone into remission after repeated administration of the drug. However, in many instances, repeated drug use has been associated with the emergence of methotrexate-resistant variants within the tumour cell population. These cells fail to respond to further antifolate therapy and the clinical course of the disease is resumed (Bertino *et al.*, 1963). In an attempt to cope with an emerging drug-resistant tumor cell population, combination chemotherapies were developed in which other drugs such as 5-fluorouracil (which is converted in cells to FdUMP, an inhibitor of thymidylate synthase) were used in concert with antifolates. Paradoxically, studies in tissue culture have yielded conflicting reports as to whether methotrexate and 5-fluorouracil (5FU) are additive or antagonistic in their action (Ullman *et al.*, 1977). In spite of this apparent contradiction, the use of methotrexate and 5FU in combination has been relatively successful in the management of many different cancers (for a review see Morrow, 1983). Nevertheless, drug-resistant tumour cell lines still emerged (Vesely and Cihak, 1973).

In spite of the various limitations in their use, folic acid

antagonists have proven to be one of the best available choices of chemical substances in cancer chemotherapy. For this reason, there is considerable interest in studies designed to increase our understanding of the mechanisms of resistance to these compounds. The results of numerous investigations which attempted to reconstruct in tissue culture, the conditions under which methotrexate is used clinically, have revealed that drug-resistance can be acquired in three general ways: 1) an increase in the level of dhfr enzyme in the cell (Shields, 1978); 2) a decrease in cell permeability to the antifolate (Frei et al., 1984) or 3) a decrease in the affinity of the target enzyme for methotrexate (Simonsen and Levinson, 1983). The first mechanism of resistance is the best understood at the molecular level. In these cell lines, enzymatic activities were elevated as much as 3000 fold and result from an increase in the number of molecules per cell (Bastow et al., 1984). Littlefield (1969) has shown that hybrids between wild-type and overproducing lines were intermediate in activity which suggests that the increase in enzyme activity was not due to a diffusible repressor. This indicated that overproduction of dhfr unlike the situation which has been observed in procaryotes, can not be ascribed solely to regulatory mutations (Smith and Calvo, 1982). It was revealed subsequently that many of these MTX-resistent lines arose as a consequence of gene amplification (Heintz and Hamlin, 1982; Schimke, 1984).

Alt et al., (1978) demonstrated that in at least two cases overproduction of dihydrofolate reductase in response to a methotrexate challenge resulted from an increase in copy number of the structural gene for the enzyme. In one of these variants the

dihydrofolate reductase gene had been amplified over two hundred fold and was localized within a homogeneously staining chromosomal region. Furthermore, Nunberg et al., (1978) using in situ hybridization studies have confirmed that the amplified dihydrofolate reductase genes were specifically localized to these homogeneously staining regions. This type of methotrexate resistant cell retains its phenotype in the absence of any selection (Shimke, 1984). In contrast to these isolates, another type of gene amplification event, which is located on double minute chromosomes, resulted in an unstable drug resistance phenotype (Kaufman et al., 1979).

Several models have been advanced to explain the occurrence of gene amplification events in methotrexate-treated eucaryotic cells. Varshavsky (1981) has suggested that replicon "misfiring" might lead to precocious synthesis of chromosomal domains adjacent to DNA replication origins. The extra copies of the dihydrofolate reductase gene which accumulate during growth in the presence of the drug confer a selective advantage to host cells. A prediction of this model is that the dihydrofolate reductase amplified region must be in close proximity to an origin of DNA replication. This was shown to be the case in Chinese hamster ovary cells (Heintz and Hamlin, 1982). Once the dihydrofolate reductase gene region has been duplicated by this mechanism, it is supposed that subsequent amplification events occur by unequal mitotic crossing over (Banerjee and Benedict, 1979).

Recently Barclay et al., (1982) have shown that the depletion of intracellular pools of thymidylate by antifolate drugs results in a large increase in the rate of mitotic crossing-over in yeast. In this study, an increase in recombination frequency of over 500 fold was

observed in yeast populations treated with methotrexate (Barclay et al., 1982) and in cells cultured in the presence of FdUMP, the metabolically active derivative of 5-fluorouracil (Kunz et al., 1980). Thus, these authors have suggested that methotrexate might induce resistance to itself by promoting genetic rearrangements in target cells.

Although it is a somewhat perilous exercise, in general terms, to extrapolate results directly from one species to another, studies in yeast have been used as a basis for designing similar experiments in animal cells. Since yeasts are intermediate in biological complexity relative to bacteria and mammals, they are being used for basic studies in eukaryotic biology including those of gene expression, DNA replication, recombination, transposition, chromosome segregation, chromatin structure and control of cell mating type (for a review see Petes, 1980; Struhl, 1983).

Thus, the bakers yeast Saccharomyces cerevisiae is an excellent experimental system for genetic and biochemical analysis of folate metabolism. Yeast cells can be easily manipulated by classical microbiological techniques and more recently by the very powerful recombinant DNA methods. Thus, it has been possible to reveal some aspects of the genetic consequences of folate depletion in this organism, which have been difficult to analyze directly in human cells.

For example, since yeast is a facultative anaerobe, lesions occurring in either the nuclear or the mitochondrial genome can be readily distinguished. Damage to yeast mitochondria is easily monitored (Goldwaite et al., 1974). Thus, this system is ideal for

studying the effects of the cloned dhfr gene under folate deprived conditions. Another important consideration in choosing yeast as a model system for studies of folic acid biosynthesis in eukaryotes, is the finding that some folate interconversion enzymes exist in 2 forms; an enzymatic activity isolated in the cytoplasm and an analogous function associated with the mitochondrion (Luzzati, 1975). Thus it is advantageous in these experiments to be able to distinguish easily the expression of nuclear from mitochondrial gene products.

One of the difficulties encountered in studying folate metabolism in S. cerevisiae has been the paucity of useful information on yeast dhfr. Although an extensive genetic map of the 17 chromosomes on S. cerevisiae is available (Mortimer and Schild, 1980) the map position of the dhfr locus remains unknown. Furthermore, no dihydrofolate reductase deficient mutants of S. cerevisiae (which would be of considerable utility in biochemical studies) are known to exist. Use of the cloned gene would greatly facilitate both the isolation of a dhfr gene disruption and determination of the position of the gene in the yeast genome.

Yeast is also well suited for studies of dihydrofolate reductase expression due to the sophisticated genetic system which has been developed in this organism over many years. By the analysis and manipulation of yeast genes in vitro and in vivo using recombinant DNA technology, the control of the expression of numerous yeast genes is now well understood, at the molecular level (Andreadis et al., 1984; Holland and Holland, 1979; Mantsala and Zalkin, 1984; Zaret and Sherman, 1982). At the present time there exist simple and general methods for the isolation and subsequent amplification on multicopy

plasmids of virtually any yeast gene. The cloning of specific DNA segments allows one to study the expression and organization as well as the regulation of individual and co-regulated gene sets. The details and applications of these techniques have been extensively reviewed (Botstein and Davis, 1982).

A prerequisite to understanding the mode of action of a cloned gene *in vivo* is that the said gene be returned to the organism of origin. Relatively simple methods have been developed by which cloned DNA can be introduced into yeast spheroplasts, or into intact cells treated with lithium chloride. This DNA can be maintained in the host cell in various ways: 1) integrated into a specific location in the genome; 2) as an extrachromosomal element on a multicopy plasmid ;or 3) stably maintained on "minichromosomes" containing one of several cloned yeast c entromeric regions (for a review see, Hinnen *et al.*, 1978).

As mentioned previously, one of the advantages of yeast as an experimental system resides in the extensive catalogue of yeast mutants which are available for study. For example, there are nearly 100 different loci in *S. cerevisiae* which modify the response of strains to mutagenesis by chemicals or radiation (for a review see Haynes and Kunz, 1981). Enhanced sensitivity to either UV light or ionizing radiation occurs in strains carrying mutant alleles of these genes (Resnick, 1969). These radiation sensitive genes have been classified into three epistatic groups, which correspond to three distinctive modes of DNA repair (for a review see, Game, 1983).

The *Rad* 6 and *Rad* 18 genes of *S. cerevisiae* are 2 of the at least 9 genes thought to be involved in the error-prone dark repair system

of UV induced pyrimidine dimers (Game, 1983). Even though the precise function of the Rad 6 gene product is unknown, it has been shown that mutagenesis in S. cerevisiae by both chemical and physical agents requires a functional RAD 6 gene (Lawrence and Christensen, 1976; Prakash, 1974). An unusual property of both rad 6 and rad 18 mutant strains is that they are strongly inhibited by the antifolate trimethoprim. These findings are intriguing since there is no obvious relationship between radiation sensitivity and folate metabolism.

Furthermore, mutant strains carrying both rad 6 and rad 18 exhibit a high frequency of spontaneous mutation to trimethoprim resistance accompanied by metabolic suppression of UV-induced death. This suggests that there are 2 activites associated with these mutations: 1) wild-type radiation repair and resistance to trimethoprim; and 2) induced mutagenesis.

In general, the primary focus of this study was to begin the characterization of the cloned dihydrofolate reductase gene of yeast in the radiation sensitive mutant and other yeast strains. By transforming rad 6 and rad 18 mutants with the dhfr gene on multicopy plasmids, it should be relatively easy to determine whether the trimethoprim sensitivity of these strains arises as a consequence of decreased dhfr activity. It was hoped that by the use of the cloned gene on integrating vectors and multicopy plasmids it would be possible to gain insight into the regulation of folate metabolism in this simple eukaryote.

Materials and Methods

The strains of Escherichia coli and Saccharomyces cerevisiae used throughout the course of this study are summarized in Tables 1 and 2.

Media and Buffers

For routine growth of bacterial strains either L medium or B medium was used. L medium contained per litre: 10 g bacto-tryptone, 5 g bacto-yeast extract and 5 g NaCl, whereas B medium contained per litre: 10 g bacto-tryptone, and 8 g NaCl. Minimal media (MIN) contained per litre: 10.5 g K_2HPO_4, 4.5 g KH_2PO_4, 1.0 g $(NH_4)_2SO_4$, 0.5 g sodiumcitrate.$2H_2O$, 1.0 ml of a 20% $MgSO_4.7H_2O$ stock solution and 10.0 ml of a 20% dextrose stock solution*. Appropriate nutrients were added at a final concentration of 50 ug/ml with the exception of thiamine hydrochloride** which was added at a final concentration of 5 ug/ml. Antibiotics were added to media at 48°C after autoclaving at final concentrations of: ampicillin 50 ug/ml, chloramphenicol 30 ug/ml, neomycin sulphate 200 ug/ml and tetracycline 150 ug/ml. Where needed, trimethoprim was added to MIN medium at a final concentration of either 2 ug/ml, 100 ug/ml or 500 ug/ml. Unless otherwise indicated, bacto-agar was added at a final concentration of 2% for plates and 0.6% for soft agar.

YPDP medium was used for routine growth of yeast strains. This contained per litre: 10 g bacto-yeast extract, 20 g bacto-peptone, 20 g dextrose, and 1.5 g KH_2PO_4. Minimal medium (SD) contained per

* added after autoclaving.
** filter sterilized separately.

Table 1 *Escherichia coli* strains used.

Strain	Genotype	Source
JF1754	*his* B, *leu* B, *met* B, *hsd* r-, *hsd*m+, *lac*, *gal*, *rec* A,	J.Freisen
JM103	*lac*, *pro*, *thi*, *str* A, *sbc* B 15, *hsd* R 4, *sup* E, F'*tra* D 36, *hsd* m+ *pro* AB, *lac* 1 Z M15, *hsd* r-	Bethesda Research Laboratories

Table 2 Yeast strains used in this study.

Strain	Genotype	Source
AH 2	Mat a, ρ+, leu 2-3, leu 2-112, his 4	R.Storms
LP2730-1A	Mat a, ρ+, leu 2-3, leu 2-112, his 3-1, trp 1-289, ura 3-52, rad 6-1	L.Prakash
LP2729-4B	Mat a, ρ+, leu 2-3, leu 2-112, his 3-1, trp 1-289, ura 3-52, rad 18-2	L.Prakash

litre: 6.75 g bacto-yeast nitrogen base without amino acids and 20 g glucose. Appropriate nutrients were added at a final concentration of L-histidine-HCl 20 ug/ml, L-leucine 30 ug/ml, L-tryptophan 20 ug/ml and uracil 20 ug/ml. Trimethoprim was added to SD medium as required. YPGP medium contained 3% glycerol in place of dextrose. dTMP and Geneticin (G418) were added to molten media at 48°C after autoclaving. Unless otherwise stated, the dTMP concentrations were as follows: in YPDP 100 ug/ml and YPGP 200 ug/ml. G 418 was added to both YPDP and YPGP at a final concentration of 150 ug/ml.

Plasmid DNA was maintained or dissolved in either TES buffer or TE buffer. TES buffer contained per litre: 20 mM Tris-HCl pH 7.5, 10 mM NaCl and 0.1 mM EDTA pH 7.5, whereas TE buffer contained per litre: 10 mM Tris-HCl and 1mM EDTA adjusted to a pH of either 7.5 or 8.0. Yeast cells were washed in a 0.1M phosphate buffer (pH 7.0).

Chemicals

Restriction endonucleases, nuclease Bal 31 and T4 DNA ligase were obtained from Boehringer Mannheim. Nucleotide reagents, M13 single stranded primer and Klenow fragment were purchased from P. L. Biochemicals Inc. and Bethesda Research Laboratories. Trimethoprim, sulfanilamide, methotrexate, and ampicillin were from Sigma Chemical Co. All culture media and remaining chemicals were purchased from Difco and BDH Chemicals with the exception of Glusulase which was obtained from Endo Laboratories.

Rapid Plasmid Preparation

The method used to characterize plasmid DNA in E. coli was a

modified procedure of Birnboim and Doly (1979). Cells from a single clone were grown overnight with gentle shaking at 37°C in L media supplemented with the appropriate antibiotic. Approximately 1.0 ml of culture was transferred to a 1.5 ml microfuge tube and the cells were harvested by centrifugation at 12K for 30 sec (Beckman Microfuge 12). The supernatant was decanted and the cells resuspended in 0.2 ml of freshly prepared cold lysis solution (50 mM glucose, 10 mM EDTA (pH 8.0), 25 mM Tris-HCl (pH 8.0) and 2.0 mg/ml lysozyme) by vortexing.

This cell suspension was left on ice for at least 30 min, after which 0.4 ml of alkaline SDS solution (0.2 M NaOH and 1% SDS) was added to the tubes. The contents were mixed occasionally by inversion and left on ice. After 5 min, 0.3 ml of cold 3.0 M Na acetate (pH 5.0) was added to this viscous solution and the microfuge tubes were left on ice for at least 30 min or until a heavy white precipitate formed. The suspension was then centrifuged at 12K for 5 min at 4°C and the supernatant was transferred to a clean microfuge tube. 1.0 ml of isopropanol prechilled to -20°C was added to the supernatant and the microfuge tubes were then placed at -20°C for at least 30 min or at -70°C for 15 min.

The precipitate was recovered by centrifugation at 12K for 2 min at 4°C and the pellet was dissolved in 100 ul of 0.1 M Na acetate (pH 5.0). One ml of 95% ethanol was added to the suspension and the sample was left at -70°C for 10 min. The precipitate was recovered as before and the resulting pellet was washed with 70% ethanol prechilled to -20°C one or two times. The ethanol was decanted and the pellet dried in an incubator at 37°C for 15 min. The DNA was then resuspended in 20 ul of TE buffer.

Treatment with 1.0 ul of a 10 ug/ml DNAse-free RNAse solution (in 10 mM Tris HCl pH 7.5, 15 mM NaCl) for 15 min at -20°C was optional, depending on the future disposition of the sample. This DNA was stored at 4°C until it was used in restriction map analysis, tranformation or cloning.

Large Scale Preparation of Plasmid DNA

For the isolation of plasmid DNA in larger amounts a modified alkali SDS procedure was used (Maniatis et al., 1982). Cells from a single bacterial colony harbouring the desired plasmid were grown overnight with gentle shaking at 37°C in L media supplemented with the appropriate antibiotic. 100 ul of the overnight culture was added to 25 ml of fresh media and grown at 37°C until cells entered late log phase (OD_{600} = 0.6A). These cells were then used to inoculate 500 ml of supplemented L media and grown as before to an OD_{600} of 0.4A (approximately 3 hr). To allow for an increase in the ratio of plasmid/chromosomal DNA either chloramphenicol (30 ug/ml) or spectinomycin (300 ug/ml) was added to the culture which was then incubated for an additional 12 - 16 hr.

Bacterial cells were harvested by centrifugation at 10K for 10 min at 4°C in an I.E.C. B-20A centrifuge (A-54 rotor). The supernatant was decanted and the bacterial pellet resuspended in 8.0 ml of freshly prepared lysis solution (50mM glucose, 10 mM EDTA pH 8.0, 25mM EDTA pH 8.0 and 5 g/ml lysozyme) prechilled to -20°C. The suspension was transferred to a 45 ml Oakridge tube and left at room temperature for 5 minutes. All subsequent steps were carried out on ice unless otherwise stated. 16 ml of freshly made alkaline SDS solution (0.2 N

NaOH, 1% SDS) was added to the suspension and the test-tube was inverted occasionally to mix the contents thoroughly.

After 10 min, or until the contents became viscous, 12 ml of 3.0 M Na acetate (pH 5.0) prechilled to -20°C was added. After a minimum of 15 min the sample was centrifuged at 16K for 20 min at 4°C in an I.E.C. B20A centrifuge (A-211 rotor). A minimum of 25 ml of supernatant was transferred to a fresh Oakridge tube and isopropanol was added to 0.54 v/v. The solution was throughly mixed and left on ice for at least 15 min. DNA was collected by centrifugation at 16K for 30 min at 4°C as before. The supernatant was decanted and the pellet washed with 30 ml of 75% ethanol prechilled to -20°C. Care was taken to ensure that the pellet was not disturbed during this step. The ethanol was decanted and the pellet dried in a 37°C incubator, after which the pellet was resuspended in 7.0 ml of TE buffer. Further purification of plasmid DNA was carried out by cesium-chloride density gradient centrifugation.

Preparation of Cesium-Chloride Density Gradients

7.0 ml of plasmid DNA solution was transferred to a 16 x 76mm ultra-clear ultracentrifuge tube which contained 7.5 g of CsCl. The tube was covered with parafilm and inverted until all CsCl was dissolved. 0.5 ml of a 5 mg/ml ethidium bromide (EtBr) solution was added to the top of the tube and any air bubbles were displaced with paraffin oil. Care was taken to ensure that the ultra-centrifuge tube assembly was air-tight before the tube was inverted to mix the EtBr with the CsCl solution. All subsequent steps were done in the dark, to prevent photolysis of the DNA/EtBr complex.

The plasmid preparation was then centrifuged at 40K in an I.E.C. B-60 ultra-centrifuge (A-321 rotor) for a minimum of 48 hrs at 23°C.

Recovery of Plasmid DNA

The ultra-centrifuge tube was mounted onto a retort stand and the DNA bands were viewed under long wavelength UV light. A 1.0 ml disposable syringe with a large gauge needle was inserted just below the plasmid DNA band (lower band). To facilitate removal of the plasmid DNA a smaller-gauge needle was inserted into the ultra-centrifuge tube immediately below the cap assembly. EtBr was removed by extracting the plasmid DNA with equal volume of 1-butanol at least 3 times. The DNA sample was then dialyzed against 3 changes of 100 volume excess TE buffer for at least 6 hours each at 4°C. After this the DNA concentration and purity were determined in a Beckman DU-7 Spectrophotometer. The biological activity of the DNA was determined by its transformation frequency and then stored at 4°C until use.

Preparation of Competent Cells

E. coli cells from a single clone were added to 2 ml of L media broth and grown overnight with gentle shaking at 37°C. Depending upon the volume of competent cells required the overnight culture was then diluted 1:100 in fresh L media and incubated at 34°C to an OD_{600} of 0.6 - 1.0.

Cells were harvested by centrifugation at 2.7K for 5 min at 4°C in an I.E.C. clinical centrifuge (model Centra-7R). The supernatant was decanted and the pellet resuspended in one-half the growth volume of 50 mM $CaCl_2$ prechilled to -20°C. The suspension was kept on ice for

at least 20 min, after which the cells were collected and the pellet gently resuspended in 1:10 original growth volume of 50mM $CaCl_2$ as before. These cells were stored on ice or at 4°C for period of up to 48 hrs prior to being used. Since competent cells are extremely fragile, care was taken to avoid vortexing or centrifugation of cells except as stated previously.

Transformation of E. coli

For each transformation, 200 ul of competent cells were needed to which 1.0 ug of plasmid DNA from rapid preparation or approximately 50 ng of plasmid DNA from large scale preparations were added. The suspensions were left on ice for at least 60 min after which the entire 200 ul sample was spread onto the appropriate agar plate. These plates were then incubated at 37°C for a minimum of 6 hrs to determine the number of transformants /ug DNA.

Restriction Endonuclease Digestion

Plasmid DNA was digested with restriction enzymes in the appropriate buffer under the conditions described by the manufacturer or as suggested by Maniatis et al., 1982.

Ligation of DNA Fragments

The conditions for "sticky-end" and "blunt-end" ligations have been described previously (Maniatis et al., 1982). The molecular concentration of DNA was determined by use of the following equation.

$$\text{p mol ends/ ug DNA} = \frac{2 \times 10_6 \times N}{M}$$

where 2 = the number of ends per linear molecule, 10_6 = conversion factor, N = the number of cut sites + 1 for linear DNA, or the number of cut sites for circular DNA and M = the number of base pairs x 666 g/mol (at 50% AT). For maximum cloning efficiency a 3 fold p mol end excess of fragment DNA over vector DNA was used.

Agarose Gel Electrophoresis

The standard method used to separate, identify and purify DNA fragments was electrophoresis through agarose gels. As little as 1.0 ng of DNA can be detected by direct examination of the gel in UV light (Sharp et al., 1973).

To prepare agarose gels the appropriate amount of agarose which was dependent upon the size of the DNA fragments to be separated (see Maniatis et al., 1982) was added to TBE buffer (90 mM TRIS-borate, 90 mM Boric acid, 2 mM EDTA) and the suspension was boiled 3 times. The gel was poured into the desired mould, the comb inserted and the gel allowed to solidify. Sufficient TBE buffer to cover the gel to a minimum depth of 1.0 mm was added after which the comb was carefully removed to minimize damage to the wells. The samples were then loaded into the appropriate pockets of the submerged gel as quickly as possible to minimize diffusion of the DNA samples into the agarose surrounding the pockets. The gels were run at a constant voltage of 70 V until the bromophenol blue portion to the tracking dye (25% glycerol, 0.2% xylene cyanol, 0.2% bromophenol blue) had migrated approximately two-thirds down the length of the gel. This was generally between 2 - 5 hrs, dependent on the agarose concentration of the gel and the size of DNA fragments being separated.

After the electrophoresis was complete the gel was placed into TBE buffer containing 0.2 ug/ml of EtBr for approximately 5 min. The gel was rinsed with tap water to remove any surface EtBr and then photographed.

Gel Photography

The stained gel was fluoresced under UV illumination provided by a Chromato-Vue Transilluminator (Model TM-15) and photographed using an Omega-View 45E camera fitted with a Nikkor-W 150 mm lens and a light-pass UV block Wratten filter. The film used was Polaroid High Speed 4 X 5 land film, Type 57 (ASA 3000). Generally an exposure time of 2 - 5 sec and a developing period of 30 - 45 sec was sufficient to obtain good resolution of the bands.

Restriction Map Analysis

Once photographed the gel was ready to be analysed. All linear measurements were made from the origin (cathode end) to the mid-point of the migrating band. The distance each band had migrated (in cm) was recorded. Actual DNA fragment sizes were determined using a commercially obtained " phage marker DNA" as described (Maniatis et al., 1982).

Localization of the Yeast DFR 1 Gene

Approximately 10 ug of pIUD-1 DNA was digested with either Bam H1 or Sal 1 to completion under previously mentioned conditions. DNA was ethanol precipitated and collected as before, except that 0.2 M NaCl was used in place of 0.3 M Sodium Acetate. The pellet was resuspended

in 180 ul of Bal 31 reaction buffer (12 mM $CaCl_2$, 12 mM MgCl, 600 mM NaCl, 20 mM Tris HCl pH 8.1, 1 mM EDTA) placed at 30°C and allowed to equilibrate for 5 min. 10 units of Bal 31 nuclease was added to the sample which was then mixed throughly and immediately placed back at 30°C. 10 ul aliquots were removed at t = 0 and at 30 sec intervals thereafter. To stop Bal 31 digestion 3 ul of 200 mM EDTA was added to each aliquot which was then placed on ice.

