National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

Canada

UNIVERSITY OF ALBERTA

IN VIVO DISRUPTION AND *DE NOVO* AMPLIFICATION OF THE *DFR1* GENE ENCODING DIHYDROFOLATE REDUCTASE IN *SACCHAROMYCES CEREVISIAE*

BY

© TUN HUANG

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

DEPARTMENT OF GENETICS

EDMONTON, ALBERTA

SPRING 1993

National Library of Canada

Acquisitions and Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale du Canada

Direction des acquisitions et des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file *Votre référence*

Our file *Notre référence*

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-81975-8

Canada

Huang.

UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR: TUN HUANG

TITLE OF THESIS: *IN VIVO* DISRUPTION AND *DE NOVO* AMPLIFICATION OF THE *DFR1* GENE ENCODING DIHYDROFOLATE REDUCTASE IN *SACCHAROMYCES CEREVISIAE*

DEGREE: DOCTOR OF PHILOSOPHY

YEAR THIS DEGREE GRANTED: 1993

PERMISSION IS HEREBY GRANTED TO THE UNIVERSITY OF ALBERTA LIBRARY TO REPRODUCE SINGLE COPIES OF THIS THESIS AND TO LEND OR SELL SUCH COPIES FOR PRIVATE, SCHOLARLY OR SCIENTIFIC RESEARCH PURPOSES ONLY.

THE AUTHOR RESERVES OTHER PUBLICATION RIGHTS, AND NEITHER THE THESIS NOR EXTENSIVE EXTRACTS FROM IT MAY BE PRINTED OR OTHERWISE REPRODUCED WITHOUT THE AUTHOR'S WRITTEN PERMISSION.

Tun Huang

10116-87 Ave. Edmonton, Alberta

Date: March 31, 1993

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *IN VIVO* DISRUPTION AND *DE NOVO* AMPLIFICATION OF THE *DFR1* GENE ENCODING DIHYDROFOLATE REDUCTASE IN *SACCHAROMYCES CEREVISIAE* submitted by TUN HUANG in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Genetics.

Dr. P. J. Hastings (Co-supervisor)

Dr. R. C. von Borstel (Co-supervisor)

Dr. H. McDermid

Dr. R Hodgetts

Dr. E. A. Cossins

Dr. J. Bell for Dr. L. Chasin

Date: March 31, 1993

To my loving wife, Yanling, who made it possible for me to complete my pursuit of a Ph.D. through her love, sacrifice, and persevering encouragement.

ABSTRACT

Dihydrofolate reductase (DHFR), the target of the antifolate drug methotrexate, plays an important role in cellular growth and metabolism. Development of methotrexate resistance due to a number of mechanisms including *dhfr* gene amplification has been a recurrent clinical limitation to the use of this drug in chemotherapy. DNA amplification is also a common genetic change in cancer cells. *Saccharomyces cerevisiae* is an excellent eukaryotic model system, but studies on these biological phenomena in this organism had been limited by the lack of a DHFR-deficient mutant and a suitable amplification marker. This thesis describes a yeast DHFR-deficient (*dfr1*) mutant and amplification of the yeast *dhfr* gene (*DFR1*) in methotrexate-resistant mutants. The *dfr1* mutant is auxotrophic for dTMP, adenine, histidine and methionine, and is respiratory-deficient. Unlike wild-type strains treated with methotrexate, its growth requirements are not satisfied by folinic acid in complex medium. Two types of additional mutations (*fou* and *DIR*) that modify the *dfr1* mutant phenotype have been identified and partially characterized. The *fou* mutation confers a folinic acid-utilizing phenotype on the *dfr1* mutant in complex medium. The *DIR* mutation confers a respiratory-sufficient phenotype on the *dfr1* mutant as well as on a *met7* mutant. Besides *DFR1* amplification, mutations at genetic loci other than *DFR1* have been identified which contribute to the methotrexate-resistant phenotype. All these mutants should be useful for studies on folate metabolism and the molecular mechanism(s) of antifolate drug resistance. *De novo DFR1* amplification is a common causal mechanism in methotrexate-resistant mutants, isolated under the selective conditions described in this study. The molecular characteristics of the *DFR1* amplicons characterized so far resemble those in mammalian systems. For example, both chromosomal and extrachromosomal *DFR1* amplicons have been found. The extrachromosomal elements seem to be circular. To my knowledge, this is the only yeast amplification marker with such properties. Thus, it is now possible to take advantage of the yeast *S. cerevisiae* as a model system to study the mechanisms that underlie the process of gene amplification, or, perhaps more generally, that safeguard genome integrity, which may be more difficult to study in complex mammalian cells.

ACKNOWLEDGEMENTS

The author would like to thank Drs P. J. Hastings, F. C. von Borstel, and B. J. Barclay, who provided me the opportunity to work on the research project presented in this thesis, the guidance throughout this research work, and the assistance in preparation of this thesis.

My thanks extend to Drs H. McDermid and D. Pligrim as well as M. Maduro for their assistance in experiments with pulsed-field gel electrophoresis, and Dr. E. A. Cossins and S. Lin for their helps in experiments with dihydrofolate reductase activity assays and for the helpful discussion on folate metabolism.