To physically map the location of the dihydrofolate reductase gene, the sizes of the Bal 31 digestions were monitored by agarose gel electrophoresis as described previously. The remainder of each sample was then blunt end ligated and used to transform bacteria. Resultant transformants were tested for sensitivity to TRM (2ug/ml) and digested with the appropriate restriction endonuclease to ensure that Bal 31 digestion had taken place (see Results).

Yeast Transformation

Transformation of yeast cells with plasmid DNA followed a modified procedure of Hinnen et al., (1978). This method yielded between 1 - 100 transformants with plasmids which require integration into the yeast genome or 100 - 10,000 transformants with plasmids which are capable of automonous replication per ug DNA.

To 80 ml of YPDP media approximately 10^7 yeast cells from an overnight culture were added. This suspension was grown with shaking at 30°C until the cell density reached 1 X 10^7 cells/ml. Cells were harvested by centrifugation at 5K for 5 min (Sorval SS34 rotor) and the pellet was washed once with 1 M sorbitol. The cells were then resuspended in 8 ml of 1 M sorbitol containing 1% glusulase and the

suspension was incubated with gentle shaking at 30°C for 30 - 90 min, or until spheroplast formation was approximately 50% complete. Spheroplasts were harvested by centrifugation at 2.5K for 5 min as before. Cells were washed twice with 1 M sorbitol and then once with 20 ml STC (1 M sorbitol, 10 mM Tris HCl ph 7.5, 10 mM $CaCl_2$). The pellet was then gently resuspended in 0.5 ml STC.

Plasmid DNA was added to the competent cells and after 15 min 10 volumes of PTC (20% PEG 4000, 10 mM Tris HCl pH 7.5, 10 mM $CaCl_2$) was added. The suspension was incubated for 15 min after which cells were harvested by low speed centrifugation (2.5K for 5 min), washed once in STC and resuspended in 2 ml STC. 100 ul of competent cells was added to 10 ml of regenerating agar (1 M sorbitol, 3% agar, 2% by volume YDP media, 2% glucose, 0.67% yeast nitrogen base) at 48°C containing the appropriate supplements to allow for the selection of transformants. The suspension was then gently mixed to ensure a homogenous cell distribution and immediately poured onto the appropriate SD agar plates. Putative transformants were scored after incubation for 3 - 5 days at 30°C.

Radial Gradients

Approximately 10^6 exponential yeast cells were added to 20 ml of YPDP top agar supplemented with 5 mg/ml sulfanilamide (Sulf). To ensure a homogeneous cell distribution, the suspension was gently vortexed and immediately poured onto a YPDP agar plate. 200 ug of methotrexate (MTX) and 5 mg Sulf were added to a centre well to establish a gradient of drug concentration which decreased toward the periphery of the plate. After incubation at 30°C for 48 hrs, the

induction of cytoplasmic petites was scored by overlaying the YPDP agar plates with 0.1% 2,3,5 triphenyltetrazolum chloride (TTC) to identify respiratory deficient mutants (ρ-).

Methotrexate Sensitivity

Sensitivity of yeast cells to MTX was determined two ways: 1) radial gradients of antifolate drugs; 2) by culturing log phase cells in MTX-Sulf supplemented YPD media.

Yeast cells were grown overnight in YPD media at 30°C to a cell density of 2 - 4 X 10^5/ml. Cells were harvested and inoculated into fresh flasks containing YPD media supplemented with 5 mg/ml Sulf and MTX at concentrations of 7.5, 15, 25, 50, 75, 100, 175, 250, and 500 ug/ml. The flasks were immediately placed into a rotary shaker water bath and incubated for 12 hrs at 30°C. Samples were chilled on ice to 4°C and disrupted by brief sonication to disperse cell clumps. Cell density was determined by counting using a Coulter counter (model ZF) equipped with a 100 um aperture. The suspensions containing 0 or 500 ug/ml MTX and 5 mg/ml Sulf were diluted, plated on YPD agar plates and incubated at 30°C for 24 hrs.

Viable cell number was recorded and any putative ρ - colonies (white) were streaked onto YPG agar plates to confirm their ρ - phenotype. Cell morphology in the presence and absense of antifolate drugs was analyzed microscopically.

Trimethoprim Sensitivity

Sensitivity of yeast cells to trimethoprim (2,4-diamino-5-[3,4,5,-trimethoxy benzyl] pyrimidine) was determined in two ways;

1) by spotting between 10^2 and 10^3 well washed cells onto SD plates supplemented with the appropriate amino acids with and without trimethoprim (TRM); and 2) by culturing log phase starved cells in TRM-amino acid supplemented SD media. With either method it was important to avoid contaminating the cell suspensions with traces of yeast extract-peptone media as well as high cell concentrations since both conditions diminish or abolish the sensitivity of 2 radiation sensitive mutant strains (rad 6 and rad 18) to the antifolate TRM (Lawrence and Christensen, 1979).

To obtain log phase starved cells, a modified Mayer and Goin (1984) procedure was employed. Cells were preconditioned by inoculating 2 - 4 X 10^4 cells/ml into amino acid supplemented SD media and incubated at 30°C in a rotary shaker water bath overnight to produce a midlog phase culture. These cells were harvested by low speed centrifugation, washed 3 times with saline (0.8%), resuspended in saline contained 0.1% glucose and incubated for a period of at least 16 hrs at 30°C in a rotary shaker water bath to produce a midlog phase starved culture. These measures were taken to deplete intracellular folate pools and C1 metabolites that might interfere with TRM sensitivity (Game et al., 1975). Amino acid supplemented SD media was used to dilute a stock TRM solution (2 mg/ml) to prepare solutions containing 25, 50, 75, 100, 150, 200, 250, 300, and 500 ug/ml of the drug. The log phase starved cells were inoculated into these solutions (15ml) to yield suspensions of 2 X 10^5 cells/ml. These suspensions were immediately placed in a rotary shaker water bath and incubated for 24 hrs at 30°C.

The samples were then chilled on ice and disrupted by brief

sonication to disperse cell clumps. Cell density was determined by counting using a Coulter counter as before. The suspensions which contained 0 and 500 ug/ml TRM were diluted, plated on to amino acid supplemented SD agar plates and incubated for 2 days. Viable cell numbers were recorded and any putative ρ^- colonies were streaked onto YPG agar plates to confirm their ρ^- phenotype.

Petite Induction by Ethidium Bromide

Exponential yeast cells were grown in the dark with gentle shaking at 30°C in the presence of 30 ug/ml Ethidium Bromide (EtBr). Aliquots were removed at 1 hr intervals, diluted and plated on YPDP agar plates which were incubated at 30 °C for 2 - 3 days. Viable cell numbers were recorded and any white colonies were scored as putative ρ^- mutants. The ρ^- phenotype was confirmed as described previously.

M13 Cloning

After cloning the yeast dihydrofolate reductase DNA fragment in to M13mp8 and M13mp19 a series of deletions were done to: 1) localize the functional domain of DFR 1; and 2) determine the primary base sequence of DFR 1. These recombinant phage were characterized in several ways; 1) spotting 10 ul of viral supernatant onto a lawn of JM103 cells spread on L agar plates and on TRM (2ug/ml) supplemented MIN agar plates; and 2) by agarose gel electrophoresis of single stranded viral DNA (ssDNA).

Transfection of E. coli

To plate transfected cells 2 - 100 ng of recombinant phage DNA

was added to 300 ul of competent cells in a 13 X 100 mm glass test-tube and placed on ice for at least 40 min. The mixture was then heat shocked for 2 min at 42°C immediately after which 10 ul of 100 mM IPTG, 50 ul 2% Xgal, 0.2 ml fresh exponentially growing JM103 cells and 3 ml of soft B agar were added. These components were quickly mixed and plated directly onto B plates which were incubated at 37°C for 6 - 18 hrs. Turbid plaques and the colour reaction were seen after approximately 4 and 6 hr respectively.

Preparation of Template DNA

Two ml of exponentially growing JM103 were placed into a sterile 50 ml plastic conical test-tube. A sterile toothpick was used to "pick" a plaque and transfer the phage to the culture which was shaken vigourously for 3 hr at 37°C. 0.1 ml of this culture was added to a fresh sterile test-tube containing 1.0 ml of exponential JM103 cells and 9 ml of B media. After further incubation for 6 hr, as before, cells were harvested by centrifugation at 2.7K for 15 min in an I.E.C. clinical centrifuge (model Centra-7A).

Initial characterization of the recombinant phage was done by withdrawing 10 ul of the supernatant and placing it into a 1.5 ml microfuge tube containing 1 ul of 2% SDS and 3 ul of 0.1% bromophenol blue solution. The sample was loaded onto a 0.7% agarose gel (in TBE buffer), electrophoresed at 70 V for 5 hr and stained as described previously.

The remainder of the supernatant was decanted in to a sterile 50 ml plastic test-tube which contained 900 ul of a 20% PEG solution (in 3M NaCl). The mixure was left at room temperature for 15 min,

then centrifuged for 30 min at 10K in an I.E.C. B20A centrifuge (870 rotor). Care was taken not to disturb the pellet while removing the supernatant with a pasteur pipet after which the inside wall of the test-tube was wiped with a Kimwipe. The pellet was resuspended in 0.4 ml of TES buffer and the solution transferred to a 1.5 ml microfuge tube. 400 ul of phenol equilibrated with TES was added to the microfuge tube, the solution was vortexed for 10 sec and centrifuged at 12K for 1 min (Beckman Microfuge 12). After the aqueous phase was removed the sample was ethanol precipitated (40 ul of 3 M sodium acetate pH 6.0, 1 ml 95% ethanol) at -70°C for 30 min. The DNA was recovered by centrifugation for 5 min as before and the pellet was rinsed in 1 ml of 70% ethanol prechilled to -20°C. The supernatant was carefully removed and the pellet dried under reduced pressure. 50 ul of TE buffer was used to resuspend the DNA which was now ready for sequencing.

Sequencing Procedure

The procedure used to sequence DFR 1 was that of Sanger et al., (1977). For each sequencing reaction the phage M13 template was prepared as described (Sanger et al., 1980) and annealed to a commercially available synthetic primer (Duckworth et al., 1981) in a 1.5 ml microfuge tube. The annealings were performed by combining 4 ul of template DNA, 2 ul of single-stranded primer, 1 ul of 10 X annealing buffer (10mM Tris HCl pH 7.5, 10 mM NaCl) and 3 ul H_2O. The mixture was incubated in a tightly sealed microfuge tube at 55°C for 10 min in a 10 ml water bath after which the bath was allowed to slowly return to room temperature.

DNA synthesis reactions were carried out at room temperature in 4 1.5 ml microfuge tubes labelled A,C,G, and T. The primer-template mixture was added to a microfuge tube which contained 40 uCi of lyophilized α^{32}P dATP (New England Nuclear) and 2 ul of radioactively labelled template mix was placed on the side wall of each tube. Commercialy obtained A,C,G, and T reaction mixtures (P.L. Biochemical) were dispensed in 3 ul aliquots to their respective tubes. To these tubes 1 ul of E. coli DNA polymerase 1 (large fragment) was added and reactions were begun simultaneously after 2 sec microfugation. 1 ul of Chase solution (P.L. Biochemicals) was placed on the side of each tube and after 15 min incubation the tubes were centrifuged as before. After 15 min incubation the reactions were stopped by addition of 3 ul formamide stop solution (0.3% xylene cyanol FF, 0.3% bromophenol blue, 1 ml 100 mM EDTA pH 8.3, 9 ml deionized formamide).

The reaction tubes were placed into boiling water for 3 min immediately prior to loading 3 ul of sample onto the polyacrylamide gel. Electrophoresis of samples was carried out at 40 - 45 W in 1 X TBE buffer. Routinely when non-gradient gels were used, samples were loaded 3X at 90 - 120 min intervals, whereas samples were loaded only once and run for 4 - 6 hr when buffer gradient gels were used.

Polyacrylamide Gel Electrophoresis

Two types of polyacrylamide gels were used during the course of this study: 1) non-gradient gel; and 2) buffer gradient gel. In either instance the gels were cast between 20 X 40 cm glass-plates separated by 0.4 mm spacers. The glass plates were thoroughly washed with a mild non-abrasive detergent, rinsed several times "ultra-pure"

distilled water and then wiped with 90% ethanol.

Non-gradient Gel

For 60 ml of an 8% acrylamide solution the following were thoroughly mixed; 25.2 g urea, 12 ml 40% acrylamide/bisacrylamide (38:2), 6 ml 10 X TBE buffer, 0.6 ml 10% ammonium persulfate, distilled H_2O to a final volume of 60 ml. The solution was filtered into a 125 ml flask and then placed on ice until the temperature of the solution reached 15°C. Immediately prior to pouring the gel 30 ul of TEMED was added and the solution swirled gently to ensure thorough mixing. The gel was formed as previously described (Sanger and Coulson, 1977) by pouring the gel solution down one side of the gel mould. When the gel mould was completely filled the sharks-tooth comb was inserted at the top of the gel. The gel was left flat on the bench top until polymerization occurred.

Buffer Gradient Gel

Gradient gels used in the study were formed by a modified procedure of Biggin et al., (1983). 35 ml of 0.5 X TBE gel mix (25.2 g urea, 12 ml 40% acrylamide-bisacrylamide (38:2), 3 ml 10 X TBE buffer, 0.2 ml 10% ammonium persulfate, 60 ul TEMED and distilled H_2O to a final volume of 60 ml) was drawn into a 50 ml disposable syringe and put on ice. A 25 ml graduated glass pipette was used to first draw up 12 ml of the 0.5 X TBE gel mix followed by 12 ml of a 5.0 X TBE gel mix (10.5 g urea, 5 ml 40% acrylamide-bisacrylamide (38:2), 6.25 ml 10 X TBE, 0.1 ml 10% ammonium persulfate, 2.5 g sucrose, 0.1 ml bromophenol blue, 30 ul TEMED and distilled H_2O to 25 ml). Care

was taken to minimize mixing of the two solutions while pipetting. This resulted in a two-phase solution separated by a diffuse boundry which was slightly mixed by allowing several air bubbles to run up the pipette. The gradient was poured by running the gel mix down one side of the gel mould until the mould was approximately half full. After the gradient was poured, the gel mould was declined to a nearly horizontal position approximately 1 inch above the bench top to arrest the flow of the gel mix. The syringe containing the 0.5 X TBE mel mixed was now used to finish pouring the gel. The bromophenol blue edge gave a visual indication of how the buffer concentration gradient formed. After the gel was cast the comb was inserted and the gel was left to polymerize.

Gel Processing and Autogradiography

After electrophoresis was complete the gel was removed from the sequencing apparatus. The glass plates were carefully separated to prevent damage to the fragile gel. Wet areas were dried and a piece of plastic wrap was placed over the gel. Kodak® XR-5 film was used to expose the gel for 12 - 18 hrs at -70°C.

The film was developed in Kodak® GBX developer for 5 min and fixed in Kodak® GBX fixer. Primary nucleotide base sequence was determined as described by Sanger and Coulson (1978).

Results

The yeast dihydrofolate reductase gene (tentatively named DFR 1) was isolated previously from a J. Freisen yeast genomic library. The selection technique exploited the differential sensitivities of E. coli dhfr and the yeast enzyme to the antifolate trimethoprim. DNA/DNA hybridization studies confirmed that the 8.8 kbp yeast DNA fragment isolated from the trimethoprim resistant clone was of yeast origin (Barclay et al., in prep.).

Prokaryotic dhfrs are strongly inhibited by trimethoprim, whereas analogous eukaryotic enzymes show a much greater resistance to the drug in vitro (Baccanari et al., 1975). Preliminary experiments were done to quantitate this difference in drug resistance between wild-type bacterial strain (JF1754) and the same strain transformed with the dhfr plasmid pDR420. Table 3 shows that TRM (2 ug/ml) in the culture medium completely inhibited the host strain whereas bacterial cells containing plasmid pDR420 grew substantially at this drug concentration. Provision of higher amounts of TRM inhibited growth of both cell types. To avoid the possibility of rearrangements in the plasmid DNA sequence as a consequence of thymine depletion (Barclay and Little, 1978), the drug concentration at which E. coli cells were screened for a TRM-resistant phenotype was standardized at 2 ug/ml.

When exponential bacterial cells are grown in minimal medium containing TRM but lacking exogenous C1 metabolites (Cohen and Barner, 1954) the bacteria enlarge without dividing, and die. This "unbalanced growth" is due to a depletion of the intracellular folate pools which results in a thymineless condition (Angehrn and Then, 1973). Figure 3 shows E. coli cells growing under normal and

Table 3 Expression of the cloned yeast dihydrofolate reductase gene in *E. coli*.

[TRM] ug/ml	Survival (%) JF1754	JF1754/pDR420
0	100	100
2	<1.0	89.0
100	<1.0	2.3

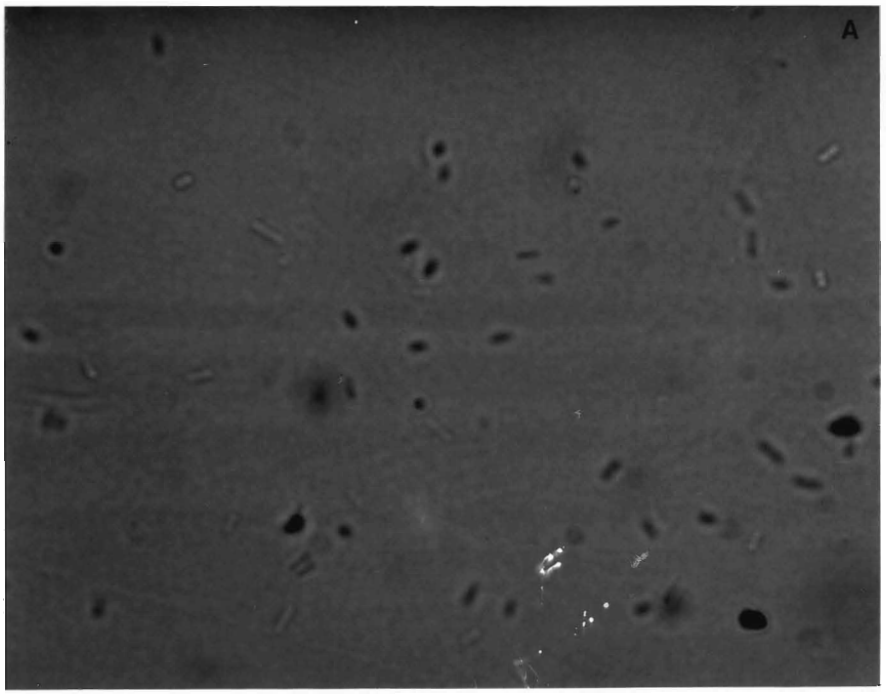
Cells were grown under the conditions described in Methods.

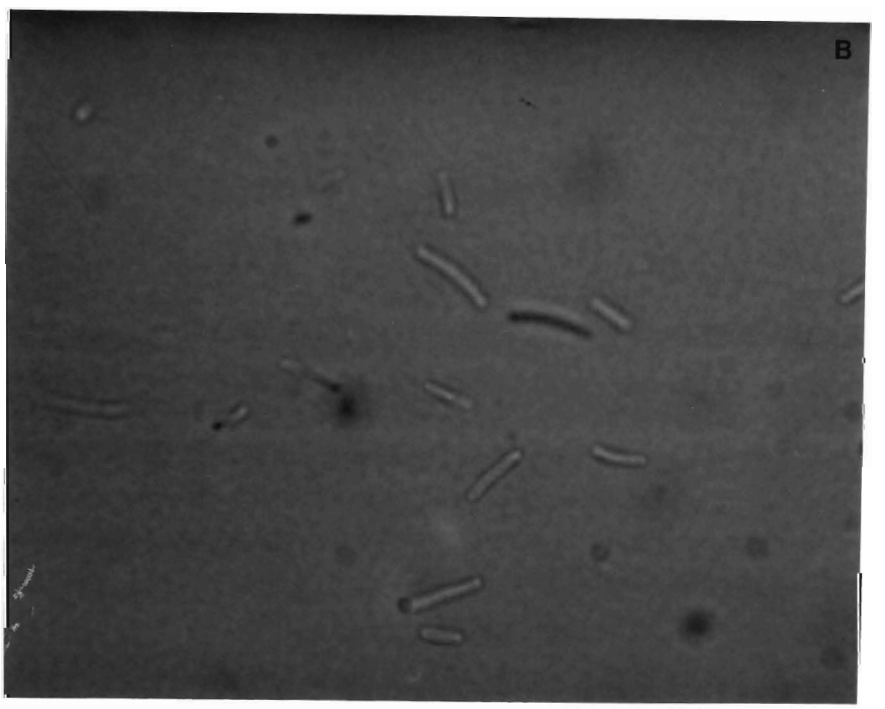
Figure 3 Morphology of bacterial cells treated with trimethoprim.

Exponential cells of E. coli strain JM103 were grown in minimal medium supplemented with 2 ug/ml TRM, as described previously. After 8 h incubation the cells were examined by light microscopy and photographed (see methods).

A) Exponential cells incubated in minimal media (X2000).

B) Exponential cells incubated in minimal media contained 2 ug/ml TRM (X2000).


A


B

folate-depleted conditions. Instead of the characteristic morphology of balanced exponential growth, TRM treated cells became elongated and filamentous. This is characteristic of E. coli cells in which DNA synthesis has been inhibited.

As shown in Figure 4, the recombinant plasmid pDR420 contains 2 Sal 1 DNA fragments of 4.0 and 4.8 kbp. The smaller fragment corresponds to the vector pACYC 184 and the larger fragment contains the yeast insert and a portion of the tetracylene (Tc) gene. The 4.8 kbp DNA segment contained a Bam H1 site which when digested with the enzyme generated fragments of 2.8 kbp and 2.0 kbp. The latter which contained the DFR 1 gene was sub-cloned on a 1.8 kbp Bam H1-Sal 1 fragment. It was this 1.8 kbp yeast DNA fragment which was used for future constructions.

Plasmid Constructions.

In order to study the effects of the cloned yeast dhfr gene in yeast the construction of several plasmids was required. The first construction involved the directional cloning of the 1.8 kbp Bam H1-Sal 1 dhfr DNA fragment into the 5.5 kbp yeast integrating vector YIP5. Initially, the recombinant plasmids were used to transform E. coli and DFR 1 transformants were characterized by their resistance to TRM. As a second control, the 3 TRM resistant colonies were cured of their plasmid by growth under non-selective conditions (Hsiao and Carbon, 1981) and it was determined that the TRM resistance was plasmid encoded. Figure 5 shows that the restriction map of the 3 recombinant yeast dihydrofolate reductase plasmids pIUD 1, pIUD 2 and pIUD 3 in lanes 4, 5 and 6 respectively were identical. In all cases

Figure 4 Characterization of the plasmid pDR420.

1 ug of purified pDR420 DNA was digested with the restriction endonucleases Bam H1 (lane 2), Sal 1 (lane 3) and Bam H1 and Sal 1 (lane 4) as described previously. The DNA fragments were separated by agarose gel electrophoresis (0.7%) in TBE buffer for 90 min at 70mV. DNA bands were stained with EtBr, visualized under UV light and photographed as described previously. λDNA digested with Hind III was used as standard (lane 1).

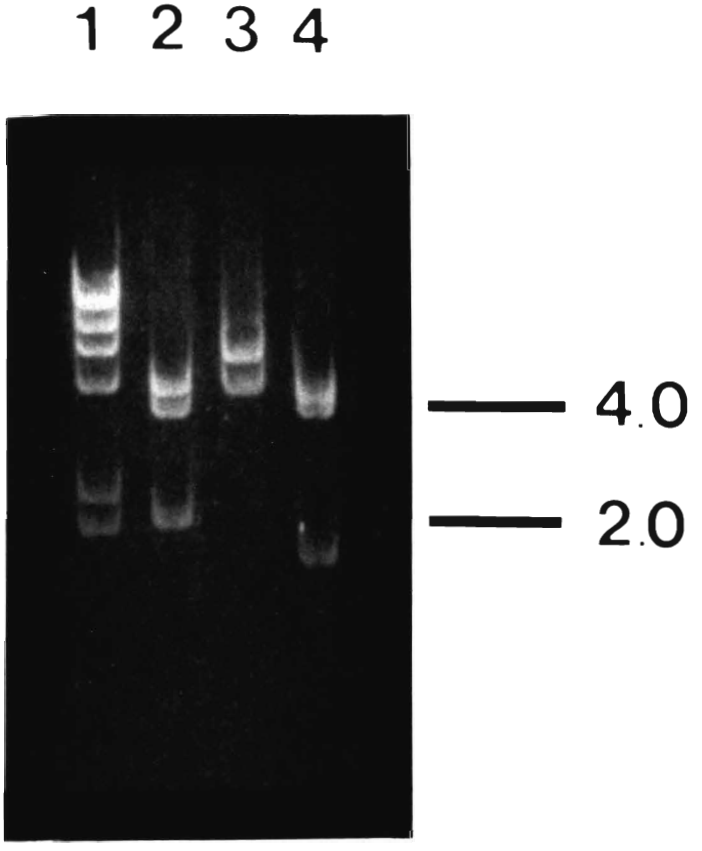
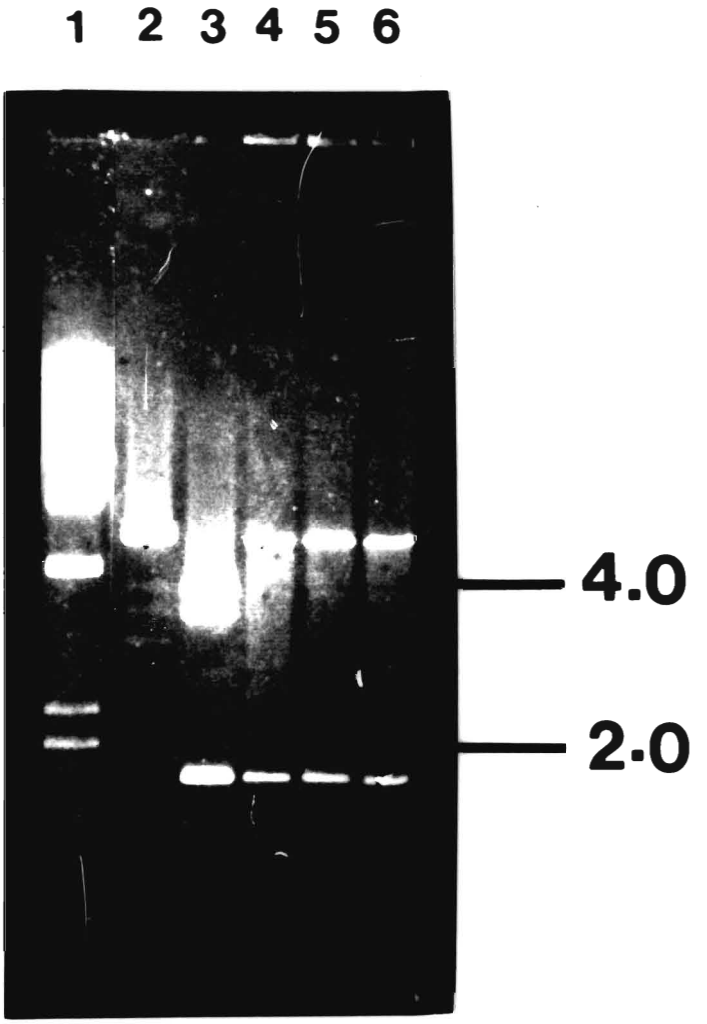

1 2 3 4
4.0
2.0

Figure 5 Characterization of the DFR 1 - yeast integrating plasmid pIUD 1.

The yeast dhfr fragment donor pDR420 and the shuttle vector YIP5 were first digested with Bam H1 and then Sal 1, the fragments were ligated and plasmids used to transform E. coli as described previously (see Methods). Plasmid DNA from 3 TRM resistant transformants was digested as above. DNA fragments were separated by agarose gel electrophoresis (0.7%) and analyzed as before.

Lane 1	λphage	DNA digested with Hind III.
Lane 2	YIP5	DNA digested with Bam H1 and Sal 1.
Lane 3	pDR420	DNA digested with Bam H1 and Sal 1.
Lane 4	pIUD1	DNA digested with Bam H1 and Sal 1.
Lane 5	pIUD2	DNA digested with Bam H1 and Sal 1.
Lane 6	PIUD3	DNA digested with Bam H1 and Sal 1.


1 2 3 4 5 6
4.0
2.0

a 5.2 kbp fragment, which corresponded to the vector YIP5 (lane 2) and a 1.8 kbp fragment which corresponded to the dhfr Bam H1-Sal 1 DNA fragment of pDR420 (lane 3) were present.

The second construction involved the non-directional cloning of the 2.0 kbp Bam H1 dhfr fragment into the 13.2 kbp autonomously replicating shuttle vector pYF91. Resultant recombinant plasmids were screened in E. coli, as before, by their resistance to TRM as well as the ability of the LEU 2 gene of pYF91 to complement the leu B mutant E. coli strain JF1754 (Storms et al., 1979). Of the 16 TRM resistant, Leu+ plasmids isolated (pDR501 to pDR516), 15 had the yeast dihydrofolate reductase fragment in the same orientation as pIUD 1 (with respect to the ampicillin gene) and only pDR511 contained the insert in the opposite orientation (Figure 6). This suggested that the dhfr DNA fragment in addition to the structural gene for yeast dihydrofolate reductase, also contained any necessary regulatory elements which allowed the gene to express in bacteria.

For studies designed to physically map the yeast dihydrofolate reductase gene and determine its primary nucleotide base sequence using the Sanger et al., (1977) dideoxy-chain termination method, two recombinant phages (Messing and Vieira, 1982) containing DFR 1 were constructed. Figure 7 shows the cloning strategy for these constructions. They were characterized initially by their colourless plaque phenotype on IPTG X-gal plates (see Methods) and secondly by their resistance to TRM. Restriction map analysis confirmed that these recombinant bacteriophage, M13mp8B13 and M13mp19B13 contained the yeast dhfr fragment in the orientation shown. All attempts to clone DFR 1 in the opposite orientation into the M13 viral vectors

Figure 6 Schematic diagram of the construction of the chimeric plasmids pIUD 1 and pDR509, 511.

The 1.8kbp dhfr DNA fragment from pDR420 was cloned into the yeast integrating plasmid YIP5 as described in the legend to Figure 5. A TRM resistant, Ap+, URA 3 clone was characterized as described and named pIUD 1.

Non-directional cloning of the yeast dhfr fragment from pDR420 into the shuttle vector pYF91 was accomplished as described. Characterization of 16 TRM resistant Ap+, LEU2 clones included restriction map analysis to determine the orientation of the dihydrofolate reductase fragment in the vector.

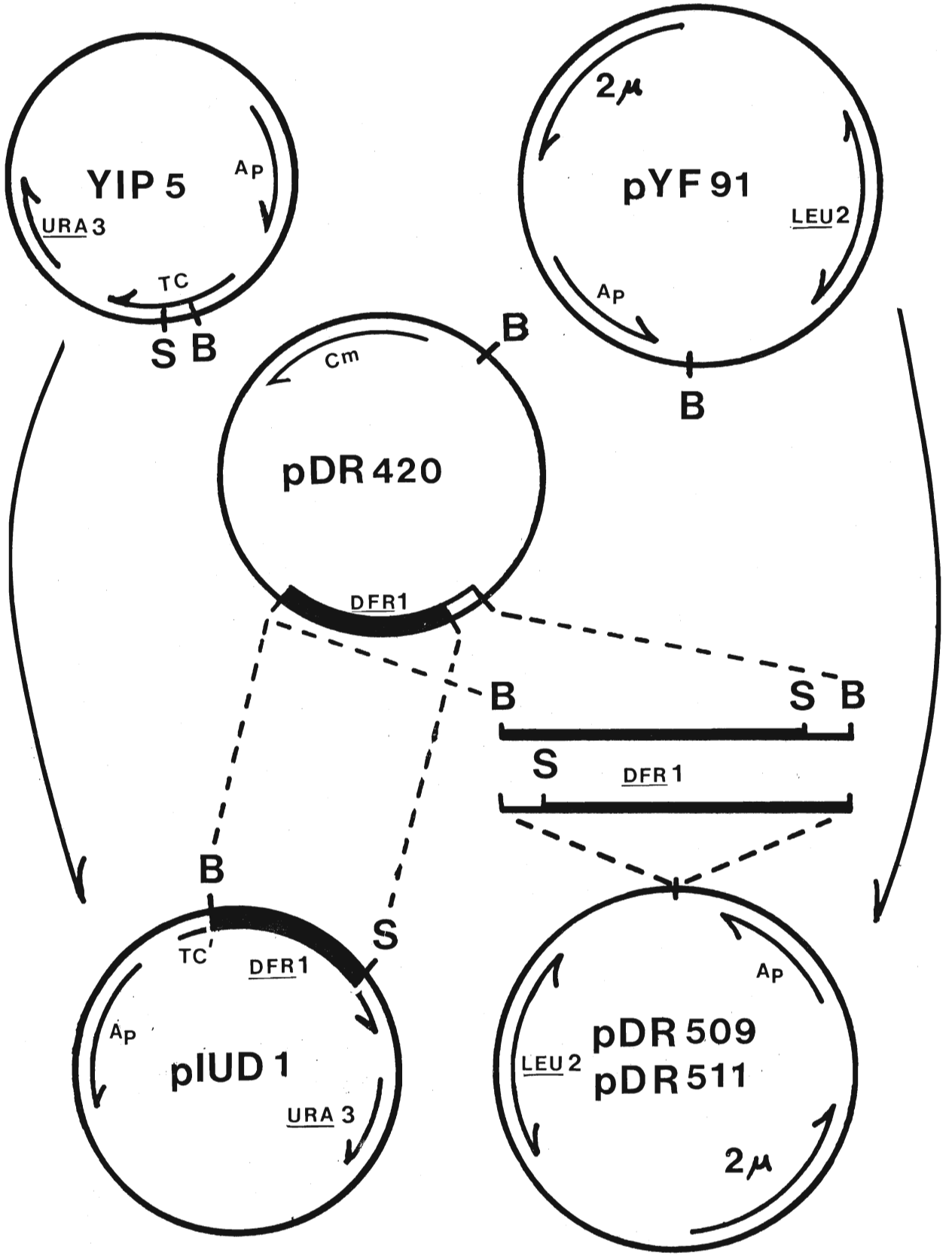
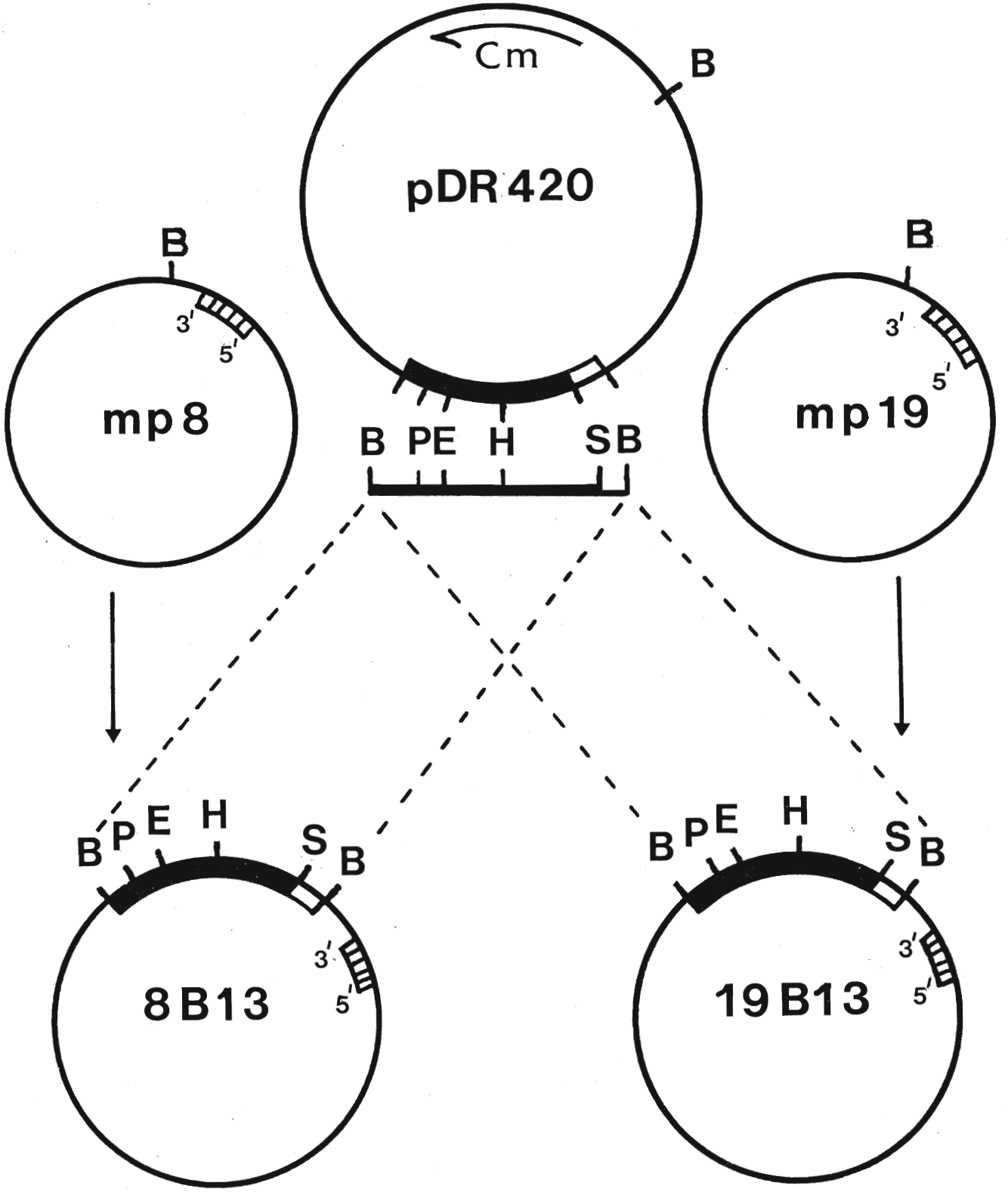

YIP 5
Ap
URA 3
TC
S B
2μ
pYF 91
LEU2
Ap
B
B
Cm
pDR 420
DFR1
B
S B
S
DFR 1
B
S
TC
DFR1
Ap
pIUD 1
URA 3
Ap
pDR 509
pDR 511
LEU 2
2μ

Figure 7 Schematic diagram of the construction of the chimeric phage M13mp8B13 and M13mp19B13.

The 2.0 kbp dhfr DNA fragment from pDR420 was cloned into the M13 vectors mp8 and mp19 as described previously. Recombinant phage were used to transfect E. coli and putative M13-dhfr clones were characterized by their resistance to TRM in culture.


Cm
B
pDR 420
B
3'
5'
mp 8
B PE H SB
B
3'
5'
mp 19
B P E H S B
8 B13
3'
5'
B P E H S B
19 B13
3'
5'

were unsuccessful. Although the reason for this bias is unclear, preferential insertion of cloned DNA fragments into M13 phage has been observed previously (Messing et al., 1981).

In order to study the expression of the cloned yeast dihydrofolate reductase in mammalian systems, it was necessary to construct a number of plasmids. Transduction of mammalian cells with DNA is extremely inefficient without a selectable marker (Parker and Stark, 1979). Thus, it is essential that the transducing vectors to be used contain specific genes which allow transduced cells to be grown preferentially. The two vectors used, pSV2-neo and pSV2-gpt have been described previously (Mulligan and Berg, 1981). Figure 8 shows the cloning strategy and the resultant recombinant plasmids pDN21 and pDG27.

The first construction (pDN21) was initially characterized in E. coli by its Ap+, Neo+, and TRM+ phenotype, whereas pDG27 exhibited an Ap+, TRM+ phenotype. Since it was not feasible to study the effects of yeast dhfr expression in mammalian cells in our laboratory, these plasmids were sent to Columbia University for further characterization. Preliminary studies revealed that the cloned yeast dihydrofolate reductase gene failed to complement the dhfr deficiency of a fol- Chinese hamster cell line (L. Chasin, pers. comm.).

Table 4 summarizes the plasmids constructed during the course of this study. In all cases the DFR 1 allele was present and the plasmids were initially characterized in E. coli.

Localization of the Cloned DFR 1 Gene

The initial series of experiments designed to physically map

Figure 8 Schematic diagram of the construction of the chimeric plasmids pDN21 and pDG27.

The 2.0 kbp dhfr DNA fragment from M13mp8B13 was cloned in pSV-neo and pSV-gpt as described previously. Recombinant plasmids were used to transfect E. coli and putative dhfr DFR 1 transformants were characterized as before.

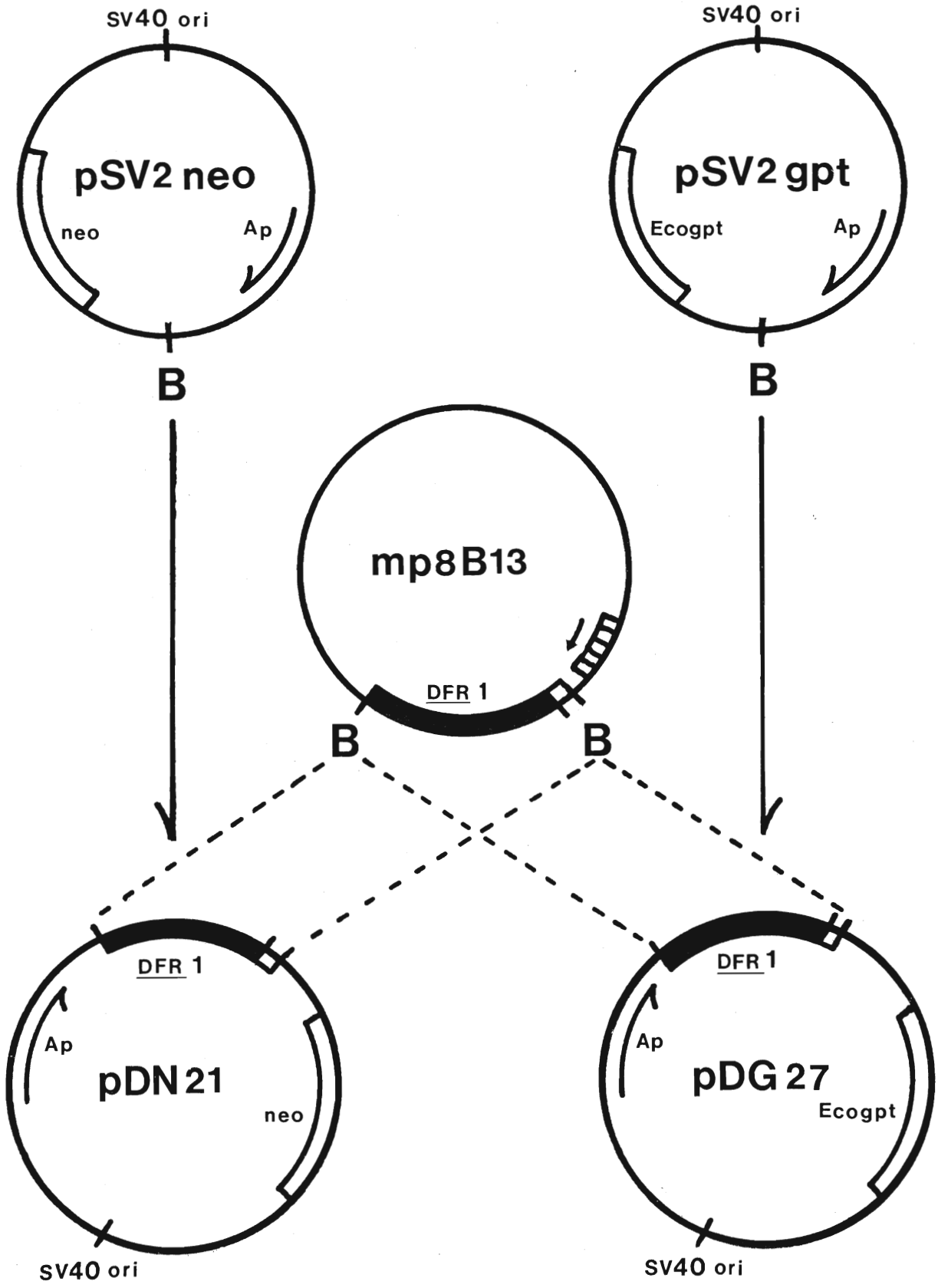

SV40 ori
pSV2 neo
neo
Ap
B
SV40 ori
pSV2 gpt
Ecogpt
Ap
B
mp8B13
DFR 1
B
B
DFR 1
Ap
pDN21
neo
SV40 ori
DFR 1
Ap
pDG 27
Ecogpt
SV40 ori

Table 4 Plasmids used in this study.

Plasmid	Characteristics	Source
pDR420	ori, cm+, DFR 1	G. Taylor
pIUD 1	ori, Ap+, URA3, DFR 1	this study
pDR509 pDR511	ori, Ap+, 2u, LEU2, DFR 1	this study
M13mp8B13 M13mp19B13	DFR 1	this study
pDN21	ori, Ap+, neo+, SV40 ori, DFR 1	this study
pDG27	ori, Ap+, gpt+, SV40 ori, DFR 1	this study

the location of the cloned yeast dhfr gene utilized the M13-DFR 1 recombinant phage. Figure 9 shows the relative size of the various M13mp8B13 and M13mp19B13 bacteriophage deletions which were constructed. These clones were then tested for sensitivity to TRM (2 ug/ml) by means of a spot test. Figure 10A shows that all 10 M13 derivatives formed plaques when spotted on a lawn of exponential JM103 cells growing on B agar media. However, when these same phage were plated on MIN agar media supplemented with 2 ug/ml TRM only 3 clones, M13mp8B13, M13mp19B13 and M13mp19B13ΔP grew.

These data suggested that the Bam H1-Pst 1 region of the 1.8 kbp dhfr fragment was not necessary for yeast dihydrofolate reductase expression in E. coli (Figure 11). Thus, the functional unit of DFR 1 was localized to a 1.6 kbp fragment between Pst 1 and Sal 1 restriction cut sites. Before any further characterization of the dhfr gene could be done it was necessary to construct a more accurate restriction map of the fragment.

Figure 12 shows the DNA fragment sizes obtained when various restriction endonucleases were used alone and in combination to digest pIUD 1 DNA. These results are summarized in Figure 13. The restriction map of pIUD 1 was constructed by combining the published restriction map of YIP5 (Scherer and Davis, 1979) and data obtained from this experiment. Thus, one end of the yeast DNA fragment necessary for expression in E. coli was localized to the 150 bp Pst 1-Eco R1 fragment. However, this procedure could not be used to map the terminus of the DFR 1 gene in the other direction due to the lack of suitable restriction endonuclease cut sites.

The ability of Bal 31 nuclease to digest duplex DNA was exploited

Figure 9 Characterization of the M13mp8B13 and M13mp19B13 deletions.

M13mp8B13 and M13mp19B13 DNA was digested with the restriction endonucleases Hind III, Eco R1 and Pst 1 under conditions described previously. The samples were religated and used to transfect the bacterial strain JM103. Single stranded DNA from appropriate phage was isolated as before and subjected to agarose gel electrophoresis (0.7%) at 70 mV for 5 hr.