I would also like to thank my laboratory colleagues Tani Hsu, P. manivasakam, H. Ray, and M. Hamilton for their assistance and helpful discussion throughout this study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACP1	a gene encoding the general acid monophosphatase in *S. cerevisiae*
ADH2	a gene encoding the glucose-repressed isozyme of alcohol dehydrogenase in *S. cerevisiae*
ADH4	a gene encoding an isozyme of alcohol dehydrogenase in *S. cerevisiae*
BFB	breakage-fusion-bridge
C1	one-carbon
CAD	a gene encoding a multifunctional protein containing the three following activities: carbamyl-phosphate synthetase, aspartate transcarbamylase, and dihydroorotase.
CHEF	contour-clamped homogeneous electric field electrophoresis
CHO	Chinese hamster ovary
CUP1	a gene encoding a small, metallothionein-like, copper-binding protein
$CUP1^R$	a genetic locus conferring copper resistance in *S. cerevisiae*
DFR1	a gene encoding dihydrofolate reductase in *S. cerevisiae*
dfr1	a mutant allele at the *DFR1* locus
dfr1::URA3	a *dfr1* disruption mutant allele
DHF	dihydrofolate
DHFR	dihydrofolate reductase
dhfr	a gene encoding dihydrofolate reductase
DIR	a genetic locus conferring a DHFR-independent respiratory phenotype in *S. cerevisiae*
DMs	double minute chromosomes
dTMP	2'-deoxythymidine 5'-monophosphate
dUMP	2'-deoxyuridine 5'-monophosphate
E. coli	*Escherichia coli*
ECR	expanded chromosomal regions

EtdBr	ethidium bromide
FA	Folinic acid
FISH	fluorescent *in situ* hybridization
fol1	a mutation resulting a folate-deficient phenotype
fol2	a mutation resulting a folate-deficient phenotype
Folinic acid	5-formyl-tetrahydrofolate:calcium salt
Fou	Folinic acid utilization
FPGS	folylpolyglutamate synthetase
GGH	gamma-glutamyl hydrolases
HSR	homogeneously-staining regions
kb	kilobase(s) or kilobase-pairs
MC	Mortimer's synthetic complete medium
met7	a mutation resulting in auxotrophic for methionine and adenine
MTX	methotrexate
NADP	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
pABA	*p*-aminobenzonic acid
PALA	N-phosphonacetyl-L-aspartate
rDNA	ribosomal DNA
S. cerevisiae	*Saccharomyces cerevisiae*
SD	a yeast synthetic minimal medium
SG	a yeast nonfermentable synthetic medium
Tm	trimethoprim
tup	dTMP uptake mutation
TS	thymidylate synthase
THF	tetrahydrofolate
URA3	a gene encoding orotidine 5'-phosphate decarboxylase in *S. cerevisiae*

X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
YEPD	a yeast complex complete medium
YPG	a yeast non-fermentable complex medium

INTRODUCTION

The enzyme dihydrofolate reductase (DHFR) catalyzes the reduction of dihydrofolate to tetrahydrofolate. This reaction is generally considered to be the sole pathway that supplies intracellular tetrahydrofolate and is, therefore, essential for maintenance of sufficient intracellular reduced folate pools (Blakley, 1969; Blakley and Benkovic, 1984). Tetrahydrofolate functions as a carrier of one-carbon units. Tetrahydrofolate-mediated one-carbon metabolism is essential in many cellular processes, including purine and pyrimidine nucleotide biosynthesis, amino acid metabolism, and initiation of protein biosynthesis in prokaryotes and in eukaryotic mitochondria and chloroplasts (Blakley, 1969; Blakley and Benkovic, 1984). In addition, a reduced folate is required for light-dependent DNA repair (Sancar, 1990).

DHFR is clinically important in two respects. On the one hand, DHFR inhibitors such as methotrexate and trimethoprim have been used as chemotherapeutic agents in clinical treatment of a number of malignancies, notably leukemia, and certain bacterial and protozoan infections, respectively. On the other hand, overproduction of DHFR protein, often by *dhfr* gene amplification, leads to resistance to these chemotherapeutic agents and therefore limits their further use in treatment (Schimke, 1986). A great deal of effort has been made to attempt to explain the basis of the development of this phenomenon. It is hoped that an understanding of the mechanism(s) of resistance to DHFR inhibitors might provide some insights into strategies to prevent the resistance or to develop new therapies that selectively kill resistant cells.

Analysis of the effects of inhibition of DHFR activity by folate antagonists has shown that folate antagonism has profound genetic consequences such as chromosomal DNA breakage and enhanced genetic exchanges (e.g., see Section I.G. of "LITERATURE REVIEW"). It is conceivable that these genetic alterations and the drug-selected gene amplification may arise as different outcomes of the physiological effects of folate depletion on nucleotide precursor biosynthesis and DNA metabolism. It is therefore important to understand how the metabolic pathway of folate metabolism is normally involved in nucleotide and DNA synthesis and what aspects of defects in their metabolism lead to the genetic alternations.

It is now apparent that the phenomenon of gene amplification can be found throughout the phylogenetic tree and occurs during developmental and differentiation processes, in response to anti-proliferative drug treatment, and in the genesis and

progression of tumor cells. Amplification serves as a mechanism that allows a cell to produce a large amount of gene products in a period, in response to environmental change or developmental need. This genetic event has attracted a great deal of attention during the last few years. Despite the active research and rapid progress in this field, the mechanisms that control the process of gene amplification remain poorly understood. However, recent work (Stark et al., 1989; Hartwell, 1992) has promised the prospect that studies on gene amplification may shed important insights into genome architecture and mechanisms that control normal cell growth or maintain genome integrity.

Because the yeast *Saccharomyces cerevisiae* is readily amenable to both genetic and molecular manipulations, this simple eukaryote should be an ideal model system for investigation of the role of DHFR activity in cell function and the mechanism of gene amplification. However, studies on these issues in yeast cells had been limited by the failure in isolation of a yeast mutant deficient in DHFR activity and demonstration of yeast *dhfr* gene amplification. Thus, the primary goals of this work were to isolate a DHFR-deficient mutant and to develop the yeast *dhfr* gene system for gene amplification studies. Reported in the following are the successful construction of a yeast DHFR-deficient (*dfr1*) mutant by gene replacement and the experimental evidence that demonstrates the amplification of the yeast *dhfr* gene (*DFR1*) in methotrexate-resistant mutants.

LITERATURE REVIEW

I. Folate metabolism and the function of dihydrofolate reductase

I.A. Biochemical pathways involved in folate metabolism

Folate metabolism, as outlined in Figure 1, consists of several metabolic pathways that include the *de novo* pathway for folate biosynthesis; reduction of folate to tetrahydrofolate; biogenesis and interconversion of substituted tetrahydrofolate derivatives; and the metabolic pathways that utilize the tetrahydrofolate cofactors (Blakley and Benkovic, 1984). Bacteria, fungi and plants have the *de novo* pathway for folate biosynthesis; while animals obtain their folate from dietary sources (Blakley and Benkovic, 1984).

I.B. Chemistry of tetrahydrofolate derivatives

Folate is found in many organisms and cell types (Cossins, 1984). The general structure of tetrahydrofolate is shown in Figure 2. It consists of three distinct moieties: (1) a pteridine ring (6-methylpterin); (2) *p*-aminobenzoic acid (pABA); (3) glutamic acid. In substituted tetrahydrofolate derivatives (see Table 1), the one-carbon units are carried either on the N-5 or on the N-10 position, or then form a bridge between N-5 and N-10. These one-carbon units are found at three levels of oxidation corresponding to formic acid, formaldehyde, and methanol. The intracellular substituted reduced folates are predominantly polyglutamyl derivatives (McGuire and Coward, 1984).

I.C. Tetrahydrofolate-mediated one-carbon metabolism is essential for several major cellular processes

Tetrahydrofolate is the carrier of one carbon units in one-carbon transfer reactions. Tetrahydrofolate-mediated one-carbon metabolism plays an important role in several major metabolic pathways, including purine and pyrimidine nucleotide biosynthesis, amino acid biosynthesis and interconversion, and initiation of protein biosynthesis in prokaryotes and in eukaryotic mitochondria and chloroplasts (Blakley and Benkovic, 1984). In addition, a reduced folate is required for light-dependent DNA repair (Sancar, 1990).

I.D. Biogenesis of substituted tetrahydrofolate derivatives

Tetrahydrofolate derivatives have various one-carbon unit substitutions and glutamyl conjugation (Figure 2 and Table 1). The substitutions can be generated either by the addition of one carbon units derived from donors such as serine, glycine,