A

Lane 1	M13mp8
Lane 1	M13mp8B13
Lane 3	M13mp8B13 Δ H
Lane 4	M13mp8B13 Δ E
Lane 5	M13mp8B13 Δ P

B

Lane 1	M13mp19
Lane 2	M13mp19B13
Lane 3	M13mp19B13 Δ H
Lane 4	M13mp19B13 Δ E
Lane 5	M13mp19B13 Δ P

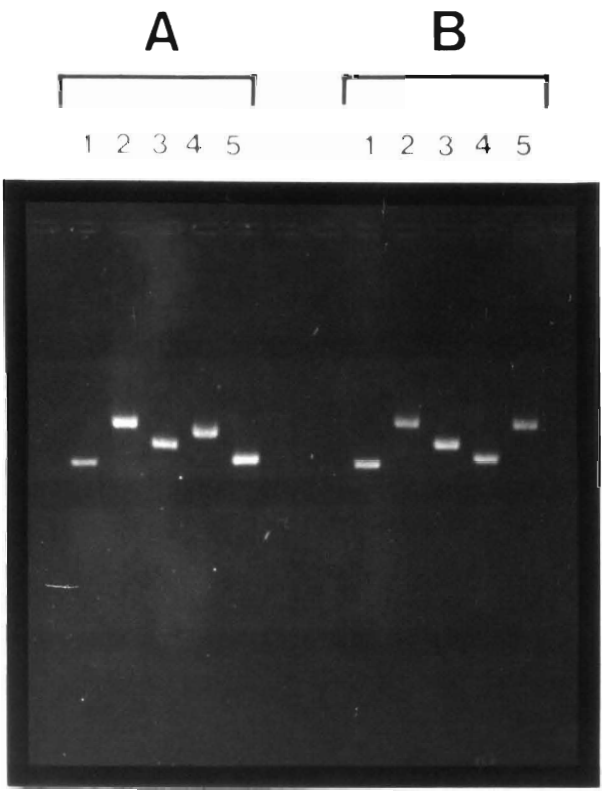
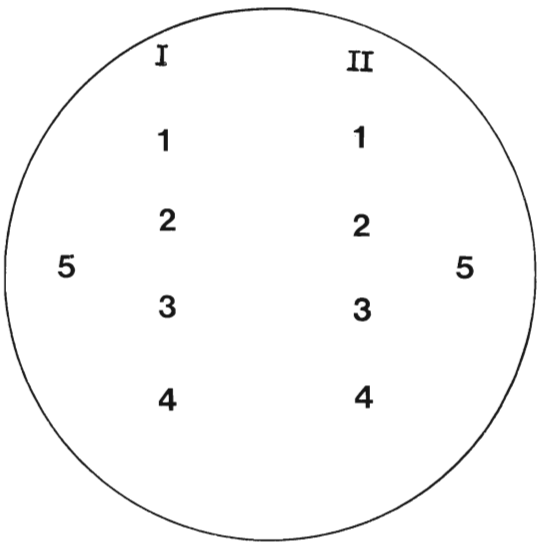

A
B
1 2 3 4 5
1 2 3 4 5

Figure 10 Trimethoprim sensitivity of the M13 based deletions

A lawn of cells of the bacterial strain JM103 (10^8) was spread on a B plate (A) and on a Min plate (B) supplemented with 2 ug/ml TRM. 10 ul of each ssDNA isolated as described in the legend to Figure 9 was spotted on both plates and incubated for 24 - 72 hr at 37°C.

I
1 mp8B13
2 mp8B13 Δ H
3 mp8B13 Δ E
4 mp8B13 Δ P
5 mp8

II
1 mp19B13
2 mp19B13 Δ H
3 mp19B13 Δ E
4 mp19B13 Δ P
5 mp19B13



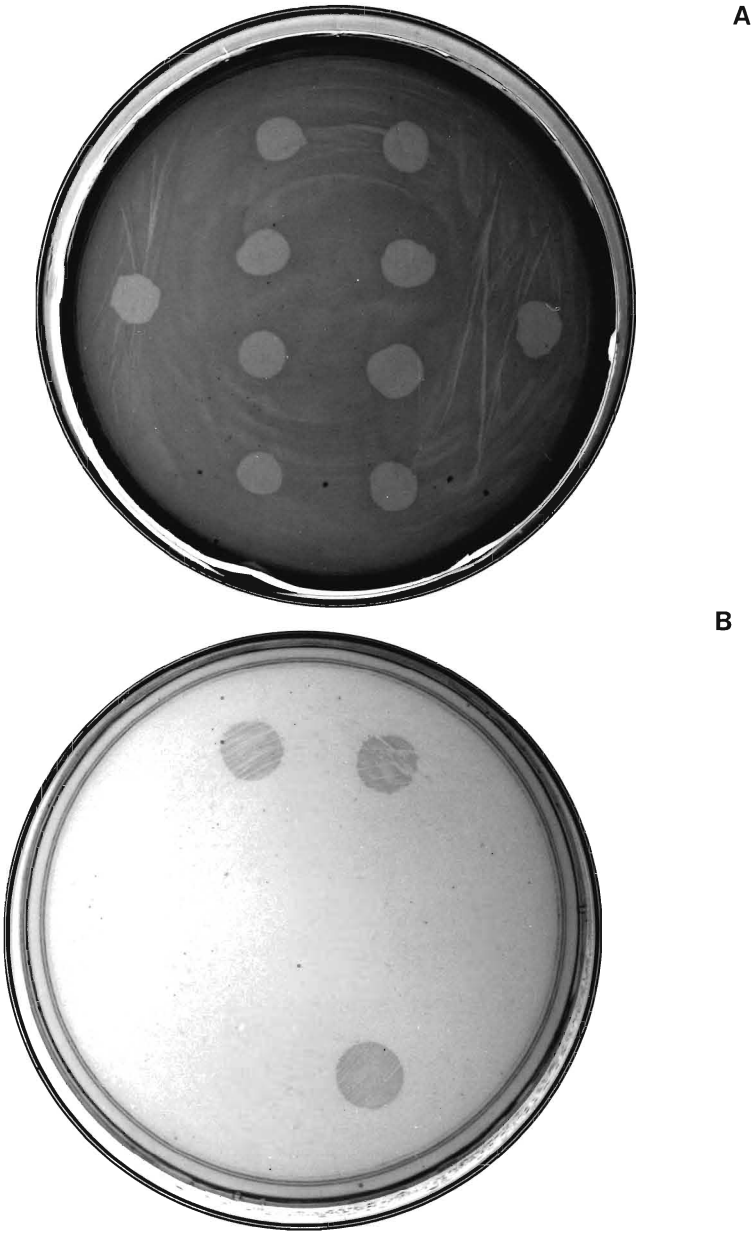

A
B

Figure 11 Schematic representation of the M13 constructions and related trimethoprim sensitivity.

Data from Figures 9 and 10 were correlated to demonstrate the region of the cloned dhfr DNA fragment necessary for the expression of yeast dihydrofolate reductase in E. coli. ssDNA was isolated and characterized as described in the legends to Figures 9 and 10.

Restriction Endonucleases

Bam H1 (B)
Eco R1 (E)
Hind III (H)
Pvu II (Pv)
Pst 1 (P)
Sal 1 (S)

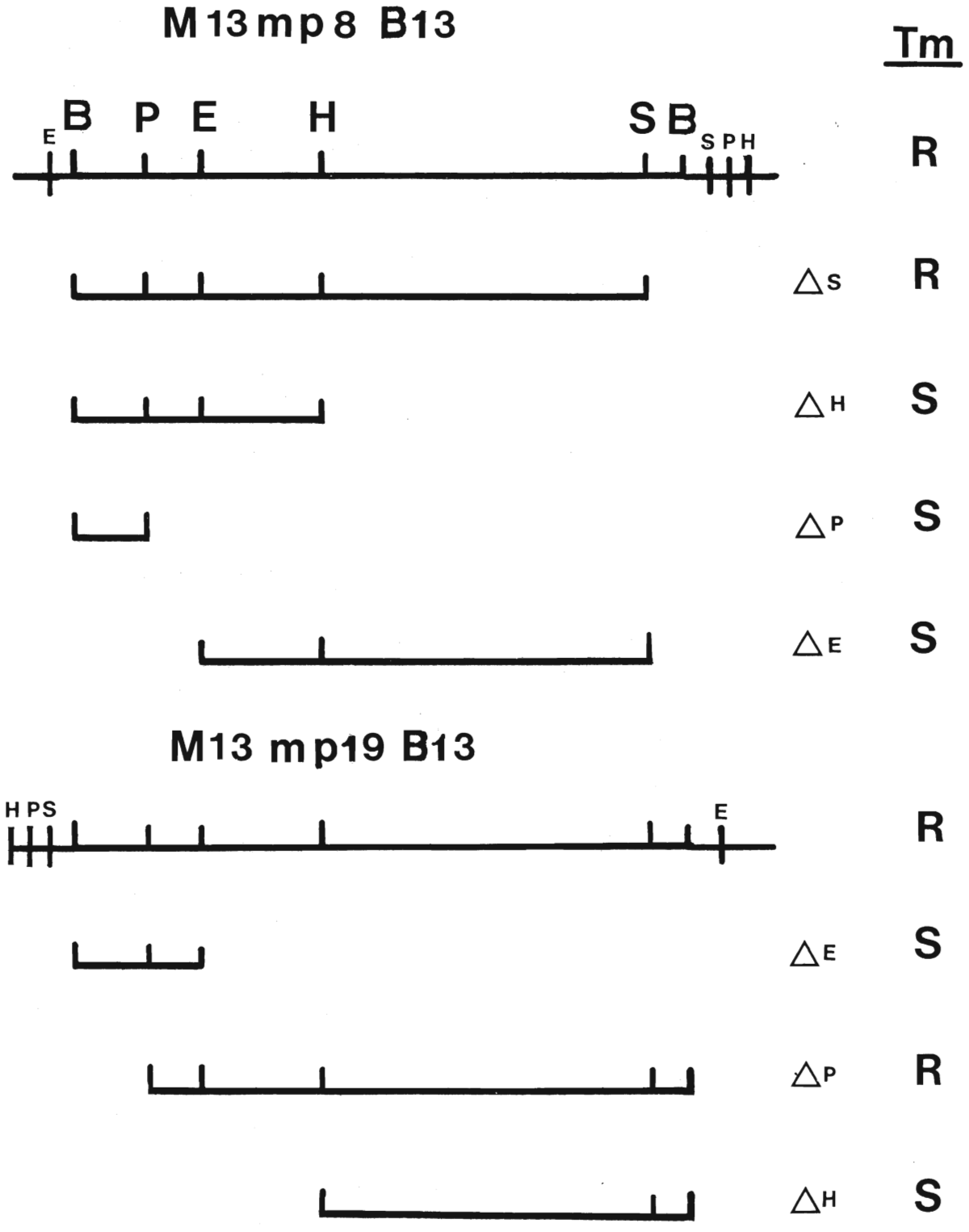

M13mp8 B13
Tm
E
B
P
E
H
S
B
S
P
H
R
△S
R
△H
S
△P
S
△E
S
M13 mp19 B13
H
P
S
E
R
△E
S
△P
R
△H
S

Figure 12 Characterization of the DFR 1 fragment

pIUD 1 DNA was digested with various restiction endonucleases as described previously. The fragments were separated on a 2% agarose gel at 50 mV for 3 hr and their sizes determined by comparison to Hind III digested λ phage DNA.

Lane	DNA	Enzyme(s)
1	pIUD 1	Bam H1
2	pIUD 1	Bam H1 Sal 1
3	pIUD 1	Bam H1 Eco R1
4	pIUD 1	Bam H1 Pst 1
5	pIUD 1	Bam H1 Hind III
6	pIUD 1	Hind III Pst 1
7	λ phage	Hind III
8	pIUD 1	Hind III Eco R1
9	pIUD 1	Sal 1
10	pIUD 1	Sal 1 Hind III
11	pIUD 1	Sal 1 Pst 1
12	pIUD 1	Sal 1 Eco R1

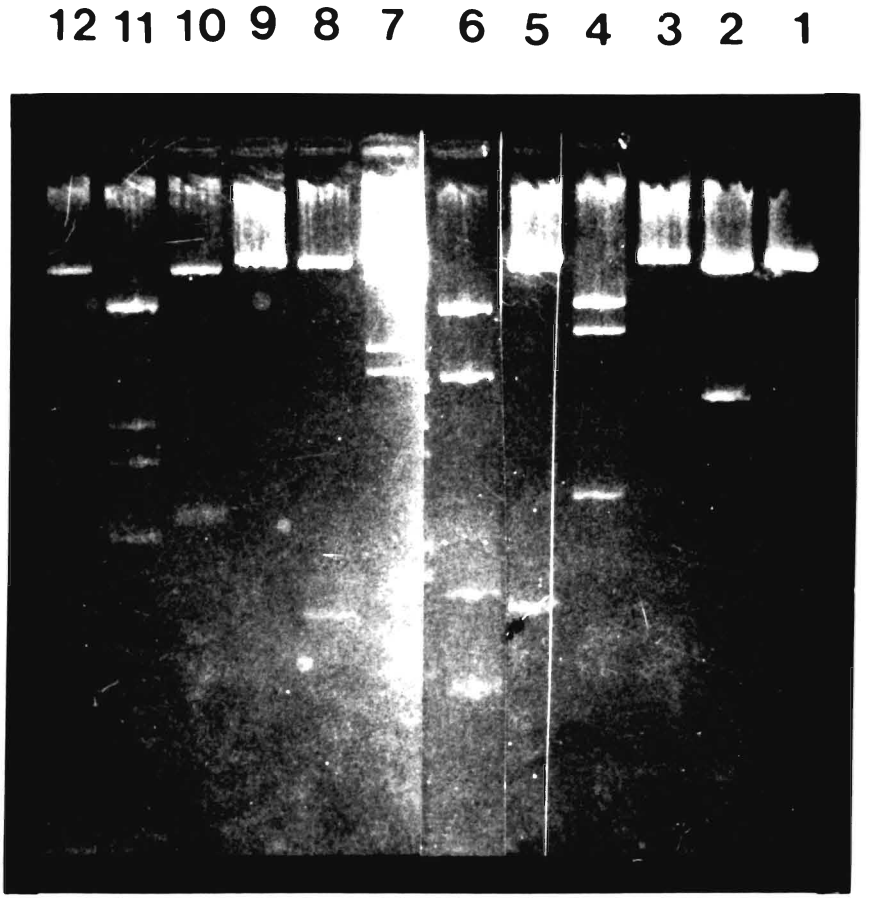
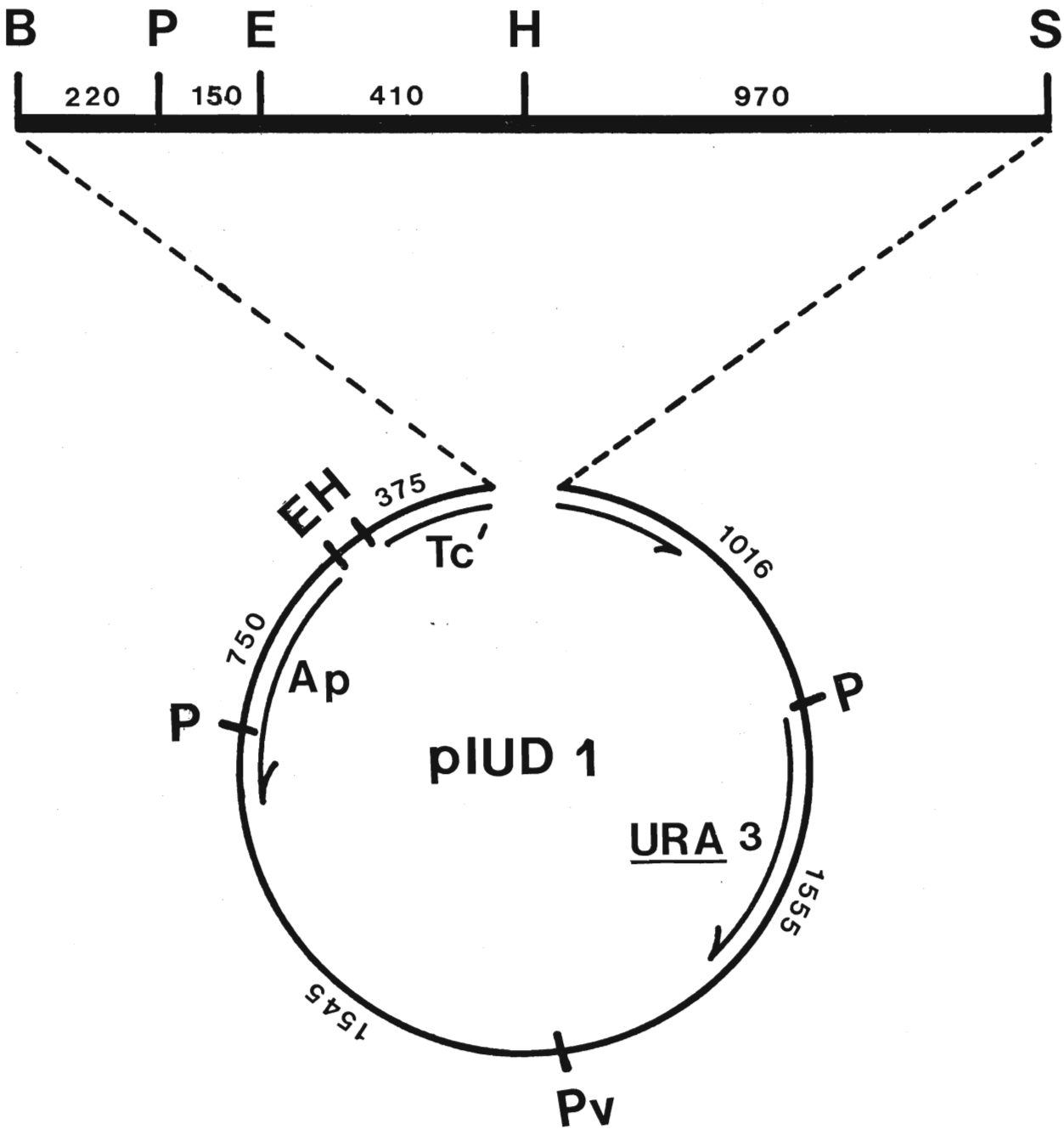

12 11 10 9 8 7 6 5 4 3 2 1

Figure 13 Restriction map of the recombinant plasmid pIUD 1

Data obtained from the restriction map analysis of DFR 1 and the published restriction map of YIP5 were combined to accurately map plasmid pIUD 1 by standard techniques as described previously (see Methods). Distances between restriction endonuclease sites given in base pairs (bp).


B
P
E
H
S
220
150
410
970
EH
375
Tc
1016
750
Ap
P
P
pIUD 1
URA 3
1555
1545
Pv

to locate the end of the DFR 1 functional unit within the 970 bp HindIII-Sal 1 fragment (Legerski et al., 1978). Nuclease Bal 31 contains both highly specific exonuclease and endodeoxyribonuclease activity for double stranded DNA and potent endonuclease activity for single stranded DNA or RNA. pIUD 1 DNA was first digested to completion with Sal 1 restriction endonuclease. The DNA was then treated with Bal 31 as described previously (see Methods). The results are summarized in Figure 14.

In order to further localize the yeast dhfr gene, DNA from the above experiment was ligated and used to transform E. coli strain JF1754. Transformants were characterized by their sensitivity to TRM. Table 5 shows that the TRM resistant phenotype of plasmid pIUD 1 transformants was not lost until a deletion of approximately 1500 bp of plasmid DNA occurred. This corresponds to a deletion of 750 bp from the Sal 1 cut site on the Pst 1-Sal 1 DNA fragment.

These data showed that the limit of the functional unit of the DFR 1 gene was located approximately 220 to 370 bp from the Hind III cut site on the Hind III-Sal 1 fragment. This suggested that the smallest fragment on which DFR 1 could be cloned and still retain dihydrofolate reductase activity in E. coli was between 630 and 930 bp.

Although 2 Fol- loci have been identified in S. cerevisiae, the location of the gene which in fact encodes yeast dhfr has remains unknown. A plasmid which carries a yeast gene and no DNA replication origin will integrate at its genomic locus by homologous recombination (Hinnen et al., 1978). When the integration takes place, any additional plasmid marker becomes tightly linked to the gene and its

Figure 14 Bal 31 nuclease treatment of plasmid pIUD 1.

DNA from pIUD 1 was first digested with Sal 1 and then treated with Bal 31 nuclease for 30 sec intervals as described previously. Samples were loaded on a 0.7% agarose gel and subjected to electrophoresis at 70 mV for 2 hr.

Lane	Bal 31 Treatment (sec)	
1	0	
2	30	
3	60	
4	90	
5	120	
6	150	
7	---	Hind III digested λphage DNA
8	180	
9	210	
10	240	
11	270	
12	300	

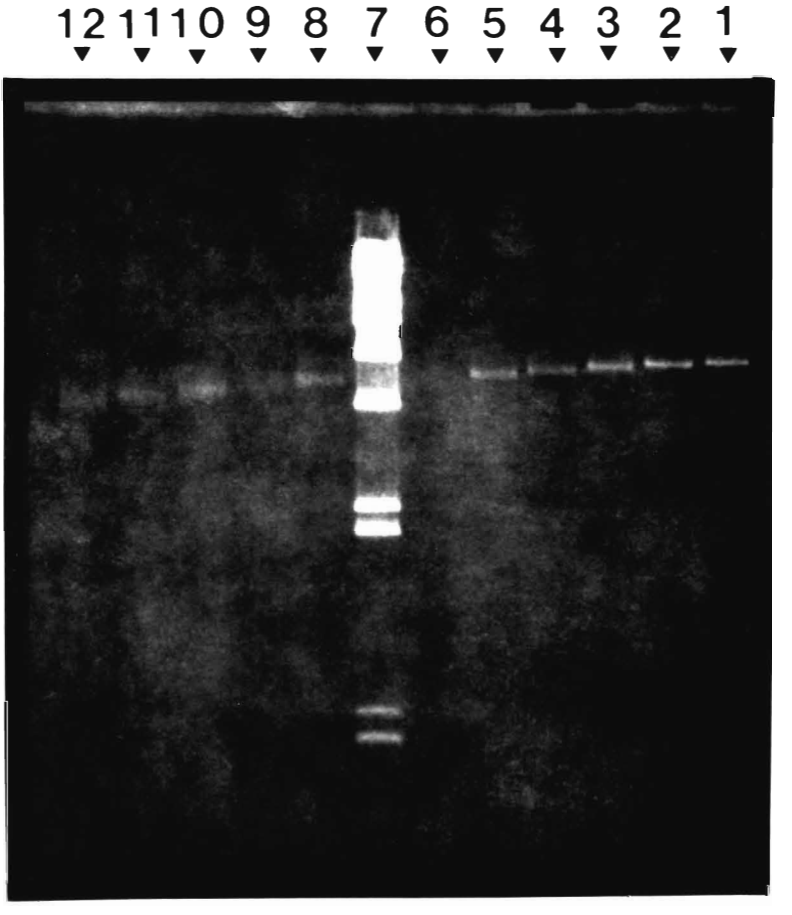

12 11 10 9 8 7 6 5 4 3 2 1

Table 5 Characterization of Bal 31 treated plasmids.

Bal 31 (sec)	Average Plasmid Size (bp)	Phenotype of Transformants (TRM)	Number Scored
0	7100	R	50/50
30	7000	R	37/37
60	6550	R	21/21
90	6200	R	43/43
120	5900	R	19/19
150	5600	S	33/33
180	5375	S	25/25
210	5175	S	18/18
240	5075	S	15/15
270	4975	S	13/13
300	4925	S	4/4

pIUD 1 DNA was digested to completion with Sal 1 restriction endonuclease and treated with nuclease Bal 31 as described in Methods. The DNA was ligated and used to transform bacterial strain JF1754. Transformants were characterized by resistance to ampicillin (50ug/ml) and trimethoprim (2ug/ml).

neighboring markers on the genome. The new linkage between markers can then be examined by tetrad analysis after the integrant is mated with a suitable strain.

In order to map the location of DFR 1, pIUD 1 DNA was used to transform yeast strain M12B. Three transformants were sent to J. Game (University of California at Berkeley) for the necessary matings and tetrad analysis. All 3 of these integrants mapped to the same location in the nuclear genome. Figure 15 shows that the cloned yeast dhfr gene is closely linked to Met 7 on chromosome 15 of S. cerevisiae (Game, pers. comm.).

In order to determine more accurately the location of the structural gene encoding yeast dhfr and any attendant regulatory regions, the DNA sequences of several segments of 1.6 kbp Pst 1-Sal 1 fragment were determined by the method of Sanger et al. (1977). Figure 16 shows the sequencing strategy employed and the primary base sequence obtained. Of particular interest was the stretch of 8 T residues located 46 bp from the Eco R1 cut site which was preceded by an opal stop codon (TGA) and a GC rich region.

To determine the direction of transcription of DFR 1, all 6 reading frames of this region were translated into the corresponding amino acids. By comparison with the amino acid sequences of other dihydrofolate reductases determined previously, a potential 3' terminus of DFR 1 was identified on the EcoR1-Pst 1 fragment with a "stop" codon 9 amino acids from the Eco R1 cut site (Figure 17). Previously, it has been shown that the 4 dhfr sequences examined have a phenylalanine residue at amino acid position 178 of Mus musculus (Freisheim et al., 1977; Nunberg et al., 1980; Smith and Calvo, 1980;

Figure 15 Map location of DFR 1 on chromosome 15 of S. cerevisiae

A pIUD 1 integrant was used to map the location of the yeast dihydrofolate reductase gene by standard techniques described elsewhere (Fogel et al., 1983).