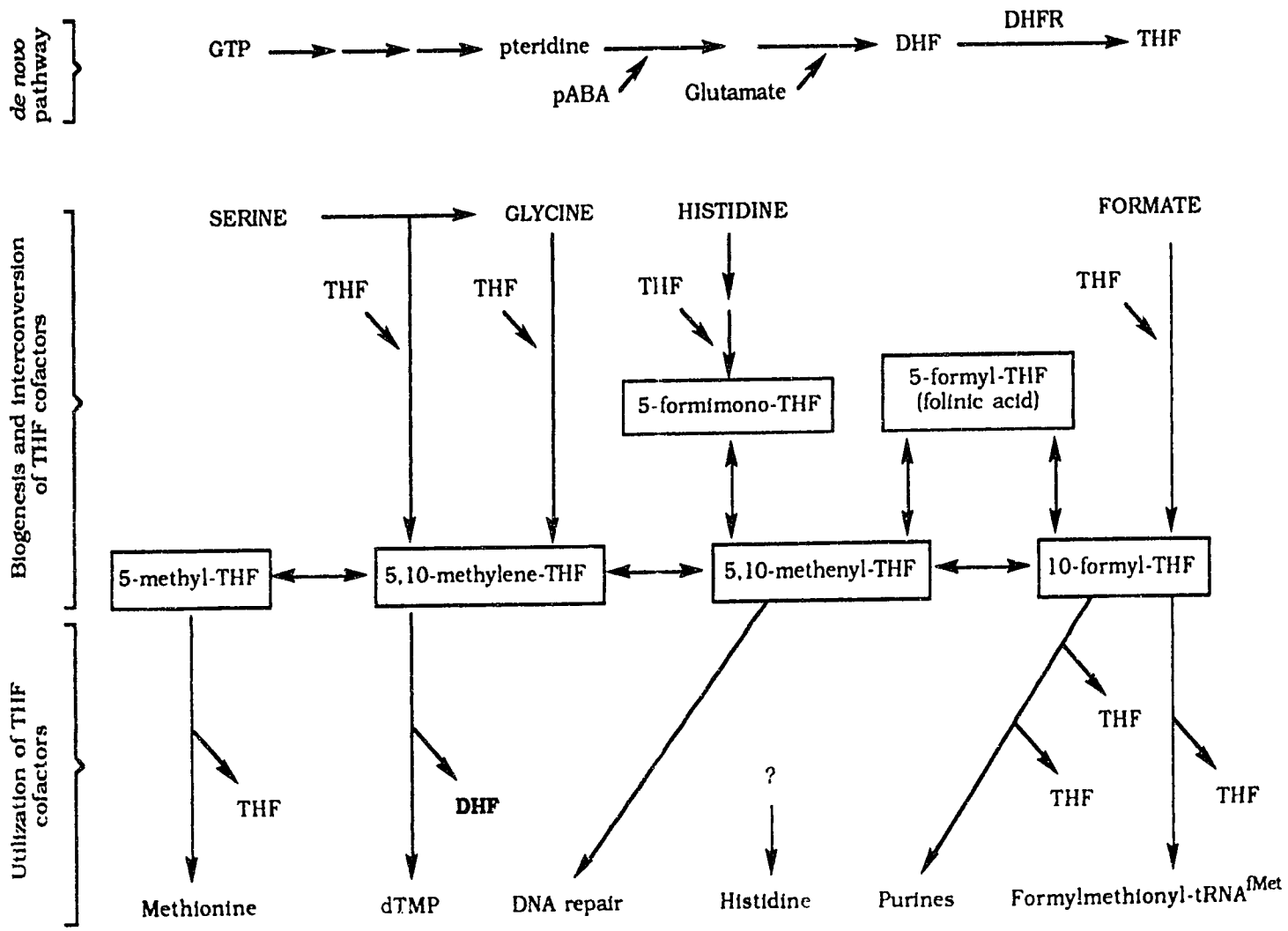


FIG. 1. An overview of biochemical pathways involved in folate metabolism

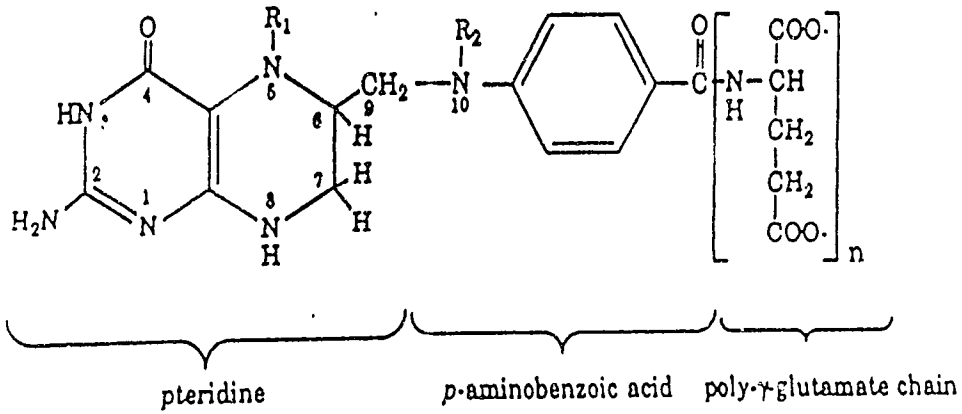


FIG. 2. Structure of tetrahydrofolate

Table 1. Substituted tetrahydrofolate derivatives

Name	Abbreviation	R1	R2
5,6,7,8-tetrahydrofolate	5,6,7,8-THF	H	H
5-methyl-tetrahydrofolate	5-CH_3-THF	CH_3	H
5-formyl-tetrahydrofolate	5-CHO-THF	CHO	H
10-formyl-tetrahydrofolate	10-CH_3-THF	H	CHO
5,10-methylene-tetrahydrofolate	5,10-CH_2-THF	-CH_2-	
5,10-methenyl-tetrahydrofolate	5,10-CH=THF	-CH=	

histidine and formate to carrier tetrahydrofolate, or by interconversion between substituted tetrahydrofolates. Some major reactions involved in these pathways are shown in Figure 1. The intracellular substituted tetrahydrofolate coenzymes are predominantly folate polyglutamates containing 3 to 7 glutamic acid residues (see Figure 2; reviewed in McGuire and Coward, 1984). Two enzyme activities vary the number of the glutamic acid residues. The formation of folate polyglutamates is catalyzed by folylpolyglutamate synthetase (FPGS), while the degradation of the polyglutamate tail of folates is catalyzed by gamma-glutamyl hydrolases (GGH). The essential role of folate polyglutamation is not fully understood (see McGuire and Coward, 1984 for a review). Polyglutamation appears to contribute significantly to the intracellular retention of folate cofactors. CHO mutants deficient in folylpolyglutamate synthetase are auxotrophic for the C1 metabolic end products: glycine, adenine and thymidine (McBurney and Whitmore, 1974a; 1974b; Taylor and Hanna, 1977). This auxotrophic phenotype is similar to that of CHO DHFR-deficient mutant (Urlaub and Chasin, 1980). In addition, most folate-requiring enzymes have higher affinity with their polyglutamated cofactors than the corresponding monoglutamates. It has been suggested that such different affinity of some key enzymes to their differentially polyglutamated cofactors or substrates may have an important role in regulation of tetrahydrofolate-mediated one-carbon metabolism (McGuire and Coward, 1984; Shane, 1989).

I.E. <u>DHFR activity is essential for biogenesis of tetrahydrofolate</u>

Cellular tetrahydrofolate is generated in a reaction catalyzed by dihydrofolate reductase (DHFR; E.C. 1.5.1.3), which is found in a variety of organisms ranging from bacteria to mammals, and is usually found in large amounts in rapidly dividing cells (Blakley, 1969; Blakley and Benkovic, 1984). The DHFR-catalyzed reduction of dihydrofolate to tetrahydrofolate is generally considered to be the sole pathway for biogenesis of intracellular tetrahydrofolate. The importance of dihydrofolate reductase activity in the maintenance of sufficient intracellular tetrahydrofolates is best appreciated in replicating cells which have an absolute requirement for dTMP for DNA synthesis. In most of tetrahydrofolate-mediated one-carbon transfer reactions, except the methylation of dUMP to dTMP, unsubstituted tetrahydrofolate is released. In the latter reaction, catalyzed by thymidylate synthase, the coenzyme 5,10-methylene tetrahydrofolate donates both an active one-carbon (C1) moiety at the level of formaldehyde and two hydrogens from the pteridine ring for the reduction of the transferred C1 moiety to a methyl group. As a consequence, dihydrofolate is the by-

product of thymidylate synthesis. Thus, thymidylate synthesis imposes on a proliferating cell population a requirement for regeneration of tetrahydrofolate from dihydrofolate. The DHFR-catalyzed reaction is the regeneration mechanism which reduces dihydrofolate as rapidly as it is produced by thymidylate synthase and resupplies tetrahydrofolate for dTMP synthesis, thus avoiding depletion of intracellular tetrahydrofolate derivatives needed for other biosynthetic reactions.

Sufficient DHFR activity that avoids depletion of intracellular tetrahydrofolate pools is essential in all cell systems. However, this is especially critical in yeast cells, since, in common with most fungi, yeast cells lack thymidine kinase and thus possess no salvage pathway for the anabolism of thymidylate from thymine or thymidine (Grivell and Jackson, 1968). Therefore intracellular dTMP is derived solely from dUMP in the folate-dependent *de novo* biosynthetic pathway.

I.F. Compartmentation of folate metabolism

The basic pathways involved in folate metabolism have been elucidated (Blakley, 1969; Blakley and Benkovic, 1984). Intracellular folate metabolism is highly compartmentalized within intracellular organelles, such as between the cytoplasm and mitochondria (see Appling, 1991 for a review). The realization that folate metabolism is compartmentalized has raised many interesting questions. For example, are the three distinct components: the tetrahydrofolate coenzymes, the one-carbon donors and the enzymes involved, all compartmentalized? How does a cell balance its folate and one-carbon unit requirements in the cytoplasm and mitochondria and how do these pools respond to changing metabolic states? What mechanisms are involved in the transport of folate coenzymes across organelle membranes? How are all of these processes regulated and what role does the polyglutamate tail play in these processes? Understanding these questions and many others will necessarily enrich our knowledge about folate metabolism and allow the improvement of strategies for antifolate drug therapy.

I.G. Genetic consequences of DHFR deficiency induced by antifolate drugs

DHFR inhibitors have now assumed major importance as chemotherapeutic agents in the treatment of a wide range of human diseases from cancer to certain bacterial and protozoic infections. Examples of these widely-used antifolate drugs include trimethoprim (antibacterial), methotrexate (anticancer), and pyrimethamine (antimalarial). The action of these drugs is intimately related to their effects on depletion of intracellular tetrahydrofolate cofactors, as these drugs are potent

inhibitors of the DHFR enzyme and therefore block the reduction of dihydrofolate to tetrahydrofolate. The effects of depletion of reduced folate pools by enzymatic inhibition of DHFR activity on cellular metabolism and cell growth have been the subject of extensive studies. It is believed that a detailed understanding of the basis of such effects is essential for development of rational approaches to the design of better chemotherapeutic agents or drug regimens with improved therapeutic activity.

Effective folate deprivation in *S. cerevisiae* requires the synergistic inhibition of DHFR-catalyzed reduction of dihydrofolate to tetrahydrofolate and the *de novo* pathway of folate biosynthesis (see Figure 3). This is evidenced by the observation that complete growth inhibition of *S. cerevisiae* requires the combined action of a DHFR inhibitor such as methotrexate or aminopterin and a drug such as sulfanilamide that inhibits the dihydropteroate synthetase-catalyzed condensation of pteridine and pABA to dihydropterate in the *de novo* pathway of folate biosynthesis (Barclay and Little, 1977; Little and Haynes, 1979).

In *S. cerevisiae*, antifolate drug-induced folate depletion results in nutritional requirements for dTMP, adenine, histidine and methionine (Barclay and Little, 1977; Little and Haynes 1979). These four metabolites can be substituted with exogenous folinic acid, which is subsequently converted into other tetrahydrofolate derivatives that are in turn available for the tetrahydrofolate-dependent biochemical reactions.

In yeast, starvation for these folate-dependent metabolites in antifolate-treated cells has profound effects on cell viability and the genetic integrity of both the nuclear and mitochondrial genomes. For example, thymidylate deprivation in yeast causes a rapid loss of cell viability known as "thymineless death" (Barclay and Little, 1977; Little and Haynes 1979). In the surviving cells, the physiological conditions generated by thymidylate starvation greatly enhance various forms of mitotic recombination (Kunz et al., 1980; Barclay et al., 1982; Kunz et al., 1984; Kunz et al., 1986). It is believed that these genetic consequences provoked by thymidylate starvation must involve aberrations in DNA metabolism. It has been suggested that DNA strand breaks play an important role in the development of these genetic defects (Barclay and Little, 1978; Barclay et al., 1982). However, the precise chain of molecular events leading to these effects has yet to be worked out in detail. It is now generally believed that a balanced thymidylate nucleotide pool is important for normal cell function (MacPhee, et al., 1988). However, little is known about how nucleotide pools are regulated and how folate and nucleotide metabolism interact with one another to retain balanced nucleotide pools.