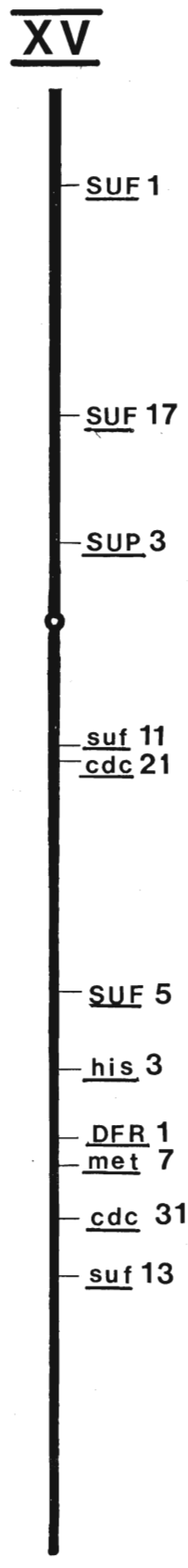

XV
SUF 1
SUF 17
SUP 3
suf 11
cdc 21
SUF 5
his 3
DFR 1
met 7
cdc 31
suf 13

Figure 16 Partial nucleotide sequence of the yeast dihydrofolate reductase gene.

M13 derivatives were used as template DNA in the Sanger dideoxy-chain termination sequencing method as described. Primary nucleotide base sequences were confirmed by either sequencing the same fragment 3 times or by sequencing both strands of a particular fragment.

Hind III
A A G C T T CAAGGAACGATTTTGTCCACGATAAAGAGAGATCAGATAGTCCAAAGTAATT

CATTGGCAAACCGCAATAATGAACCTAGAAAAGCAATTTTAACGGAGCATCTGGAAAGAACTCT

ACGTGATTGGGGGTGGCGAAGTCTCTATAGTCAAACTCTTCTCCATTACAGATCATTGGCTCAT

CACGAA_____

Eco R1
G A A T T C ACTCTATACAATCGTAAATGAAACCTCTCCGCCCGTATATTTTTTTTAATAT

GTTAAATAGTGATAGAACTGATAAGCCTCATTTTCTTTTATTGGGCTCCAAGACGCGAACTGTT
Pst 1
CGTAGGGTAACCGTTTGAGACCTAAACGACCTTTCAGCCTCAC C T G C A G TATTTCTT

CAACAACGCCTGTCGGCTATGTTAAATAATAGCAATCGTTTGTGATCACCATTGTCGAATTTGA

CGCGCTTAAACCAAAAACCATTGTTTTGGCCTCGTTCCCTGCATTCAACAAAAGAGCAAGGTAT

GCCGTCAAACAGTCGTTAAAAGAGAACGGTTTATCAAACTACTCTTGTTTTGTACTTCTGCTGT
Bam H1
CC G G A T C C

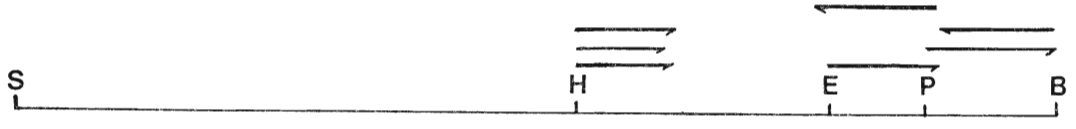


FIGURE 17 Comparision of the 3' terminus of various dihydrofolate reductase genes.

The amino acid sequence of the 3' terminus of S. cervisiae is compared with those of E. coli, L. casei, M. musculus and H. sapiens. The amino acids were aligned according to the structural equivalences described elsewhere (Volz et al., 1982). Residues that are identical with thoses in the yeast sequence are enclosed in boxes.

E. coli	cys	phe	glu	ile	leu	glu	arg	arg	+++	
L. casei	arg	phe	gln	lys	gln	lys	lys	ala	+++	
S. cerevisiae	glu	phe	thr	leu	tyr	asn	arg	lys	+++	
M. musculus	lys	phe	glu	val	tyr	glu	lys	lys	asp	+++
H. sapiens	lys	phe	glu	val	tyr	glu	lys	asn	asp	+++

Chen et al., 1984). This was also found to be the case in the potential amino acid sequence of S. cerevisiae dhfr.

Effect of Antifolates in Yeast

When yeast cells are treated with antifolates, there is a subsequent depletion of the tetrahydrofolate pools. This condition creates an auxotrophic requirement for C1 metabolites. In yeast, these folate dependent end products are adenine, histidine, methionine and dTMP. If yeast cells are starved for dTMP alone, by provision of adenine, histidine and methionine in the growth medium, there is a progressive shift in the distribution of the population within the cell cycle (Laskowski and Lehmann-Brauns, 1973). After approximately 6 hr incubation in YPD broth medium (which contains adenine, histidine and methionine, but not dTMP) supplemented with 200 ug/ml MTX and 5 mg/ml Sulf, yeast cell populations consisted entirely of parent cells with buds of equal size attached (Figure 18). This morphology is characteristic of cells in which DNA synthesis has been inhibited either by genetic lesion (Hartwell, 1974) or in cultures treated with hydroxyurea (Slater, 1974).

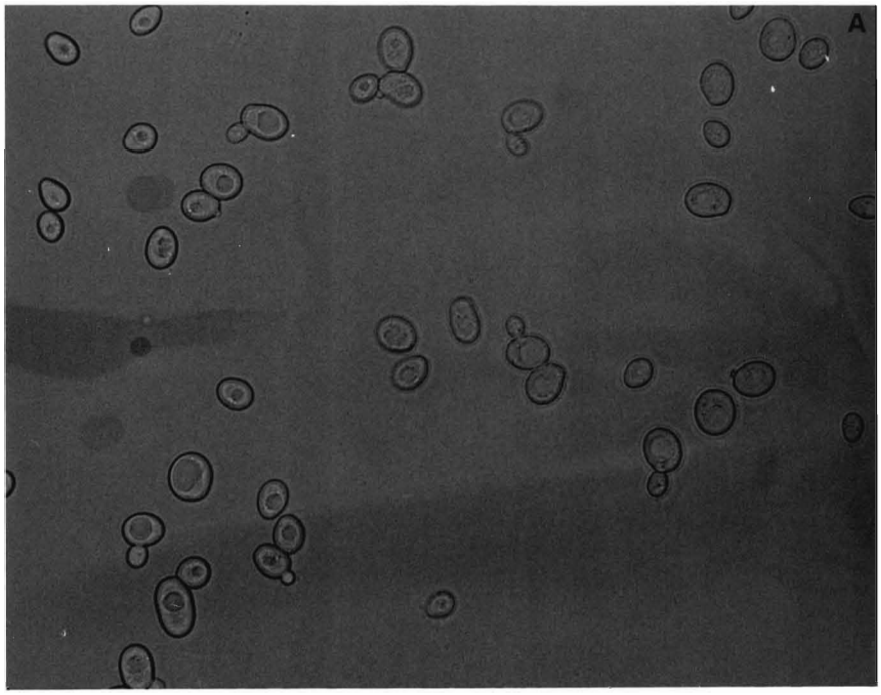
As a preliminary experiment to determine the effect of DFR 1 on a multicopy plasmid (pDR509) on dihydrofolate reductase inhibition in wild-type and 2 mutant yeast strains, a radial gradient technique was employed. In this study, the procedure was used qualitatively to assess the effects of a range of MTX concentrations on a single agar plate. A lawn of cells was spread on a YPD agar plate and 1 mg MTX and 5 mg Sulf was added to a center well. This establishes a gradient of MTX concentration which decreases towards the periphery of the

Figure 18 Morphology of yeast cells treated with methotrexate.

Exponential cells of yeast strain AH 2 were harvested by centrifugation and resuspended in YPD media. Cells were stressed for folates by the addition of 200 ug/ml MTX and 5 mg/ml Sulf. After 6 hr incubation at 30°C the cells were examined under a light microscope and photographed (see Methods).

A) Exponential cells incubated in YPD media.

B) Exponetial cells incubated in YPD media supplemented with 200 ug/ml MTX and 5 mg/ml Sulf.


A

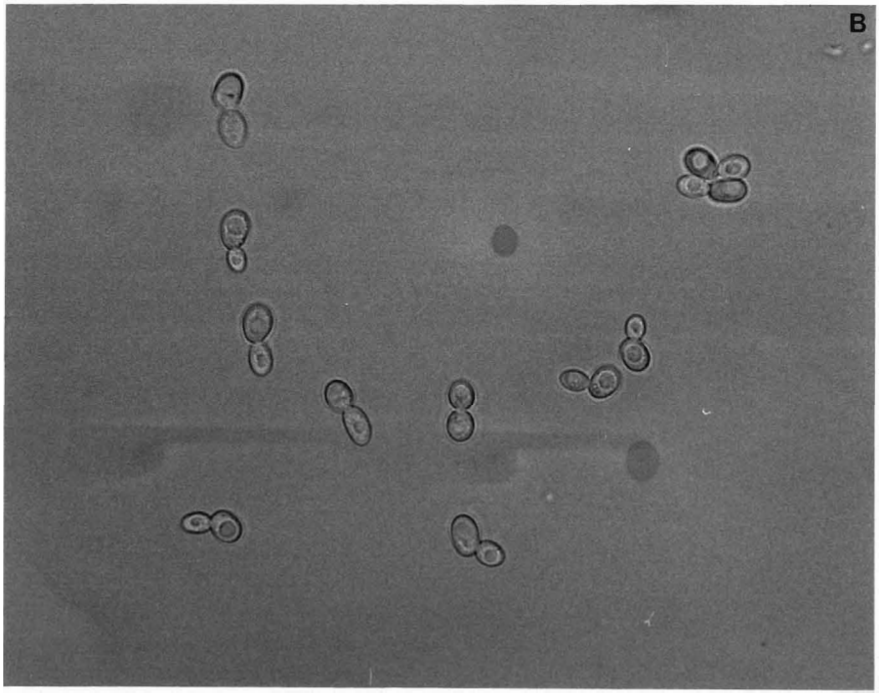

B

plate. As shown in Figures 19a, 20a and 21c, after 24 - 72 hr incubation yeast cells exhibited a zone of growth inhibition around the well. Growth occurred in this area of the plate when in yeast strains transformed with the multi-copy plasmid pDR 509 (Figure 19c, 20b, 21b). A pIUD 1 integrant in a rad 18 host showed an intermediate zone of growth inhibition (Figure 21a). Wild-type yeast strain AH2 transformed with the shuttle vector pYF91 also exhibited a zone of growth inhibition around the well similar to that of the host (Figure 19b). These data clearly demonstrate that the cloned yeast dhfr gene is expressed in yeast.

Although, the copy number of pDR509 is not known, Storms et al., (1979), have reported that the shuttle vector pYF91 is maintained at approximately 8 to 10 copies per cell. If this is also true for DFR 1 - pYF91 recombinant plasmids, these data suggest that the protection afforded to yeast cells to growth inhibition by MTX is a result of a gene dosage effect.

This conclusion is supported by the inhibition studies of the yeast strains grown in culture at various MTX concentrations. In all 3 yeast strains, a similar inhibition of growth was observed in response to this antifolate (Figures 22,23,24). However, rad 6 cells seemed to be slightly more sensitive than wild type or rad 18 cells at low levels of the drug ($\simeq$25ug/ml). Consistent with the previous results from plate tests, yeast strains containing plasmid pDR509 were more resistant to MTX than the pIUD 1 integrant in culture.

In addition to the growth inhibition observed in cells treated with MTX, there was also an absolute decrease in the survival of the 3 yeast strains (Table 6). Cells transformed with the vector pYF91,

Figure 19 Inhibition of wild-type cell growth by MTX

Approximately 10^6 cells of strain AH 2 were added to 10 ml of YPD top agar and immediately plated onto a YPD plate. To the center well 1 mg MTX and 5 mg Sulf were added. After incubation at 30°C for 24 - 28 hr the plate was overlayed with TTC as described previously (see Methods).

Plate	Strain
A	AH 2
B	AH 2/pYF91
C	AH 2/pDR509

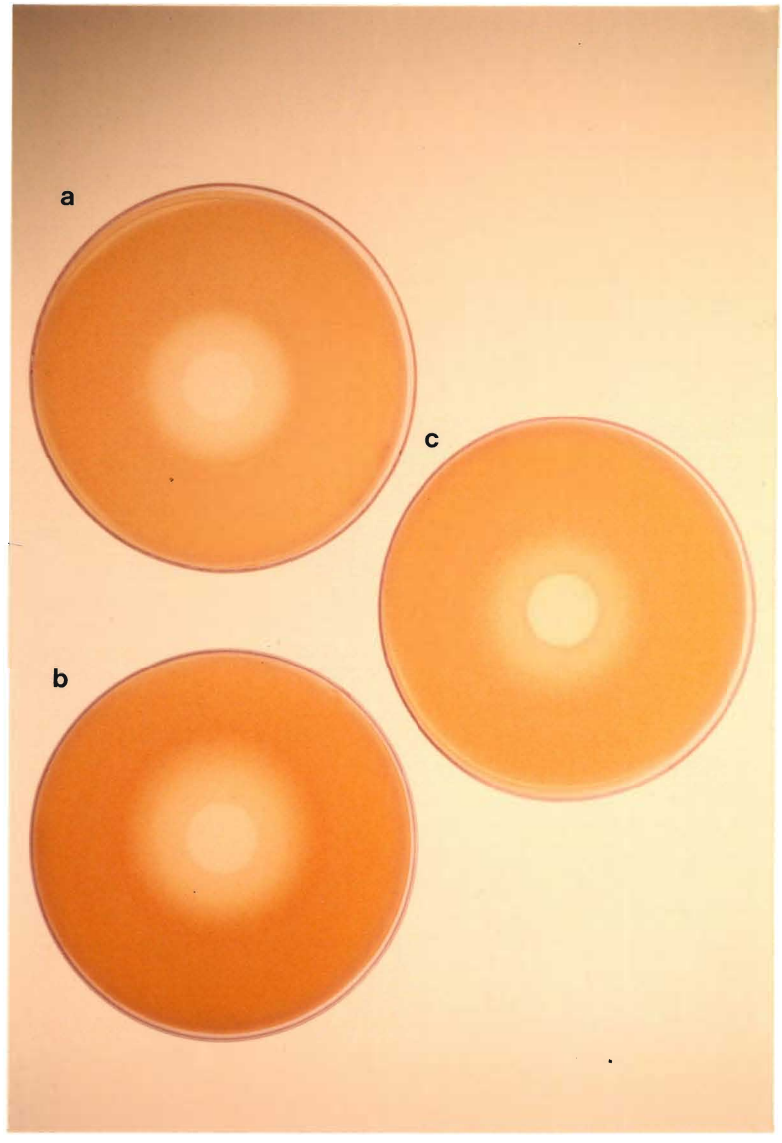

a
c
b

Figure 20 Inhibition of rad 6 cell growth by MTX

Approximately 10^6 cells of strain LLP2730-1A were plated onto a YPD plate as described in the legend to Figure 20. 1 mg MTX and 5 mg Sulf were added to the center well and after 72 hr incubation at 30°C the plate was overlayed with TTC as described previously.

Plate	Strain
A	LP2730-1A
B	LP2730-1A/pDR509

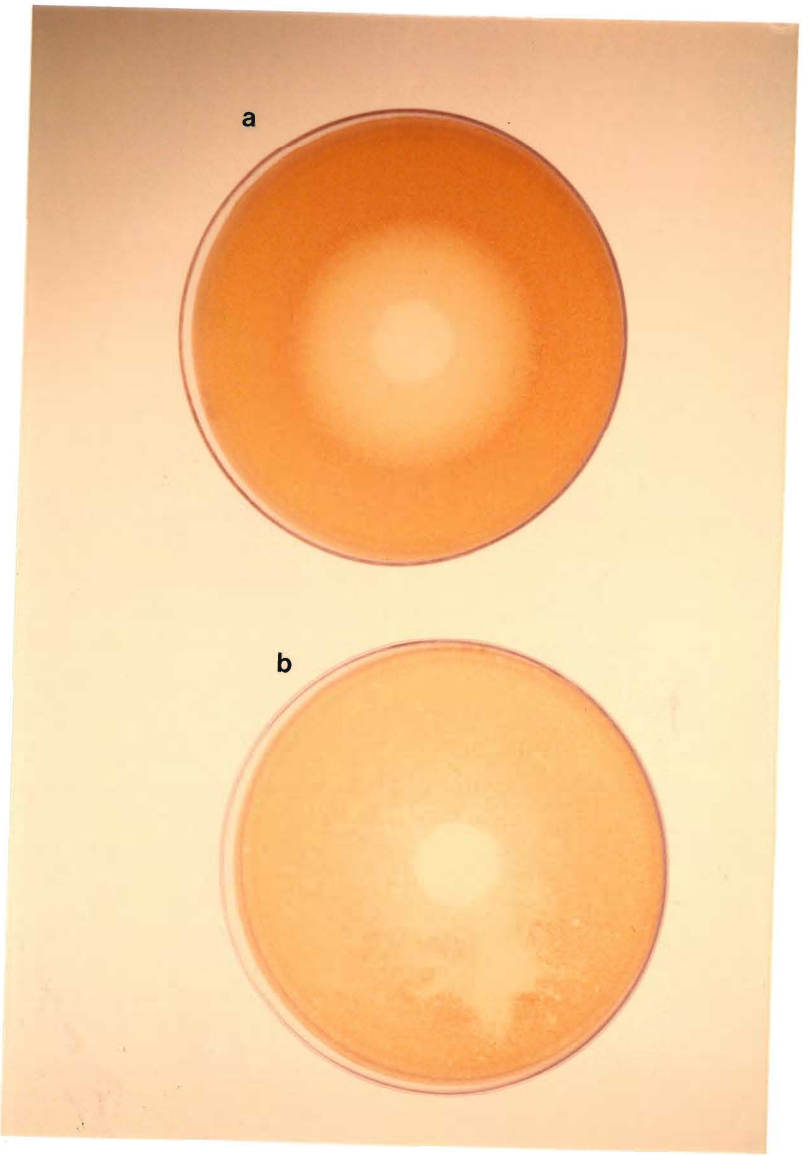

a
b

Figure 21 Inhibition of rad 18 cell growth by MTX

Approximately 10^6 cells of strain LP2729-4B were plated onto a YPD plate as before. 1 mg MTX and 5 mg Sulf were added to the center well and after 48 - 72 hr incubation at 30°C the plate was overlayed with TTC as described previously.

Plate	Strain
A	LP2729-4B/pIUD 1
B	LP2729-4B/pDR509
C	LP2729-4B

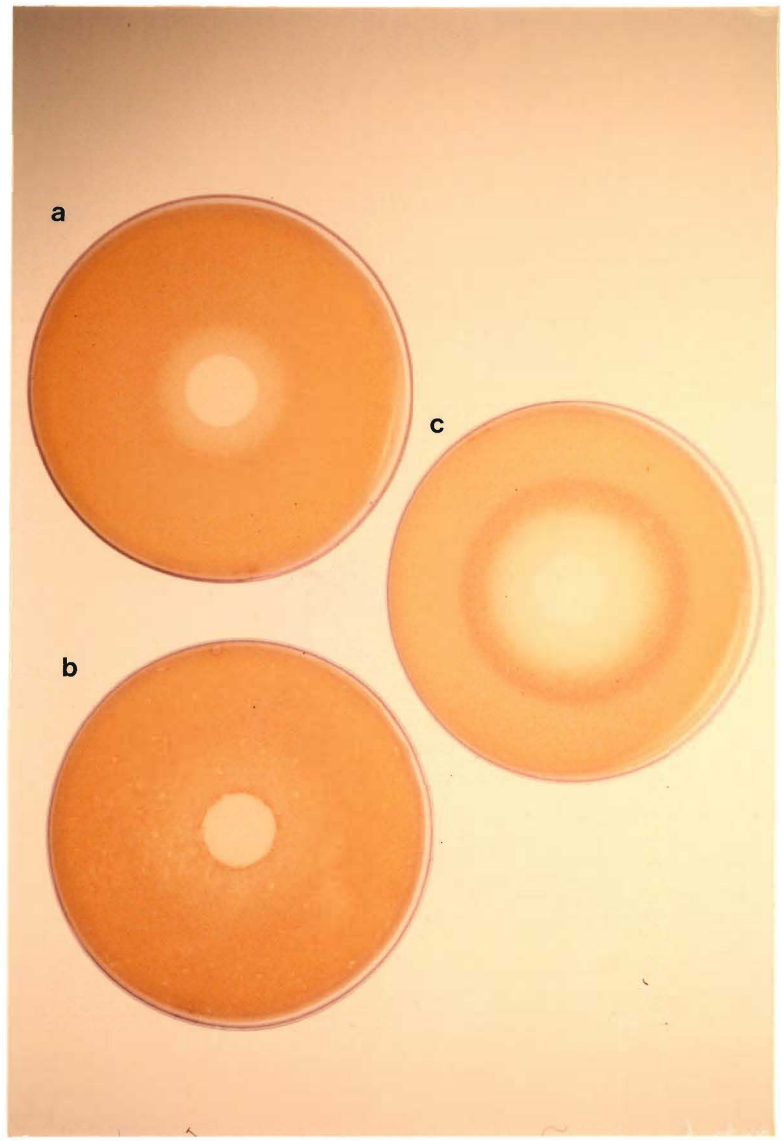

a
b
c

Figure 22 Inhibition of growth by methotrexate of a wild type-yeast strain.

Exponential cells were harvested by centrifugation and resuspended in YPD medium supplemented with various MTX cconcentrations and 5 mg/ml Sulf. Aliquots were taken after 8 hr incubation and cell density was determined by counting in a Coulter counter (see Methods).

○ Wild-type yeast strain

● Wild type yeast strain transformed with the multi-copy DFR 1 plasmid pDR509

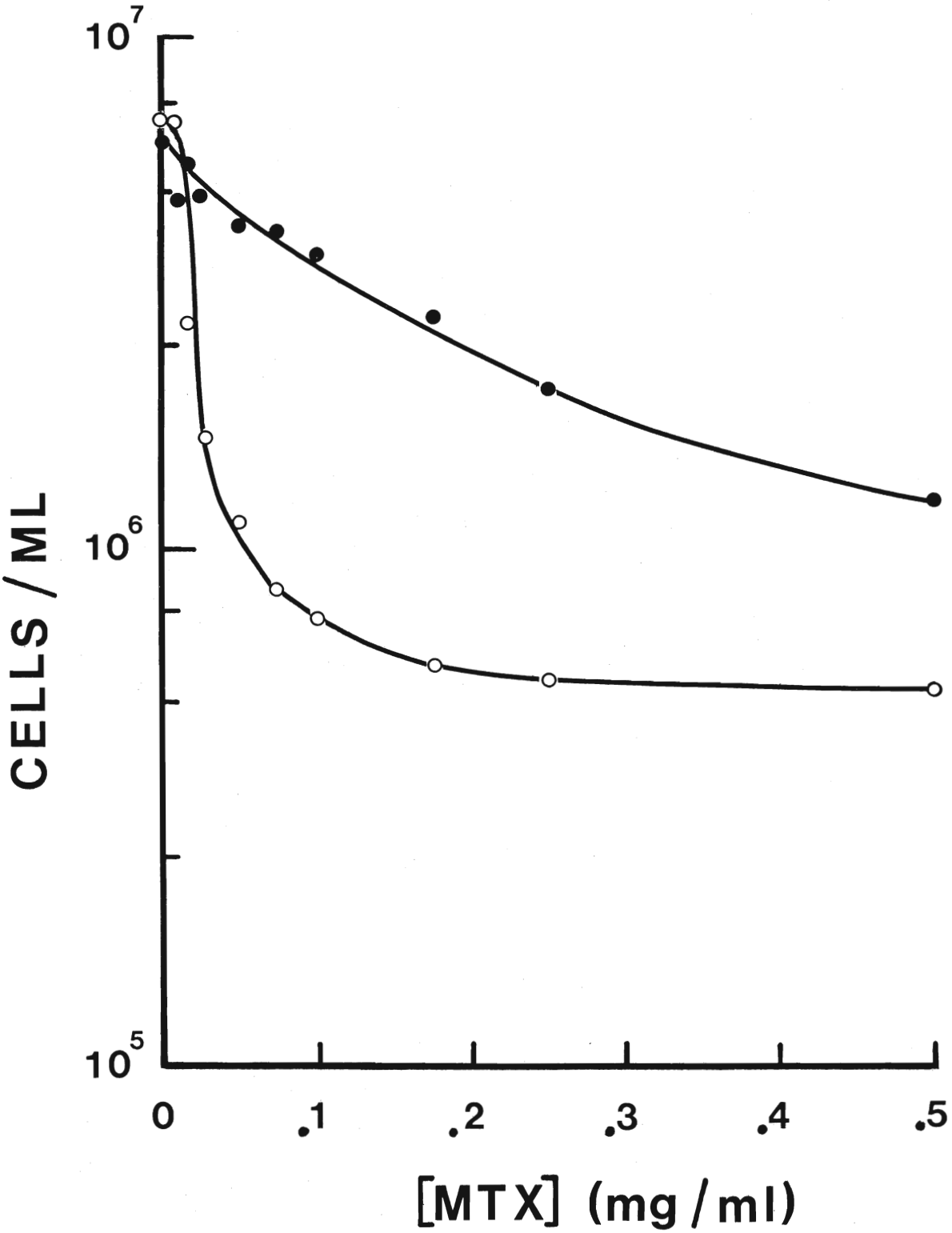

10^7
10^6
10^5
CELLS / ML
0
.1
.2
.3
.4
.5
[MTX] (mg / ml)

Figure 23 Inhibition of growth by methotrexate of a rad 6 mutant yeast strain.