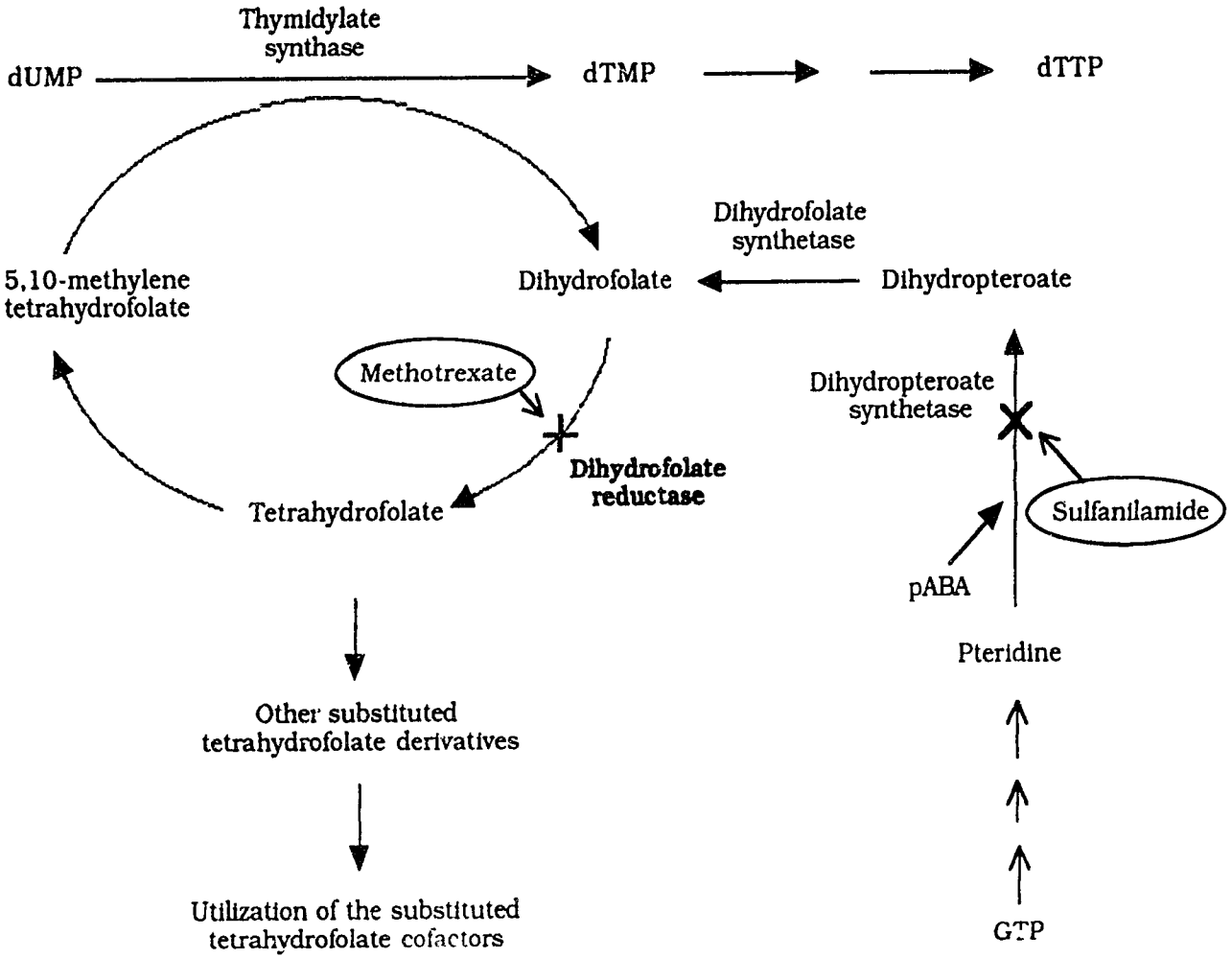


FIG. 3. Inhibition of tetrahydrofolate biosynthesis

One other well-documented genetic alteration observed in mammalian cells when DHFR activity is inhibited by methotrexate is the development of resistance to this antifolate drug by amplification of the *dhfr* gene (Alt et al., 1978). However, in spite of numerous studies, our current knowledge about the genetic control and molecular mechanism of gene amplification are still very limited. It is likely that the antifolate drug-induced physiological conditions that produce the genetic effects described above may also be associated with the development of gene amplification that in turn results in resistance to the inhibitory effects of antifolate drugs. It is therefore important to know about the specific biochemical reactions that lead to the genetic effects.

The most visible genetic effects of antifolate drug treatment in *S. cerevisiae* is the induction of cytoplasmic petites at very high frequency (Wintersberger and Hirsch, 1973a; 1973b; Little and Haynes, 1979). Two mechanisms have been proposed to explain this inhibitory effect of antifolate drugs on the mitochondrial respiration in this organism. Based on their studies on the induction effects of thymidylate starvation, Barclay and Little (1978) have suggested that respiratory petites may result from damage on the mitochondrial genomes caused by limitation of thymidylate precursor for DNA replication that in turn is the result of folate depletion. On the other hand, Wintersberger and Hirsch (1973a; 1973b) have suggested that inhibition of mitochondrial protein biosynthesis as a result of folate deficiency may contribute to the defects in respiration.

I.H. The phenotype of mutants deficient in DHFR activity

Mutants deficient in DHFR activity have been isolated in *E. coli* (Singer et al., 1985; Howell et. al.,1988; Ahrweiler and Frieden, 1988; Hamm-Alvarez et al., 1990) and in mammalian cells (Urlaub and Chasin, 1980; Urlaub et al., 1983). Interesting observations have been obtained by characterization of the mutant phenotypes. For example, the survival of an *E. coli* mutant deficient in DHFR activity requires that there is no thymidylate synthase activity within the mutant cells (Howell et. al.,1988; Ahrweiler and Frieden, 1988). Thymidine is the sole auxotrophic requirement for the *E. coli* mutants (Hamm-Alvarez et al., 1990). A novel pathway for tetrahydrofolate biogenesis in *E. coli* cells has been revealed by the observation that reduced folates can be found in the *E. coli* mutants (Hamm-Alvarez et al., 1990). The unexpected observations explain why the *E. coli* mutants require only thymine nucleotide but not other end products of folate-dependent C1 metabolism for growth. They probably also explain why DHFR deficiency is not lethal to *E. coli* cells since a reduced folate is required for the initiation of protein synthesis in this organism.

Interesting questions are also raised from characterization of the phenotype of mutants deficient in DHFR activity in mammalian cell system. Respiratory activity is generally considered to be essential for mammalian cell growth and a reduced folate is required for the initiation of protein synthesis in mitochondria. Thus, why is a mammalian DHFR-deficient mutant viable? In mammalian systems, the growth inhibitory effect of methotrexate can be overcome by provision of the end products of folate-dependent C1 metabolism or of folinic acid. However, the growth requirement of a mammalian DHFR-deficient mutant can only be satisfied by the C1 end products, but not by exogenous folinic acid (Dr. L. A. Chasin, personal communication). It is clear that many valuable insights into the role of DHFR activity in cell growth and cellular metabolism can be gained by characterization of mutants deficient in DHFR activity.

In spite of previous attempts (e.g. see Little and Haynes, 1979), a *S. cerevisiae* mutant deficient in DHFR activity had not been described prior to this study. Lack of a DHFR-deficient mutant had hindered the comprehensive studies on the role of DHFR activity in cellular metabolism and several concerns had been raised. For example, might DHFR deficiency be lethal to yeast cells? Perhaps a second mutation in the thymidylate synthase gene is required for viability as is the case in *E. coli* (Howell et. al.,1988; Ahrweiler and Frieden, 1988; Hamm-Alvarez et al., 1990). If viable, would a yeast mutant deficient in DHFR activity have a phenotype that mimics the effect of antifolate drugs on wild-type cells? In addition, as DHFR activity is detected both in the mitochondria and in the cytoplasm (Zelikson and Luzzati, 1977), does a single genetic locus encode the two enzymatic activities, or are there two different genes, one coding for the mitochondrial form, the other one coding for the cytoplasmic form? In order to address these questions and many others, part of my research project was to attempt to isolate a DHFR-deficient mutant of *S. cerevisiae*. It was hoped that by characterization of the phenotype of a yeast DHFR-deficient mutant, more insights into various aspects of the cellular functions associated with DHFR activity could be gained.

II. Mechanisms of gene amplification

II.A. Gene amplification and its biological significance

Gene or DNA amplification is described as an increase in the copy number of a gene or DNA sequence within a cell, involving less DNA than the amount contained in a whole chromosome. The phenomenon of gene amplification is observed throughout the phylogenetic tree including bacteria, plants, insects, and humans (see Anderson and Roth, 1977; Stark and Wahl, 1984 for reviews). Gene amplification plays an important

role in biological systems. Amplification may be either a normal developmentally programmed process or an abnormal process. Examples of programmed gene amplification are known in both unicellular and multicellular eukaryotic organisms (e.g., rDNA amplification in ciliates and amphibian oocytes, and chorion gene amplification in *Drosophila*; also see Stark and Wahl, 1984 for a review). Such programmed gene amplification serves as a mechanism for modulating the amount of a specific gene product such as a protein or a RNA molecule during the developmental process. Unprogrammed amplification is often observed in cells resistant to toxic agents or drugs (see Hamlin et al., 1984; Schimke, 1984a; 1984b; Stark and Wahl, 1984 for reviews). The classical example of unprogrammed gene amplification is amplification of the dihydrofolate reductase gene (Alt et al., 1978). This is the major cause of resistance to the anti-cancer drug methotrexate in mammalian cells (Schimke, 1984a). Cellular oncogenes are also found to be amplified in many different cancers, especially in highly malignant tumors, which suggests a causative role for the overexpressed c-Onc protein in tumor progression and possibly also in tumorigenesis (see Alitalo and Schwab, 1986; Stark, 1986 for reviews). Gene amplification is also proposed as a mechanism commonly employed during evolution (Maeda and Smithies, 1989). Amplification of genes not only results in abundant quantitative changes, but also new substrates for evolution because extra amplified copies can receive point mutations and evolve new function while leaving the original copies unchanged.

Because of the important implication in both clinical consideration and basic research, a great deal of research has been carried out on the mechanisms underlying gene amplification. This literature review focuses mainly on drug-selected gene amplification.

II.B. Cytogenetic and molecular characteristics of amplicons

One of the important experimental approaches used in dissecting the mechanism of drug-selected gene amplification has been the structural analysis of the amplified DNA or of chromosomes contain the amplified gene. Because cultured cell lines containing amplified drug resistance genes can be isolated relatively easily by using a variety of antiproliferative agents, e.g. DHFR gene amplification in MTX-resistant cell lines and CAD gene amplification in PALA-resistant cell lines, they have been the model systems for gene amplification studies. Observations from many studies have shown that gene amplification is a dynamic process in which the structures generated early are altered at the cytogenetic and molecular levels during cell propagation or drug selection.

In high-level resistant cell lines that were isolated by multi-step drug selection with progressively increasing drug concentrations, there are tens to hundreds of copies of the amplified gene. The presence of the high copy number of the amplified gene in the resistant cells has greatly facilitated the cytogenetic and molecular analyses of the structure of the amplified sequences and the chromosomes that contain the amplified gene. Cytogenetic analyses revealed that two types of abnormal chromosomal structures harbor amplified sequences in resistant cells selected by multi-round drug treatment (see Cowell, 1982; Stark and Wahl, 1984; Stark, 1986 for reviews). The first type consists of paired, acentric chromatin bodies, usually referred to as double minute chromosomes (DMs). The second type is the chromosomal sites of gene amplification that are described either as homogeneously-staining regions (HSR) or expanded chromosomal regions (ECR). Homogeneously-staining regions were so named because they fail to exhibit trypsin-Giemsa bands. The term expanded chromosomal regions (ECR) was used for a more general designation for the chromosomal sites of gene amplification because some chromosomally amplified regions do exhibit trypsin-Giemsa bands. In almost every case, drug-resistant cells that lack DMs have ECR at a single chromosomal site. Multiple chromosomal sites within a single cell line have been seen, but they are rare, even in lines containing many copies of an amplified gene. DMs and ECR do not usually co-exist within the same cell although either chromosomal or extrachromosomal amplification of the same gene can occurs in a single population of cells.