Exponential cells were harvested by centrifugation and resuspended in supplemented YPD medium as before. Aliquots were taken after 8 hr incubation and cell density was determined as described previously.

△ rad 6 yeast strain

▲ rad 6 yeast strain transformed with the multi-copy DFR 1 plasmid pDR509

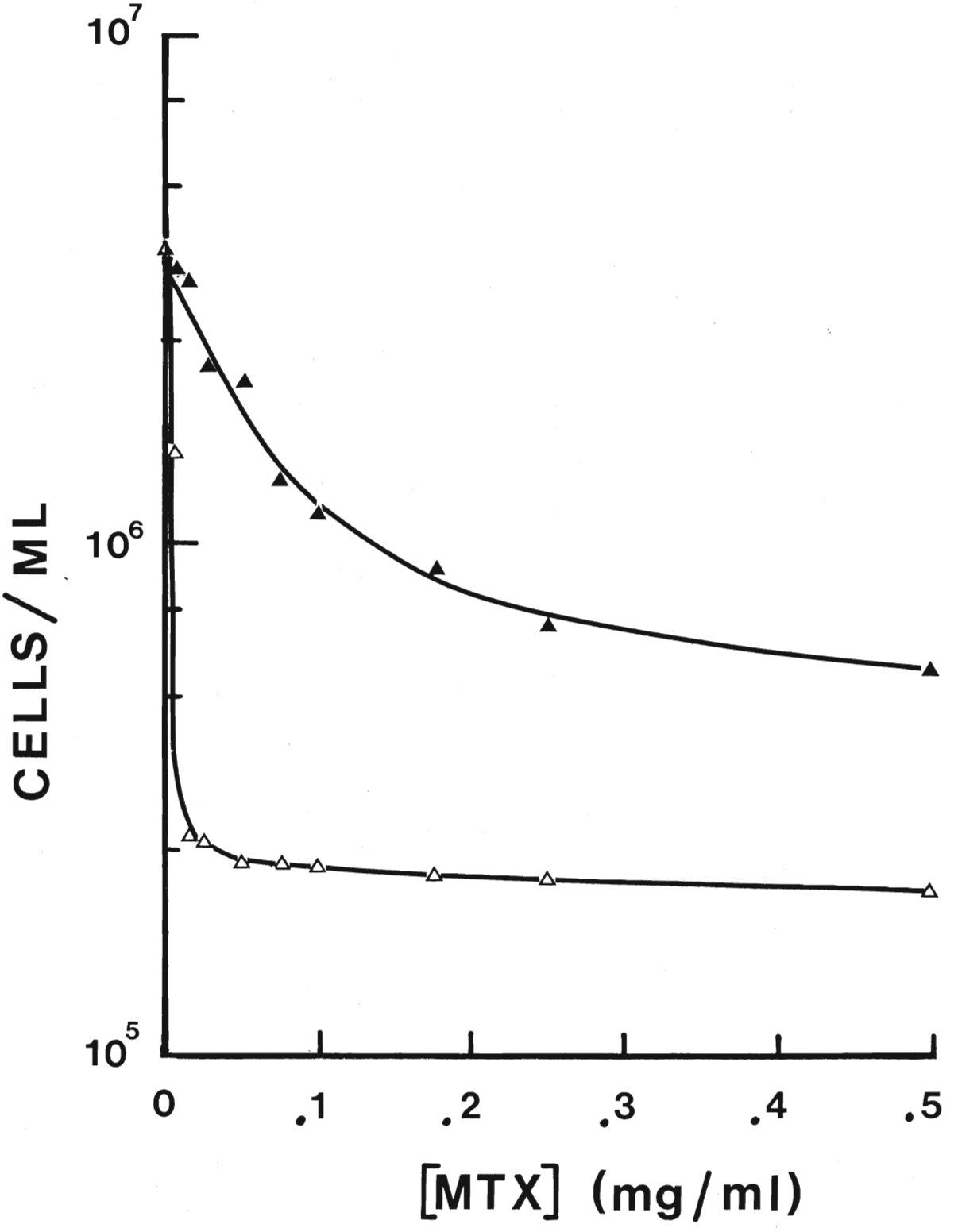

10^7
10^6
10^5
CELLS / ML
0
.1
.2
.3
.4
.5
[MTX] (mg/ml)

Figure 24 Inhibition of growth by methotrexate of a rad 18 mutant yeast strain.

Exponential cells were harvested by centrifugation and resuspended in supplemented YPD medium as before. Aliquots were taken after 8 hr incubation and cell density was determined as described previously.

□ rad 18 yeast strain

◆ rad 18 yeast strain transformed with the single copy DFR 1 plasmid pIUD 1

■ rad 18 yeast strain transformed with the multi-copy DFR 1 plasmid pDR509

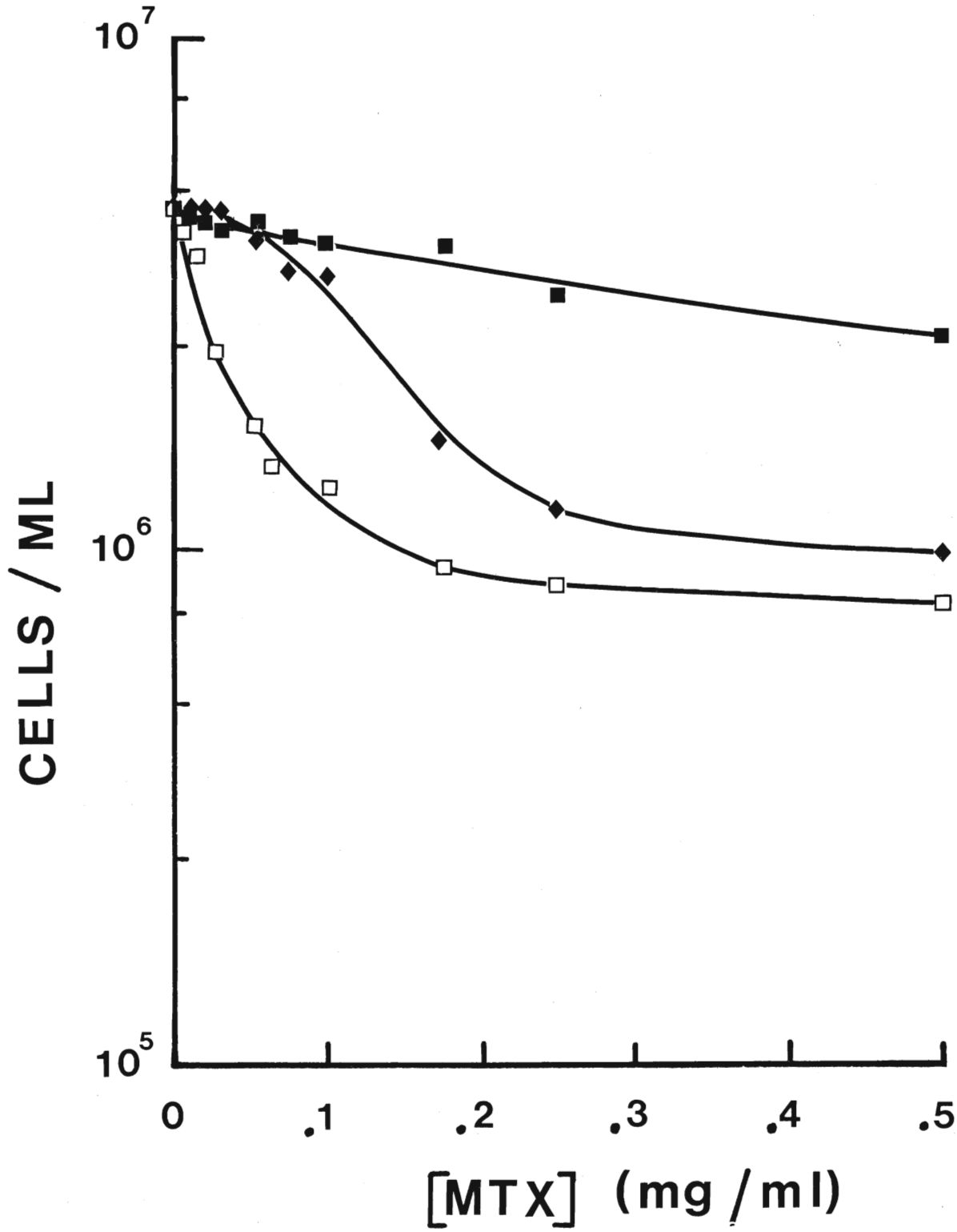

10^7
10^6
10^5
CELLS / ML
0
.1
.2
.3
.4
.5
[MTX] (mg / ml)

Table 6 Survival of various yeast strains during folate depletion.

	Survival (%)		
	No Drug	[MTX] (500 ug/ml)	[TRM] (500 ug/ml)
wt	97.2	1.3	70.4
wt + pYF91	98.8	2.9	70.1
wt + pDR509	98.6	56.1	83.4
rad 6	87.0	0.9	0.2
rad 6 + pDR509	87.9	11.0	0.3
rad 18	87.6	3.3	7.9
rad 18 + pIUD 1	87.2	9.3	8.1
rad 18 + pDR509	87.1	12.8	7.5

Cells were cultured and plated under the conditions outlined in Methods. Cell viability was determined using a Coulter counter (model ZF).

exhibited similar cell killing as the host without the plasmid. Cell viability in strains containing the DFR 1 plasmid pDR509 was greatly increased when grown at the same drug concentration (500ug/ml). A characteristic of yeast cell populations containing wild-type genes on plasmids which complement analogous mutant alleles on the chromosome is phenotypic instability. The plasmid loss in growing yeast populations occurs even when the cells are cultured under selective conditions (Struhl et al., 1979). When this is taken into account, it is likely that complete protection against cell death is conferred upon cells which have retained the DFR 1 plasmid.

As mentioned previously, wild-type yeast are refractory to the growth inhibition by trimethoprim. The results of preliminary experiments showed that yeast can grow normally in the presence of TRM up to concentrations of 1.5 mg/ml. However, wild-type cell populations can be sensitized by preincubation of the cells in saline overnight by a procedure described by Mayer and Goin, (1984) which depletes the nutrient reserves of the cells. There exist 2 mutants, rad6 and rad 18, which have been shown to be sensitive to this folate antagonist (Game et al., 1975). Since TRM is thought to be a specific inhibitor of dihydrofolate reductase, it seemed likely that these cells transformed with the DFR 1 gene might be much less sensitive to te effects of the drug.

Consistent with findings published previously, rad6 and rad 18 cell populations were more sensitive to growth inhibition by TRM than the wild-type strain at all drug concentrations tested (Figures 25, 26 and 27). The presence of DFR 1 on a multicopy plasmid had no effect on cell growth in the TRM treated populations. Moreover, no increase

Figure 25 Inhibition of growth by trimethoprim of a wild-type yeast strain.

Exponential cells were starved for folates as described previously and resuspended in MIN medium supplemented with various TRM concentrations. Aliquots were taken after 24 hhr incubation and cell density was determined by counting (see Methods).

○ Wild-type yeast strains

● Wild type yeast strains containing the multiple copy DFR 1 plasmid pDR509

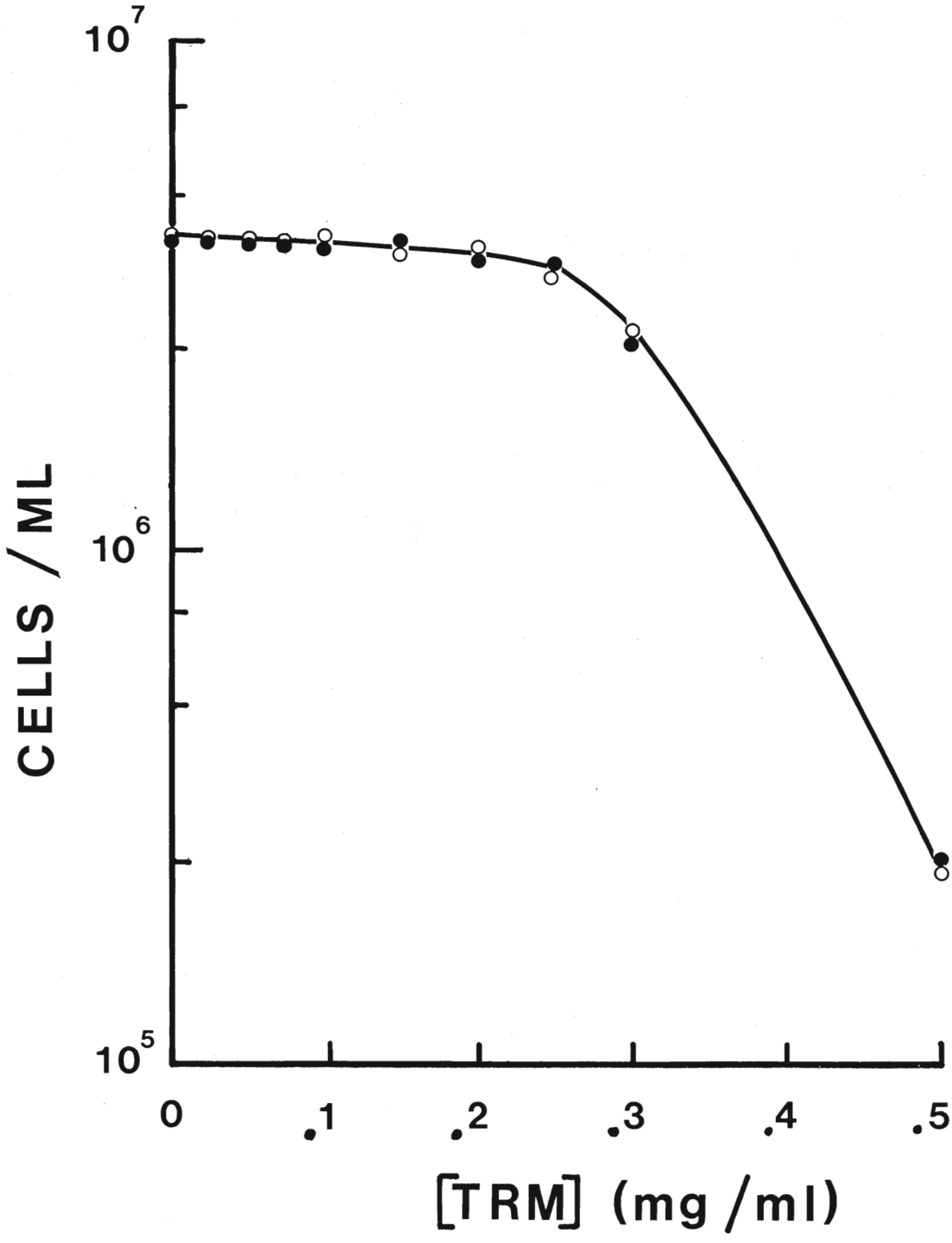

10^7
10^6
10^5
CELLS / ML
0
.1
.2
.3
.4
.5
[TRM] (mg /ml)

Figure 26 Inhibition of growth by trimethoprim of a rad 6 mutant yeast strain

Exponential cells were starved for folates and resuspended in various TRM concentrations as before. Aliquots were taken after 24 hr incubation and cell density was determined as described previously.

△ rad 6 mutant yeast strain

▲ rad 6 mutant yeast strain containing the multi-copy DFR 1 plasmid pDR509

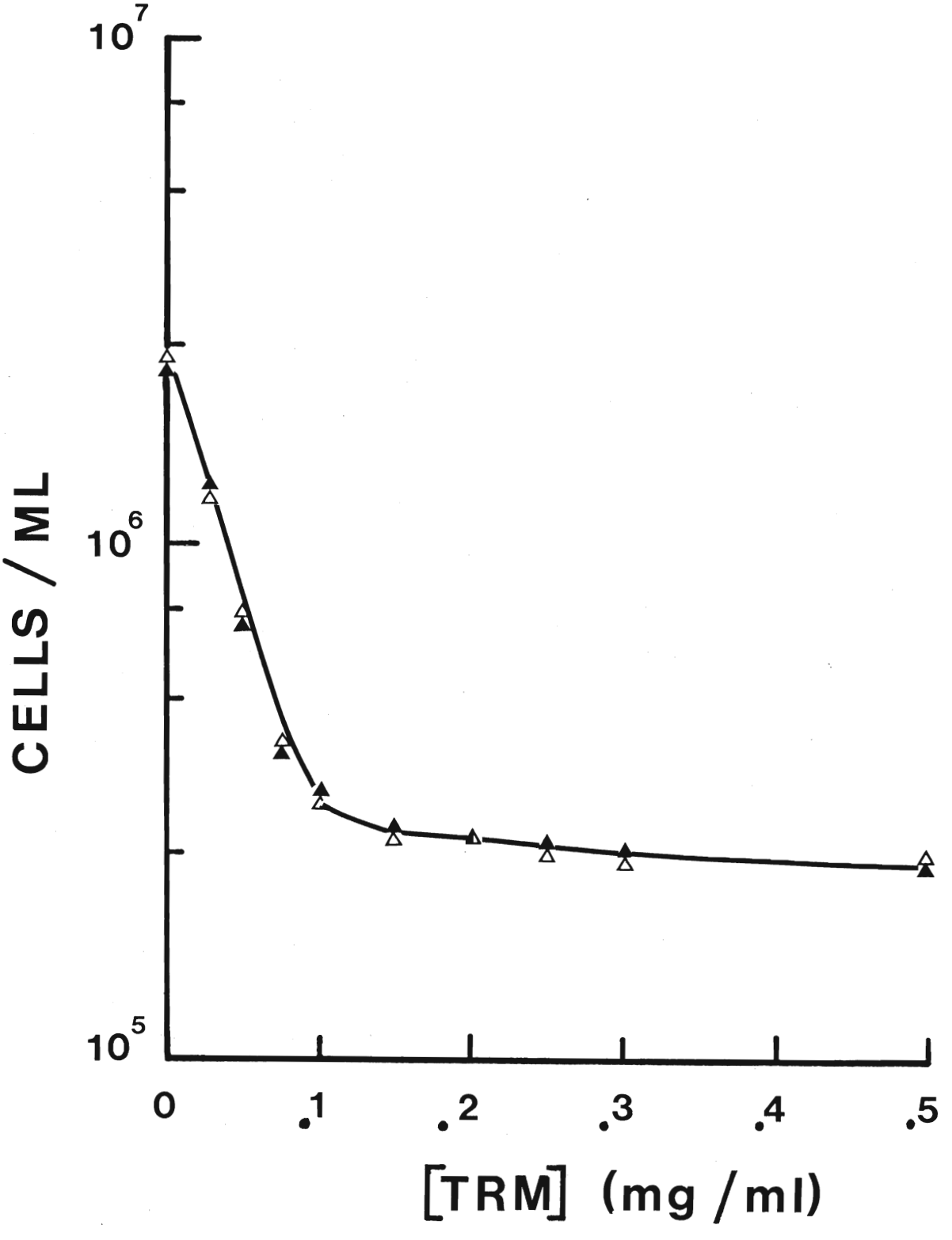

10^7
10^6
10^5
CELLS / ML
0
.1
.2
.3
.4
.5
[TRM] (mg / ml)

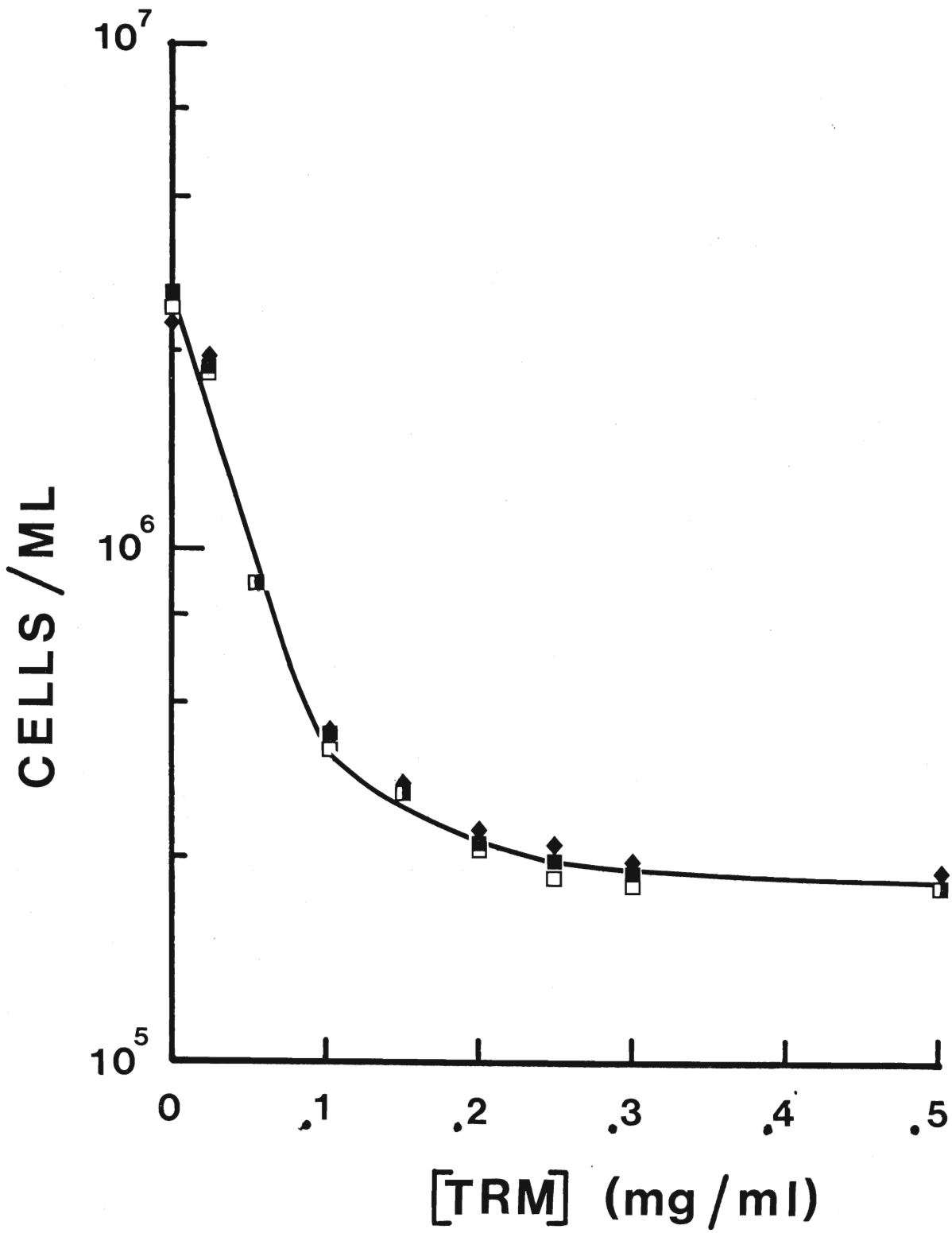
Figure 27 Inhibition of growth by trimethoprim of a rad 18 mutant yeast strain.

Exponential cells were starved for folates and resuspended in various TRM concentrations as described previously. Aliquots were taken after 24 hr incubation and cell density was determined as before.

□ rad 18 mutant yeast strain

◆ rad 18 mutant yeast strain containing the single copy DFR 1 plasmid pIUD 1

■ rad 18 mutant yeast strain containing the multi-copy DFR 1 plasmid pDR509


10^7
10^6
10^5
CELLS / ML
0
.1
.2
.3
.4
.5
[TRM] (mg / ml)

in cell viability was observed in drug treated strains harboring the cloned yeast dhfr gene compared to that of the host (Table 6). These results are somewhat surprising since the ability of increased copies of the gene to enhance resistance to TRM in host cells was the basis of the original selection procedure used to isolate the yeast dhfr gene in E. coli. In addition, the results obtained in similar experiments using the dhfr inhibitor MTX, are in direct contrast.

As mentioned previously, in addition to cell killing, inhibition of C1 metabolism by folic acid analogues such as MTX results in the formation of respiratory deficient mutants in yeast (Wintersberger and Hirsch, 1973). Cytoplasmic petites are known to arise as a consequence of a loss of a portion or all of the mitochondrial genome (Bernardi, 1978). Since yeast is a facultative anerobe, lesions occurring in either the nuclear or mitochondrial genome can be readily distinguished. It was thought that the sensitivity of the mitochondrial genome to folate depletion might be a very sensitive index of the effects of the dihydrofolate reductase gene expression under folate deprived conditions.

The rate of spontaneous petite induction for the 3 yeast strains is shown in Table 7. Consistent with previous findings, a high frequency of petites were induced in wild-type and rad 18 cell populations cultured in the presence of MTX (Wintersberger and Hirsch, 1973). In sharp contrast to these results, the rad 6 mutant showed a very low level of spontaneous petite induction (0 of approximately 2000 clones examined). Furthermore, MTX apparently failed to induce petities in this strain (0 of approximately 150 clones examined). One interpretation of this finding is that rad 6 is an obligate grande.

Table 7 Frequency of cytoplasmic petites induced by antifolate drugs.

	Petites (%)		
	No Drug	[MTX] 500 ug/ml	[TRM] 500 ug/ml
wt	1.4	86.8	1.1
wt + pYF91	1.0	86.5	0.9
wt + pDR509	1.2	90.1	0.8
rad 6	0	0	0
rad 6 + pDR509	0	0	0
rad 18	1.2	72.4	1.0
rad 18 + pIUD 1	1.0	67.8	1.4
rad 18 + pDR509	1.1	68.8	1.3

Cells were cultured and plated under the conditions given in the legend to Figure 22. Respiratory competence of clones was determined as described in Methods.

To test this hypothesis yeast strains were treated with ethidium bromide, a drug which is known to induce petites very strongly in yeast (Fukunaga and Yielding, 1981). This drug induced ρ^- in rad 6 cells at a level approximately 50% of those exhibited in rad 18 and wild-type cell populations (Table 8). Thus, even though rad 6 is not an obligate grande, it seems to be very refractory to mitochondrial damage by chemical means.

Although, the rad 6 strain could not be used to determine the effects on the induction of respiratory deficient mutants by antifolates, the rad 18 and wild-type cell populations were suitable for these types of studies. Surprisingly, the presence of DFR 1 on a multicopy plasmid did not affect the viability of any of the 3 strains tested (Table 6). Furthermore, TRM did not induce the formation of respiratory mutants in these yeast cell populations (Table 7).

Taken as a whole, these data suggest that yeast cells cultured in the presence of high concentrations of TRM do not exhibit the characteristic effects of antifolates in yeast. One interpretation of these data is that the dihydrofolate reductase enzyme may not be the target of trimethoprim in yeast.

Table 8 Induction of cytoplasmic petites by EtBr.

	Petites (%)		
Time (hrs)	wild-type	*rad* 6	*rad* 18
Control	2	0	2
1	33	17	31
2	45	34	41
3	79	50	70
4	95	53	90

Cells were grown in media supplemented with 10 ug/ml EtBr and plated as described in Methods. Respiratory competence of clones was determined as mentioned previously.

Discussion

The yeast dihydrofolate reductase gene (DFR 1) is present on a 8.8 kbp Bam H1 DNA fragment in the yeast nuclear genome (Barclay et al., in. prep.). During the course of this study a similar fragment containing the dhfr gene was cloned in a by selection of trimethoprim resistance in E. coli (Nath and Baptist, 1984).

As yeast is intermediate in biological complexity relative to bacteria and mammalian cells, studies of yeast dhfr gene expression may serve as a model system for extrapolation to higher cells. To date, of the yeast genes which have been characterized, most do not contain intervening sequences (Holland and Holland, 1979; Montgomery et al., 1980). Expression of yeast dhfr in E. coli may suggest that this gene unlike corresponding eukaryotic dhfrs of mouse and human cell lines lacks intervening sequences to any great extent (Nunberg et al., 1980; Chen et al., 1984). Therefore, it was possible to isolate DFR 1 like many other yeast genes from a genomic DNA library. An obvious advantage of this procedure as compared to cloning complementary DNA from messenger RNA, is that the DNA segment isolated from the genome may not be limited to just the structural sequence. Thus, it was hoped that along with the gene encoding yeast dhfr, any attendant regulatory regions would also be isolated.

The expression of DFR 1 is independent of its orientation on the shuttle vector pYF91. This suggests that the expression of yeast dihydrofolate reductase does not depend upon any regulatory sequences resident on the vector. Thus, the DNA insert may contain yeast regulatory regions in addition to the structural gene encoding dhfr.

In almost all cases, where the dihydrofolate reductase gene product has been characterized, it consists of a single polypeptide of about 21,000 molecular weight (Benkovic, 1980). A protein of this size corresponds approximately to a DNA coding region of 5 to 6 hundred base pairs. Therefore, the 1.8 kbp DNA fragment necessary for yeast dhfr expression in E. coli, is large enough to accommodate the structural gene and putative regulatory regions.

One of the primary aims of this study was to physically map the location of the DFR 1 gene. A useful finding was that the DNA fragment when cloned into M13 bacteriophage, conferred resistance to host cells. This is an interesting system in which a eukaryotic gene on a viral vector is expressed in a prokaryotic host. Expression of drug resistance in the M13 system was convenient to characterize deletions from the 1.8 kbp yeast DNA fragment in both M13mp8B13 and M13mp19B13. The results of these deletion experiments suggested that the Bam H1 - Pst 1 region of the yeast DNA segment was not necessary for yeast dhfr expression in bacteria. Thus, the functional unit of DFR 1 was initially localized to the 1.6 kbp DNA fragment between the Pst 1 and Sal 1 restriction sites.

The ability of nuclease Bal 31 to digest duplex DNA (Legerski et al., 1978) was exploited to create a series of deletions from the Sal 1 restriction site of the DFR 1 fragment in plasmid pIUD 1. Bal 31 treated DNA was used to transform E. coli and the resultant ampicillin resistant clones were screened for sensitivity to trimethoprim. Results of these experiments limited the functional unit of DFR 1 to a region approximately 220 to 370 bp from the Hind III cut site. These data suggested that a 630 to 930 bp DNA fragment was sufficient for

expression of yeast dhfr in E. coli. This size is consistent with dhfr genes from other organisms (for a review see Burchall, 1983). This is a reasonable estimate for the size of the DFR 1 functional unit, since it is large enough to contain a DNA coding region of approximately 550 bp as well as attendent regulatory sequences.

A second aim of this study was to begin to determine the primary nucleotide sequence of DFR 1. DNA sequence analysis of the Bam H1 - Eco R1 segment of the 1.8 kbp yeast DNA fragment containing the dhfr gene revealed a potential 3' terminus located 27 nucleotides from the Eco R1 cut site. Previously, it was shown that the dhfr amino acid sequences of E. coli, L. casei, M. musculus and H. sapiens have in common a phenylalanine residue at position 178 of the mouse sequence (Freisheim et al., 1977; Nunberg et al., 1980; Smith and Calvo, 1980; Chen et al., 1984). This was also found to be the case in S. cerevisiae.

Another potential regulatory point 3' to the structural gene is the polyadenylation site of mRNAs whose fate is to be ultimately translated in the cytoplasm. It has been reported that nearly all yeast mRNA's become polyadenylated (Hereford and Rosbash, 1977). In SV40, the sequence AAUAAA is necessary for polyadenylation (Fitzgerald and Shenk, 1981). In the chick ovalbumin system, sequences both 5' and 3' to AAUAAA have also been shown to be essential in poly A site selection (Benoist et al., 1980). Conversely, Tosi et al., (1981) have suggested that polyadenylation of the mouse amylase transcipt does not occur at the AAUAAA sequences located in the 3' region of the gene.

Zaret and Sherman, (1982) have reported that the sequence AAUAAA

may be the poly (A) sequence in yeast. However, these authors point out that since the 3' non-translated regions of most yeast genes sequenced to date are A - T rich, it is not surprising to find sequences related to AAUAAA which occur by chance alone. Interestingly, a potential poly (A) sequence was identified in the Eco R1 - Bam H1 DNA fragment. The DFR 1 gene of yeast contains an AAATAA sequence 174 bp 3' to the stop codon which is closely related to the putative poly (A) recognition site reported by Zaret and Sherman (1982).

In addition, the potential termination sequences TAGT or TATGT which have been identified in the 3' non-translated regions of most yeast genes sequenced (for a review see Andreadis et al., 1984; Mantsala and Zalkin, 1984; Zaret and Sherman, 1982) are also found in this region. This sequence usually occurs in yeast 10 - 40 bp before the first known polyadenylation site. However, there are exceptions such as, certain ribosomal proteins in which the sequence is located after the poly (A) site (Zaret and Sherman, 1982). In addition to these putative termination signals, an upstream sequence, TAG and a downstream sequence, TTT, have been often identified in the 3' non-translated regions of most sequenced yeast genes (Andreadis et al., 1984; Mantsala and Zalkin, 1984; Zaret and Sherman, 1982). For these reasons, in has been suggested that this triparte sequence:

[stop...1 - 140 (T rich) ...TAG...TAGT or TATGT..(AT rich)...TTT]

may have a role in transcription termination and/or polyadenylation in some yeast genes.

Three TATGT or TAGT sequences can be identified in the 3' non-translated region of DFR 1. In all cases these sequences were

located prior to the putative polyadenylation site AAATAA. Interesting, a triparite structure which was in perfect correspondence with those mentioned before was identified 98 bp from the stop codon. As expected the TAG sequence was preceded by a T rich region (37%). Moreover, the TATGT sequence was followed by an A - T rich region and the TTT component of the consensus termination/poly (A) sequence. These data suggest that the putative transcription termination/ polyadenylation sequences of yeast DFR 1 are similar to those identified in other yeast genes.

The molecular mechanism of mRNA transcription termination in yeast is not well characterized. Since yeast is intermediate in biological complexity between bacteria and higher eukaryotes, it is not unreasonable that yeast could follow either a prokaryotic or a eukaryotic termination system. If yeast follows the mammalian pattern, the presence of several tentative poly (A) sites in DFR 1 may suggest multiple sites of transcription termination. This has been shown to be the case in mouse dhfr, where there are at least 4 mRNA's of differing sizes (Setzer et al., 1980). Setzer attributes this size heterogeneity of the mRNA transcripts to the use of different poly (A) sites 3' to the mouse structural dhfr gene.

Although, the DNA sequences which function in transcription termination in eukaryotes are not well understood, the analogous structures in bacteria have been well characterized. As yeast is a relatively simple eukaryote, it is conceivable that transcription termination could follow the bacterial example. It is interesting to note that DFR 1 contained a GC rich region followed by a stretch of 8 T residues. These are located 46 bp 3' to the opal stop codon and

are reminiscent of transcription termination in bacteria. Analogous sequences have been identified in the corresponding region of the LEU 2 yeast gene (Andreadis et al., 1984). In some bacteria, transcription terminators consist of a GC rich region of dyad symmetry followed by 6 - 8 T residues (Rosenberg and Court, 1979). However, no such symmetry is apparent in the GC rich regions 3' to either yeast gene.

At present, approximately 20 dihydrofolate reductases of bacterial and mammalian origin have been sequenced (Appendix 1). In general, bacterial dhfr's contain about 160 amino acids whereas avian and mammalian forms have approximately 25 additional residues, which are linked in a single chain (for a review see Burchall, 1983). In contrast, R plasmid R67 encoded dihydrofolate reductase consists of 4 identical subunits (Stone and Smith, 1979). The dhfrs identified from mammalian sources show great similarity. For example, the amino acid sequences of mouse and human dhfr differ in only 21 of the 186 residues (Chang et al., 1979; Chen et al., 1984). However, plasmid encoded dihydrofolate reductases show very little amino acid conservation.

Bacterial dihydrofolate reductases are not as homogenous as mammalian enzymes, however, there is some evidence for a common ancestry (Burchall, 1983). Approximately 30 of the 160 amino acids of bacterial dhfr's are identical. Although the bacterial enzymes exhibit certain common characteristics (ie. single polypeptide of 160 amino acids), they can be distinguished from each other by the proper selection of inhibitors. For instance, E. coli dhfr is much less sensitive to inhibition by the folate antagonist methotrexate

than is the analogous Streptococcus faecium enzyme (Gleisner et al., 1974). Conversley mammalian dihydrofolate reductases exhibit very similar inhibition kinetics in response to methotrexate (Chen et al., 1984).

A small number of amino acids are common to all of the bacterial and mammalian dhfr enzymes sequenced to date. Hitchings and Roth (1980) have reported that the 20 conserved residues (excluding R67 and R388 dhfrs which have only 16 of the common amino acids) have some essential role in the catalytic activity and regulation of the enzyme. Fling and Richards (1983) have suggested that some of these residues are necessary for inhibitor and cofactor bindings.

A third aim of this study was to subclone DFR 1 into various shuttle vectors to be used in experiments on the expression of the yeast dhfr gene in a mutant Chinese hamster cell line. In addition, it was necessary to clone the yeast dhfr gene into the integrating vector YIP5 to determine the map location of this gene.

In order to study the expression of the cloned yeast dihydrofolate reductase gene in a heterologous eukaryotic sustem, DFR 1 was cloned into the mammalian transducing vector pSV-neo (Mulligan and Berg, 1981). This plasmid was sent to L. Chasin (Columbia University) for subsequent experiments in a Chinese hamster cell line. Preliminary experiments revealed that although plasmid pDN 21, which contained DFR 1 and a drug marker (G418) conferred G418 resistance to host cells, the yeast dhfr gene failed to complement the dhfr deficiency of a fol- Chinese hamster cell line (L. Chasin, pers. comm.). One interpretation of these results may be that in this heterologous system, the yeast regulatory sequences are not recognized by the

mammalian cells, thus there is no expression of the dhfr enzyme.

Although, an extensive genetic map of the 17 chromosomes of S. cerevisiae is available, the location of DFR 1 remains unknown (Mortimer and Schild, 1980). This may be due in part to the lack of a mutant deficient in dihydrofolate reductase, which would facilitate the mapping of the gene by classical mapping techniques. However, with the advent of recombinant DNA technology and more specifically, gene cloning, this problem has been overcome.

In the absence of a mutant strain, it is now possible to map a cloned yeast gene by the construction of plasmids containing other scoreable markers tightly linked to the gene of interest (Struhl, 1983). The plasmid is then integrated into the host strain and the selectable marker can then be mapped by standard classical genetic techniques. By choosing a host which has a deletion through the chromosomal region containing the selected gene, virtually all transformants contain the integration at the genomic locus of interest.

In order to map the location of DFR 1, plasmid pIUD 1 (which contains URA 3) was used to transform a yeast strain in which the URA 3 gene had been deleted from the genome (M12B). This ensures that there is no URA 3 homology between plasmid and host. Thus, most recombination will occur at the genomic location of DFR 1. Three pIUD 1 integrants were sent to J. Game (University of California at Berkeley), who mapped the yeast dihydrofolate reductase gene on chromosome XV closely linked to Met 7 (Game, pers. comm.).

Previously, 2 other mutants of genes in the folate biosynthetic pathway were isolated (Little and Haynes, 1979). Although these genes

have not been further characterized (ie. gene product identified), they have been mapped (Mortimer and Schild, 1980). On the basis of their requirements for C1 metabolites, Jones and Fink (1982), tentatively identified tmp 2 as a mutant allele of dhfr. It is now known that the tmp 2 mutants consist of 2 complementation groups, fol 1 and fol 2. The map locations of these genes have been identified and since DFR 1 maps to a different chromosome, it is unlikely that TMP 2 encodes the structural gene for yeast dihydrofolate reductase.

Although many of the details of the control and regulation of folate biosynthesis in yeast are not known, depletion of tetrahydrofolate pools has been shown to induce thymineless death (Barclay and Little, 1978). Other effects of thimidylate deprivation in yeast include: 1) induction of ρ - and mitochondrial point mutations; 2) errosion of the mitochondrial genome; and 3) DNA strand breaks (for a review see Barclay et al, 1982). Since antifolates have been shown to induce thymineless conditions (Wintersberger and Hirsch, 1973), it was of interest to determine if the presence of DFR 1 on a multicopy plasmid would afford host cells protection against thy-associated lethality and mitochondrial damage.

It was convenient to use MTX as an index of expression of yeast dhfr gene since this drug is known to inhibit the activity of dihydrofolate reductase. In all three yeast strains tested, a similar inhibition of growth was observed in response to this antifolate. However, these strains containing the yeast dhfr gene on a multicopy plasmid exhibited an enhanced resistance to MTX at the same drug concentrations. This cleary demonstrates that DFR 1 is expressed in

yeast. Furthermore, the intermediate resistance to MTX afforded to the DFR 1 integrant suggests that the level of antifolate resistance is gene dosage dependant.

As mentioned previously, the inhibition of folate biosynthesis by folate antagonists results in the induction of ρ^-. Thus, another index of yeast dhfr expression was available to study the effects of folate deprivation on DFR 1. Consistent with previous findings, a high frequency of petites were induced in wild-type and rad 18 cell populations treated with MTX. One surprising result was that the presence of DFR 1 had no effect on ρ^- formation in either of these strains. Another curious finding was the lack of petite formation in rad 6 cells, both spontaneously and upon exposure to the antifolate. One possible interpretation of these data may be that there exist two pools of folate in this organism.

If one ascribes the induction of ρ^- in cells treated with MTX to depletion of folates, due to an inhibition of dhfr, a critical question is raised, "Do mitochondria make their own folates or are they imported from the cytoplasm?". If there exists a folate pool common to both mitochondria and the cytoplasm, it is speculated that the presence of DFR 1 on a multicopy plasmid would provide enough dhfr for both systems. Thus, there should be a decrease in the frequency of MTX induced ρ^- .

In contrast the results presented here suggest that available nuclear folate pools may not be available to the mitochondria. These data support the notion put forth by Luzzati (1975), that there exist 2 forms of same folate interconversion enzymes: 1) an enzymatic activity isolated to the cytoplasm; and 2) an analogous enzyme

associated with the mitochondrian. If Luzzati is correct, there is a compartmentalization of folates and excess dhfr in the cytoplasm will have no effect on mitochondrial folate pools.

One interesting question raised by these speculations is, "Would the importation of "cytoplasmic" folates into the mitochondria have an effect on the formation of cytoplasmic petites?". These experiments may be possible in the near future, in light of the recent finding that a yeast mitochondrial precursor was sufficient to direct a mouse cytoplasmic enzyme into the yeast mitochondrial matrix (Hurt et al, 1984)

In contrast to the inhibitory effects of MTX on yeast cell growth it has been shown that wild-type yeast are refractory to the inhibition of trimethoprim up to concentrations of 1.5 mg /ml. The magnitude of this resistance is realized when it is remembered that the growth of E. coli is strongly inhibited by 2 ug/ml of this drug. However, there exist several radiation sensitive mutants of S. cerevisiae which exhibit a sensitivity to trimethoprim. The Rad 6 and Rad 18 genes of S. cerevisiae are 2 of at least 9 genes involved in the error-prone dark repair system of UV-induced pyrimidine dimers (for a review see Game, 1983). Although the precise function of the Rad 6 gene product in yeast is unknown, it has been shown that mutagenesis by physical or chemical means requires a functional Rad 6 gene (Prakash, 1974; Lawrence and Christensen, 1976).

Since there is no a priori reason to expect a correlation between the inhibition tetrahydrofolate biosynthesis and radiation sensitivity it remains puzzling that mutant alleles of these loci in addition to abolishing a mode of DNA repair, also confer sensitivity to the folate

antagonist trimethoprim.

In light of these facts it is conceivable that, rad 6 or rad 18 are allelic to DFR 1. However, comparision of the restriction maps of DFR 1 and RAD 6 suggested that these are in fact 2 different genes. Another puzzling finding was that the presence of DFR 1 on a multicopy plasmid seemed to have no effect on the TRM-sensitivity of rad 6, rad 18 and folate depleted wild-type cells. Furthermore, transformation of the rad 6 and rad 18 strains with the pDR509 did not alter their sensitivity to radiation (L. Prakash, pers. comm.). In view of the finding that DFR 1 is located on chromosome XV, it seems unlikely that either rad 6 or rad 18 are allelic to this gene.

No increase in the frequency of ρ^- was observed in cells grown in the presence of TRM, which is also uncharacteristic of an antifolate. However, this may have been due to the culture conditions which lacked all C1 metabolites. Consequently, the absence of petite induction may have resulted from this non-specific folate depletion.

Although it is unclear why TRM does not produce the characteristic effects of a folate antagonist in cell populations, one interpretation of these findings may be that the target of this drug is not dihydrofolate reductase in yeast.

SUMMARY AND CONCLUSIONS

The cloned dihydrofolate reductase gene (DFR 1) of S. cerevisiae is expressed in both bacteria and yeast. E. coli strains containing DFR 1 were at least 5 times more resistant to inhibition by the folate antagonist TRM. Expression of yeast dhfr in bacteria suggests DFR 1 lacks intervening sequences to any great extent. Furthermore, the yeast DNA fragment probably has its own promotor as evidenced by expression of the cloned yeast dhfr gene in both orientations in E. coli.

The functional unit of DFR 1 has been localized to a DNA sequence of approximately 930 bp between the Pst 1 and Sal 1 cut sites. Several potential termination/poly(A) consensus sequences were identified in 3' to an opal stop codon located 27 nucleotides from the Eco R1 cut site on the Pst 1 - Eco R1 DNA fragment.

Yeast strains transformed with the DFR 1 multicopy plasmid (pDR509) are afforded greater resistance to the folate analogue MTX than a DFR 1 integrant. This suggests a gene dosage effect. Presence of DFR 1 does not effect the induction of ρ^- in wild-type or rad 18 yeast cell populations.

rad 6 cells exhibit a very low level of petite formation both spontaneously and upon treatment with MTX. However, EtBr induced ρ^- to a level approximately 50% of that exhibited by the other 2 yeast strains.

The sensitivity of the rad mutants and wild-type yeast cells to the folate antagonist trimethoprim was unaffected by the

presence of DFR 1 on a multicopy plasmid. Furthermore, TRM did not induce petites in any strain tested which is uncharacteristic of an antifolate. This may suggest that TRM has a target other that dhfr in yeast.

Literature Cited

Alt, F. W., R. D. Kellems, J. R. Bertino, and R. T. Schimke. 1978 Selective multiplication of dihydrofolate reductase genes in Methotrexate-resistant variants of cultured murine cells. J. Biol. Chem. 253: 1357 - 1370.

Andreadis, A., Y. P. Hsu, M. Hermodson, G. Kohlhaw and P. Schimmel. 1984 Yeast LEU 2. J. Biol. Chem. 259: 8059 - 8062.

Angehrn, P. and P. Then. 1973 Nature of trimethoprim-induced death in Escherichia coli. Arzneim Forsch. 23: 447 - 451.

Baccanari, D., A. Phillips, S. Smith, D. Sinski, and J. Burchall. 1975 Purification and properties of E. coli dihydrofolate reductase. Biochemistry 14: 5267 - 5273.

Baccanari, D., D. Stone and L. Kuyper. 1981 Effect of a single amino acid substitution on Escherichia coli dihydrofolate reductase. J. Biol. Chem. 256: 1738 - 1747.

Baccanari, D. P., R. L. Tansik, S. J. Paterson and D. Stone. 1984 Characterization and amino acid sequence of Neisseria gonorrhoeae dihydrofolate reductase. J. Biol. Chem. 259: 12291 - 12298.

Banerjee, A. and W. F. Benedict. 1979 Production of sister chromatid exchanges by various cancer chemotherapeutic agents. Cancer Res. 39: 797 - 799.

Barclay, B. J. and J. G. Little. 1978 Genetic damage during thymidylate starvation in Saccharomyces cerevisiae. Mol. Gen. Genet. 160: 33 - 40.

Barclay, B. J., B. A. Kunz, J. G. Little and R. H. Haynes. 1982 Genetic and biochemical consequences of thymidylate stress. Can. J. Biochem. 60: 172 - 194.

Bastow, K. F., R. Prabhu, and Y. C. Cheng. 1984 The intracellular content of dihydrofolate reductase: Possibilities for control and implications for chemotherapy. Adv. Enzyme Reg. 22: 15 - 26.

Baugh, C. M. and C. L. Krumdieck. 1971 Naturally occuring folates. Ann. New York Acad. Sci. 186: 7 - 28.

Benkovic, S. J. 1980 On the mechanism of action of folate and biopterin-requiring enzymes. Ann. Rev. Biochem. 49: 227 - 251.

Bennet, C. S., J. A. Rodkey, J. M. Sondey and R. Hirschman. 1978 Dihydrofolate reductase: the amino acid sequence of the enzyme from a methotrexate-resistant mutant of Escherichia coli Biochemistry 17: 1328 - 1337.

Benoist, C., K. O'Hare, R. Breathnach and P. Chambon. 1980 The ovalbumin gene-sequence of putative control regions. Nucl. Acids Res. 8: 127 - 142.

Bernardi, G. 1978 The petitie mutation in yeast. Trends Biochem. Sci. 4: 197 - 201.

Bertino, J. R. 1979 Toward improved selectivity in cancer chemotherapy. Cancer Res. 39: 293 - 304.

Bertino, J. R., D. M. Donohue, B. Simmons, B. W. Gabrio, R. Silber and F. M. Huennekens. 1963 The induction of dihydrofolate reductase activity in leukocytes and erythrocytes in patients treated with amethopterin. J. Clin. Invest. 42: 466 - 478.

Biggin, M. D., T. J. Gibson and G. F. Hong. 1983 Buffer gradient gels and S label as an aid to rapid DNA sequence determination. Proc. Natl. Acad. Sci. USA 80: 3963 - 3965.

Birnboim, H. C. and J. Doly. 1979 A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7: 1513 - 1523.

Blakely, R. L. 1969 The biochemistry of folic acid and related pteridines. North Holland Publishing Co., Amsterdam.

Botstein, D. and R. W. Davis. 1982 Principles and practice of recombinant DNA research with yeast. In J. Stathern, E. Jones and J. Broach (eds.), The Molecular Biology of the Yeast Saccharomyces II. Cold Spring Harbor, New York.

Brendel, M. and W. W. Fath. 1974 Isolation and characterization of mutants of Saccharomyces cerevisiae auxtotropic and conditionally auxotrophic for 5'-dTMP. Z. Naturforsh. 29: 733 - 738.

Bresler, S., M. Mosevitsky and L. Vyacheslavov. 1970 Complete mutagenesis in bacterial population induced by thymine starvation on solid media. Nature 225: 764 - 766.

Burchall, J. J. 1983 Dihydrofolate reductase. In G.H. Hitchings (ed.), Inhibition of folate metabolism in chemotherapy. Springer-Verlag, New York.

Buttle, G. A. H., W. H. Grey and D. Stephenson. 1936 Protection of mice against streptococcal and other infections by p-aminobenzene sulfonamide and related substances. Lancet 1: 1286 - 1290.

Chang, A. C. Y., J. H. Nunberg, R. J. Kaufman, H. A. Erlich, R. T. Schimke and S. N. Cohen. 1978 Phenotypic expression in E. coli of a DNA sequence coding for mouse dihydrofolate reductase. Nature 275: 617 - 624.

Chen, M. J., T. Shimada, A. D. Moulton, A. Cline, R. K. Humphries, J. Maizel and A. W. Nienhuis. 1984 The functional human dihydrofolate reductase gene. J. Biol. Chem. 259: 3933 - 3943.

Cohen, S. S. and H. D. Barner. 1954 Studies on unbalanced growth in Escherichia coli. Proc. Natl. Acad. Sci U.S.A. 40: 885 - 893.

Cohen, S., A. Chang, H. Boyer and R. Heilling. 1973 Construction of biologically functional bacterial plasmids in vitro. Proc. Natl. Acad. Sci. USA 70: 3240 - 3244.

Condit, P. T. 1971 Chemotherapy of neoplastic disease with folate antagonists. Ann. New York Acad. Sci. 186: 475 - 485.

Diminik, L. S. and D. I. Hoar. 1983 A molecular basis for the cytotoxicity of the thymidine-less state in cultured human cells. Genetics Society of Canada Bulletin. 14: 52.

Duckworth, M. L., M. J. Gait, P. Goelet, G. F. Hong, M. Singh and R. C. Titmus. 1981 Rapid synthesis of oligodeoxyribonucleotides VI. Efficient, mechanized synthesis of heptadecadeoxyribonucleotides by an improved solid phase phosphostriester route. Nucleic Acids Res. 9: 1691 - 1706.

Fitzgerald, M. and T. Shenk. 1981 The sequence 5'-AAUAAA-3' forms part of the recognition site for the polyadenylation of late SV40 mRNA's. Cell 24: 251 - 260.

Fling, M. E. and C. Richards. 1983 The nucleotide sequence of the Trm-R DHFRase gene harbored by Tn 7. Nucleic Acids Res. 11: 5147-5157.

Fogel, S., R. K. Mortimer and K. Lusnak. 1983 Meiotic gene conversion in yeast: Molecular and Experimental Perspectives. In J. E. T. Spencer, D. M. Spencer and A. R. W. Smith (eds.), Yeast Genetics: Fundamental and Applied Aspects. Springer-Verlag, New York.

Frei, E., A. Rosowsky, J. E. Wright, C. A. Cacchi, J. A. Lippke, T. J. Ervin, J. Jolivet and W. A. Haseltine. 1984 Development of methotrexate resistance in a human squamous cell carcinoma of the head and neck in culture. Proc. Natl. Acad. Sci. USA 81: 2873 - 2877.

Freisham, J. H., K. G. Bitar, A. V. Reddy and D. T. Blankenstrip 1978 Dihydrofolate reductase from amethopterin-resistant Lactobacillus casei. J. Biol. Chem. 253: 6437 - 6444.

Freisham, J. H., C. H. Ericsson, K. G. Bitar, R. B. Dunlap and A. V. Reddy. 1977 An active center tryptophan residue in dihydrofolate reductase: Chemical modification, sequence surrounding the critical residue, and structural homology consideration. Arch. Biochem. Biophys. 180: 310 - 317.

Fried, H. M., N. J. Pearson, C. H. Kim and J. R. Warner. 1981 The genes for fifteen ribosomal proteins for S. cerevisiae. J. Biol. Chem. 256: 10176 - 10183.

Fukunaga, M. and L. Yielding. 1981 Respiration deficient mutant induction and cell killing effects in radiation sensitive mutants of Saccharomyces cerevisiae by ethidium azide. J. VOEH. 3: 5 - 10.

Galivan, J. 1979 Transport and metabolism of methotrexate in normal and resistant cultured rat hepatoma cells. Cancer Res. 39: 735 - 743.

Gallant, J. and T. Spottswood. 1964 Measurement of the stability of the repressor of alkaline phosphatase synthesis in Escherichia coli. Proc. Natl. Acad. Sci. USA 52: 1591 - 1598.

Game, J. C. 1983 Radiation sensitive mutants and repair in yeast. In J. E. T. Spencer, D. M. Spencer and A. R. W. Smith (eds.), Yeast genetics: Fundamental and applied aspects. Springer-Verlag, New York.

Game, J. C., J. G. Little and R. H. Haynes. 1975 Yeast mutants sensitive to trimethoprim. Mutation Res. 28: 175 - 182.

Gleisner, J. M., D. L. Peterson and R. L. Blakley. 1974 Amino acid sequence of dihydrofolate reductase from a methotrexate resistant mutant of Streptococcus faecuim and identification of methionine residue at the inhibitor binding site. Proc. Natl. Acad. Sci. U.S.A. 71: 3001 - 3005.

Goldthwaite, C. D., D. R. Cryer and J. Marmer. 1974 Effect of carbon source on the replication and transmission of yeast mitochondrial genomes. Mol. Gen. Genet. 133: 87 - 104.

Hartwell, L. H. 1974 Cell cycle of Saccharomyces cerevisiae. Bacteriol. Rev. 338: 164 - 198.

Haynes, R. H. and B. A. Kunz. 1981 DNA repair and mutagenesis in yeast. In J. Strathern, E. Jones and J. Broach (eds.), Molecular biology of the yeast Saccharomyces. Cold Spring Harbor, New York.

Heintz, N. H. and J. L. Hamlin. 1982 An amplified chromosomal sequence that includes the gene for dihydrofolate reductase initiates replication within specific restriction fragments. Proc. Natl. Acad. Sci. USA 79: 4083 - 4087.

Hereford, L. M. and M. Rosbash. 1977 Number and distributiion of polyadenylated RNA sequences in yeast. Cell 10: 453 - 462.

Hinnen, A., J. Hicks and G. R. Fink. 1978 Transformation in yeast. Proc. Natl. Acad. Sci. USA. 75: 1929 - 1933.

Hitchings, G. E. 1983 Function of tetrahydrofolate and the role of dihydrofolate reductase. In G.H. Hitchings (ed.), Inhibition of folate metabolism in chemotherapy. Springer-Verlag, New York.

Hitchings, G. H. and B. Roth. 1980 Dihydrofolate reductase as targets for selective inhibitiors. In M. Sandler (ed.), Enzyme inhibitors as drugs. Macmillan, London.

Hoffbrand, A. V. 1975 Synthesis and breakdown of natural folates (folate polyglutamates). Prog. Hematol. 87: 85 - 104.

Holland, J. P. and M. J. Holland. 1979 The primary structure of a glyceraldehyde - 3 - phosphate dehydrogenase gene from S. cerevisiae. J. Biol. Chem. 254: 9839 - 9845.

Holmes, A. J. and A. Eisenstark. 1968 The mutagenic effect of thymine starvation on Salmonella typhimurium. Mutat. Res. 5: 15 - 21.

Hori, T., D. Ayusawa, K. Shimizu, H. Koyama and T. Seno. 1984 Chromosome breakage induced by thymidylate stress in thymidylate synthase-negative mutants of mouse FM3A cells. Cancer Res. 44: 703 - 705.

Hrynick, W. M. and J. R. Bertino. 1971 Growth rate and cell killing. Ann. New York Acad. Sci. 186: 330 - 342.

Hsiao, C. L. and J. Carbon. 1981 Direct selection procedure for the isolation of functional centromeric DNA. Proc. Natl. Acad. Sci. U.S.A. 78: 3760 - 3764.

Jones, E. and G. Fink. 1982 In J. Strathern, E. Jones and J. Broach (eds.), Molecular biology of the yeast Saccharomyces II. Cold Spring Harbour, New York.

Kaufman, R. J., P. C. Brown and R. T. Schimke. 1979 Amplified dihydrofolate reductase genes in unstably methotrexate-resistant cells are associated with doubly minute chromosomes. Proc. Natl. Acad. Sci USA 76: 5669 - 5673.

Kirschner, K. and H. Bisswanger. 1976 Multifunction proteins. Ann. Rev. Biochem. 45: 143 - 166.

Kumar A. A., D. T. Blankenstrip, B. T. Kaufman and J. H. Friesham. 1980 Primary structure of chicken liver dhfr. Biochemistry 19: 667 - 678.

Kunz, B. A., B. J. Barclay, J. C. Game, J. G. Little and R. H. Haynes 1980 Induction of mitotic recombination in yeast by starvation for thymine nucleotides. Proc. Natl. Acad. Sci USA 77: 6057 - 6061.

Kunz, B. and B. W. Glickman. 1985 Mechanism of mutation by thymine starvation in Escherichia coli: Clues from mutagenic specificity. J. Bacteriol. 162: 859 - 864.

Lai, P. H., Y. C. Pan, J. M. Gleisner, D. L. Peterson and R. L. Blakley. 1979 Primary sequence of bovine liver dihydrofolate reductase. In R. L. Kisliuk and G. M. Brown (eds.), Chemistry and biology of pteridines. Elsevier/North Holland, New York.

Lawrence, C. W. and R. Christensen. 1976 UV-mutagenesis in radiation sensitive strains of yeast. Genetics 82: 207 - 232.

Lawrence, C. W., and R. Christensen. 1979 Metabolic suprressors of trimethoprim and ultraviolet sensitivities of Saccharomyces cerevisiae rad 6 mutants. J. Bacteriol. 139: 866 - 876.

Laskowski, W. and E. Lehmann-Brauns. 1973 Mutants of Saccharomyces able to grow after inhibition of thymidine phosphate synthesis. Mol. Gen. Genet. 125: 275 - 277.

Legerski, R. J., J. L. Hodnett and H. B. Gray. 1978 Extracellular nucleases of Pseudomonas Bal 31. III. Use of the double-strand deoxyriboexonuclease activity as the basis of a convenient method for the mapping of fragments of DNA produced by cleavage with restriction enzymes. Nucleic Acids Res. 5: 1445 - 1464.

Littlefield, J. W. 1969 Hybridization of hamster cells with high and low folate reductase activity. Proc. Natl. Acad. Sci. U.S.A. 62: 88 - 95.

Luzzati, M. 1975 Isolation and properties of a thymidylate-less mutant in Saccharomyces cerevisiae. Europ. J. Biochem. 56: 533 - 538.

Mackenzie, R. E. and C. M. Baugh. 1980 Tetrahydropteroylglutamate derivatives as substrates of two multifunctional proteins with folate-dependant enzyme activities. Biochem. Biophys. Acta 611: 187 - 195.

Maniatis, T., E. F. Fritsch and J. Sambrook. 1982 Molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Publishers. Cold Spring Harbor, New York.

Mantsala, P. and H. Zalkin. 1984 Glutamine nucleotide sequence of Saccharomyces cerevisiae ADE 4 endcoding phosphoribosylpyrophosphate aminotransferase. J. Biol. Chem. 259: 8474 - 8484.

Mayer, V. W. and C. J. Goin. 1984 Semidominance of RAD 18-2 for several phenotypic characters in Saccharomyces cerevisiae. Genetics 106: 577 - 589.

Melera, P. W., J. P. Davide, C. A. Hession and K. W. Scotto. 1984 Phenotypic expression in E. coli and nucleotide sequence of two C.H.L. cell cDNA's encoding different DHFRases. Mol. Cell. Biol. 4: 38 - 48.

Messing, J., R. Crea and P. H. Seeburg. 1981 A system for shotgun DNA sequencing. Nucleic Acids Res. 9: 309 - 321.

Messing, J. and J. Vieira. 1982 A new pair of M13 vectors for selecting either DNA strand of double-digest restriction fragments. Gene 19: 269 - 276.

Mitchell, H. K., E. E. Shell and R. T. Williams. 1944 Folic Acid. I. Concentration from spinach. J. Am. Chem. Soc. 66: 267 - 268.

Montgomery, D. L., D. W. Leung, M. Smith, P. Shalit, G. Faye and B. D. Hall. 1980 Isolation and sequence of the gene for iso-2-cytochrome c in S. cerevisiae. Proc. Natl. Acad. Sci. U.S.A. 77: 541 - 545.

Morrow, J. 1983 Eukaryotic cell genetics. Academic Press, New York.

Mortimer, R. K. and D. Schild. 1980 Genetic map of Saccharomyces cerevisiae. Microbiol. Rev. 44: 519 - 571.

Mulligan, R. C. and P. Berg. 1981 Selection for animal cells that express the Escherichia coli gene coding for xanthine-guanine phosphoribosyltransferase. Proc. Natl. Acad. Sci. USA. 78: 2072 - 2076.

Nair, M. G. and C. M. Baugh. 1973 Synthesis and biological evaluation of poly-α-glutamyl derivatives of methotrexate. Biochemistry 12: 3923 - 3927.

Nath, K. and E. W. Baptist. 1984 Cloning of a yeast dihydrofolate reductase gene in Escherichia coli. Current Genetics 8: 265 - 270.

Nunberg, J. H., R. J. Kaufman, A. C. Y. Chang, S. N. Cohen and R. T. Schimke. 1980 Structure and genomic organization of the mouse dhfr gene. Cell 19: 355 - 364.

Nunberg, J. H., R. J. Kaufman, R. T. Schimke, G. Urlaub and L. A. Chasin. 1978 Amplified dihydrofolate reductase genes are localized to a homogeneously staining region of a single chromosome in a methotrexate-resistant Chinese hamster ovary cell line. Proc. Natl. Acad. Sci. USA 75: 5553 - 5556.

Oliver, S. G. 1977 On the mutability of the yeast mitochondrial genome. J. Theor. Biol. 67: 195 - 201.

Osborn, M. J. and F. M. Huennekens. 1958 Enzymatic reduction of dihydrofolic acid. J. Biol. Chem. 233: 969 - 974.

Parker, B. A. and G. R. Stark. 1979 Regulation of simian virus 40 transcription: Sensitive analysis of the RNA species present early in infections by virus or viral DNA. J. Virol. 31: 360 - 369.

Petes, T. D. 1980 Molecular genetics of yeast. Ann. Rev. Biochem. 49: 845 - 876.

Prakash L. 1974 Lack of chemically induced mutation in repair deficient mutants of yeast. Genetics 78: 1101 - 1118.

Pritikin, W. B. and W. R. Romig. 1966 Death of Bacillus subtilis auxotrophs due to deprivation of thymine, tryptophan or uracil. J. Bacteriol. 92: 291 - 296.

Resnick, M. A. 1969 A photoreactivationless mutant of Saccharomyces cerevisiae. Photochem. Photobiol. 9: 307 - 312.

Rodkey, J. A. and C. D. Bennet. 1976 Micro-Edman degradation: The use of high pressure liquid chromatography and gas chromatography in the amino terminal sequence determination of 8 nanomoles of dhfr from a mouse sarcoma. Biochem. Biophys. Res. Commun. 72: 1407 - 1413.

Rosenberg, M. and D. Court. 1979 Regulatory sequences involved in the promotion and termination of RNA trannscription. Ann. Rev. Genet. 13: 319 - 353.

Sanger, F. and A. R. Coulson. 1978 The use of thin acrylamide gels for DNA sequencing. FEBS Lett. 87: 107 - 110.

Sanger, F., A. R. Coulson, B. G. Barrell, A. J. H. Smith and B. A. Roe. 1980 Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. J. Mol. Biol. 143: 161 - 178.

Sanger, F., S. Nicklen, and A. R. Coulson. 1977 DNA Sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74: 5463 - 5467.

Scherer, S. and R. W. Davis. 1979 Replacement of chromosome segments with altered DNA sequences constructed in vitro. Proc. Natl. Acad. Sci. USA 76: 4951 - 4955.

Schimke, R. T. 1984 Gene amplification in cultured animal cells. Cell 37: 705 - 713.

Setzer, D. R., M. McGrogan, J. H. Nunberg and R. T. Schimke. 1980 Size hetergeneity in the 3' end of dihydrofolate reductase messenger RNA's in mouse cells. Cell 22: 361 - 370.

Sharp, P. A., B. Sugden and J. Sambrook. 1973 Detection of two restriction endonuclease activities in Haemophilus parainfluenzae using analytical agarose. Biochemistry 12: 3055 - 3063.

Sheilds, R. 1978 Methorexate resistance by gene amplication. Nature 273: 269 - 270.

Simonsen, C. C., E. Y. Chen and A. D. Levinson. 1983 Identification of the type 1 Trm-R DHFRase specified by the E. coli R plasmid R483. Comparison with prokaryotic and eukaryotic DHFRases. J. Bacteriol. 155: 1001 - 1008.

Simonsen, C. C. and A. D. Levinson. 1983 Isolation and expression of an altered mouse dihydrofolate reductase cDNA. Proc. Natl. Acad. Sci. USA 80: 2495 - 2499.

Sirotnak, F. M. and R. C. Donsbach. 1974 The intracellular concentration dependence of antifolate inhibition of DNA synthesis in L1210 leukemia cells. Cancer Res. 34: 3332 - 3340.

Slater, M. 1974 Recovery of yeast from transient inhibition of DNA synthesis. Nature 247: 275 - 276.

Smith, D. R. and J. M. Calvo. 1980 Nucleotide sequence of the E. coli gene coding for dihydrofolate reductase. Nucleic Acids Res. 8: 2255 - 2274.

Smith, D. R. and J. M. Calvo. 1982 Nucleotide sequence of dihydrofolate reductase genes from trm-R mutants of E. coli. Mol. Gen. Genet. 187: 72 - 78.

Smith, N. D., R. R. Green, L. S. Ripley and J. W. Drake. 1973 Thymineless mutagenesis in bacteriophage T4. Genetics 74: 393 - 403.

Smith, S. L., P. Patrick, D. Stone, A. W. Phillips and J. J. Burchall. 1979 Porcine liver dihydrofolate reductase: purification, properties and amino acid sequence. J. Biol. Chem. 254: 11475 - 11484.

Stone, D., S. J. Paterson, J. H. Raper and A. W. Phillips. 1979 The amino acid sequence of dihydrofolate reductase from the mouse lymphoma L1210. J. Biol. Chem. 254: 480 - 488.

Stone, D., and S. L. Smith. 1979 The amino acid sequence of a trimethoprim-resistant dihydrofolate reductase specified in E. coli plasmid by R plasmid R-67. J. Biol. Chem. 254: 10857 - 10861.

Storms, R. K., J. B McNeil, P. S. Khandekar, G. An, J. Parker and J. D. Friesen. 1979 Chimeric plasmids for cloning of deoxyribonucleic acid sequences in Saccharomyces cerevisiae J. Bacteriol. 140: 73 - 82.

Struhl, K., D. T. Stinchcomb, S. Scherer and R. W. Davis. 1979 High frequency transformation of yeast: Automomous replication of hybrid DNA molecules. Proc. Natl. Acad. Sci. USA 76: 1035 - 1039.

Struhl, K. D. 1983 The new yeast genetics. Nature 305: 391 - 397.

Tosi, M., R. A. Young, O. Hagenbuchle and U. Schibler. 1981 Multiple polyadenylation sites in mouse α-amylase gene. Nucl. Acids Res. 9: 2313 - 2323.

Ullman, B., M. Lee, D. W. Martin Jr. and D. V. Santi. 1977 Cytotoxicity of 5-fluoro-2'-deoxyuridine: Requirement for reduced folate cofactors and antagonism by methotrexate. Proc. Natl. Acad. Sci. USA 75: 980 - 983.

Varshavsky, A. 1981 On the possibility of metabolic control of replicon "misfiring": Relationship to emergence of malignanat phenotypes in mammalian cell lineages. Proc. Natl. Acad. Sci. USA 78: 3673 - 3677.

Vesely, J. and A. Cihak. 1973 Resistance of mammalian tumor cells toward pyrimidine analogues. Oncology 28: 204 - 226.

Volz, K. W., D. A. Matthews, R. A. Alden, S. T. Freer, C. Hansch, B. T. Kaufman and J. Kraut. 1982 Crystal structure of Avian dihydrofolate reductase containing phenyltriazine and NADPH. J. Biol. Chem. 257: 2528 - 2536.

Wintersberger, U. and J. Hirsch. 1973. Induction of cytoplasmic respiratory deficient mutants in yeast by the folic acid analogue, methotrexate. I. Studies on the mechanism of petite induction. Molec. Gen. Genet. 126: 61 - 70.

Wormser, G. P. and G. T. Keusch. 1983 Trimethoprim/sulfamethoxazole: an overview. In G. H. Hitchings (ed.) Inhibition of folate metabolism in chemotherapy. Springer-Verlag, New York.

Zaret, K. S. and F. Sherman. 1982 DNA sequence required for efficient transcription termination in yeast. Cell 28: 563 - 573.

Zolg, J. W. and U. J. Hanggi. 1981 Characterization of a R-plasmid associated, trimethoprim-resistant dihydrofolate reductase and determination of the nucleotide sequence of the reductase gene. Nucleic Acids Res. 9: 697 - 709.

Appendix I Published Sequences of Dihydrofolate Reductases.

Source	Reference
Neisseria gonorrhoeae	Baccanari *et al.*, 1984
Tn 7	Fling and Richards, 1983
Escherichia coli forms 1 and 2	Baccanari *et al.*, 1981
E. coli MB148	Bennet *et al.*, 1978
E. coli K12	Smith and Calvo, 1980
E. coli R67	Stone and Smith, 1979
E. coli R388	Zolg and Hanggi, 1981
E. coli R483	Simonsen *et al.*, 1983
Streptococcus faecium	Freisheim *et al.*, 1977.
S. faecium II	Gleisner *et al.*, 1974
Lactobacillus casei	Freisheim *et al.*, 1978
Chicken liver	Kumar *et al.*, 1980
Mouse lymphoma L1210	Stone *et al.*, 1979
Mouse sarcoma 180	Rodkey and Bennet, 1976
Mus musculus	Chang *et al.*, 1978
Porcine liver	Smith *et al.*, 1979
Bovine liver	Lai *et al.*, 1979
Chinese hamster lung	Melera *et al.*, 1984
Human	Chen *et al.*, 1984