In addition to the cytogenetic location, the molecular nature of high-level amplification events has been characterized at the molecular level in some systems (see Stark et al., 1989; Schimke, 1988; Stark, 1986; Hamlin et al., 1984 for reviews). There is reasonable knowledge about the size (small vs large; unique size vs heterogeneous size), organization (circular vs linear; direct repeats vs inverted repeats; homogeneous repeat vs mixed repeats), and stability (stable vs unstable; with vs without selection pressure) of the units of amplification (amplicons). They appear to be extremely variable in length within and among cell lines that are resistant to the same drug and there do not appear to be preferred endpoints (hotspots) for the joining of amplified sequences (e.g. see Ardeshir et al., 1983; Federspiel et al., 1984). Amplicons are invariably much larger than the gene whose product is selected. Expanded chromosomal regions are now known to be composed of amplicons arranged either as direct tandem repeats (e.g. see Amler and Schwab, 1989) or as a mixture of tandem and inverted repeats (e.g. see Ma et al., 1988; Hyrien et al., 1988; Ford and Fried, 1986). In the absence of drug-selective pressure, extrachromosomally amplified elements are

quickly lost; intrachromosomally amplified regions are typically very stable (e.g., see Chasin et al., 1982), although gene loss from such arrays over many generations of non-selective growth has been reported (e.g. see Biedler, 1980).

To gain insight into the events that generate the first amplicon, it is crucial to identify the first amplicon or the intermediates that are as close as possible to the first event because the products presented in high-level resistant cell lines may not resemble the initial products. Therefore, amplification events after a single step selection at low drug concentration have also been characterized in several systems (Trask and Hamlin, 1989; Smith et al., 1990; Ruiz and Wahl, 1988; 1990; Windle et al., 1991). These studies have demonstrated that gene amplification can be mediated by submicroscopic, autonomously replicating, circular extrachromosomal molecules, which are referred to as episomes (Carroll et al., 1987), or amplisomes (Pauletti et al., 1990). Imperfectly inverted repeats are often found in these episomes (Carroll et al., 1988). With the use of new technologies such as fluorescent *in situ* hybridization (FISH), some recent studies have analyzed the amplification events within about 10 to 20 cell generations after the initial event (Trask and Hamlin, 1989; Smith et al., 1990; Windle et al., 1991; Toledo et al., 1992a; 1992b). The results of these studies have shown that both small and large amplicons can be produced at a very early time from the same locus under a single set of selection conditions (Giulotto et al., 1986). They can be present either as arrays within chromosomes or as acentric chromosome fragments (i.e., circular or linear extrachromosomal elements). Formation of extremely large inverted repeats at the very early stage of the amplification event has been observed (Toledo et al., 1992a; 1992b). The earliest amplification intermediates are genetically very unstable (Toledo et al., 1992a; 1992b).

Different products observed in the early and later stages of gene amplification have led to the question about their interrelationship and the chronology of the amplicon evolution during gene amplification. Recent studies have demonstrated that DMs can either be derived from submicroscopic episomes (Carroll et al., 1988), or generated by *de novo* chromosome fragmentation (e.g., see Hahn et al., 1987, 1990; Sen et al., 1989). The episomes or DMs can integrate into chromosomes to generate ECR (Carroll et al., 1988). In some cases, episomes have been shown to result from deletion of the corresponding target sequences from the chromosome (Carroll et al., 1988; Hunt et al., 1990; Ruiz and Wahl, 1990; Windle et al., 1991). In contrast, other studies demonstrate that formation of large chromosomal amplicons may be involved in the initiation of some amplification events (Giulotto et al., 1986; Gudkov et al., 1987; Saito

et al., 1989). These unstable large amplicons may then be trimmed into smaller units during the course of amplification to high copy number without generation of episomes.

In summary, further studies are needed particularly for the identification of the first products, the relationship among the different structures of the amplification products, and the precise molecular chronology of gene amplification.

II.C. The molecular mechanisms of gene amplification

The molecular mechanism(s) that lead to an increase in the copy number of a gene or DNA sequence within a cell has been a subject of intense research and speculation for many years. Generally, the primary event involved in the first step of amplification is thought to be different from the later evolution of an already amplified array. The primary event in gene amplification takes place in a single cell. Such a primary event generates extra copies of the target gene, perhaps in a acentric extrachromosomal form that will segregate unequally in subsequent cell divisions. In such a case, secondary events may then follow during growth of the initial cell to a cloned population. If the amplified DNA is intrachromosomal, secondary event(s), again taking place in a single cell, may be required to generate the additional copies of the target gene needed for survival in the selective growth conditions. Thus, identification of the first product in amplification is essential for assessment of proposed mechanisms for the primary events. Unfortunately, it is difficult to obtain information about the first event because it takes place in a single cell and therefore has not been amenable to direct study so far. In order to obtain sufficient materials for detailed structural analysis, the initial mutant clone is normally grown for far longer time periods. During the subsequent growth, a number of secondary processes may occur, which may then be manifested as the diverse structures of both low-level amplified products at the early stage and highly-amplified products of the late stage.

Thus, it is essential for each proposed molecular mechanism to account for the diverse observations and the following aspects should then be considered. If the earliest products that can be detected by current technologies represent the first events, the following questions are then raised for the formation of the first amplicons. How can the initial amplicons be either small or large? How can the first amplicon be produced as an episome or within a chromosome? How does the formation of initial episomes accompany deletion of the target site? How can these first amplicons be generated as either direct repeat or inverted repeat? Why are the initial amplicons genetically unstable? How do the initial amplicons become high copy amplified structures? How are large amounts of flanking passenger sequence retained during

amplification to high copy number? If large amplicons are precursors of small ones, how are the large amplicons trimmed into smaller ones? How are small episomes transformed into DMs? As both extrachromosomal and chromosomal amplicons are found in the early stage of gene amplification, how can extrachromosomal amplicons be converted into intrachromosomally amplified regions, and vice versa? If the episomes are integrated into chromosomes to generate chromosomal amplicons, how could this occur? How are novel joints formed during amplification? Why is it that some novel joints are present at about the same copy number as the target gene and some amplified much less? As differently-sized amplicons are not likely to be defined by specific endpoints, how are the different sequence lengths coamplified in both primary and subsequent amplicons? How is the amplified DNA translocated into a new chromosome? Generally, why are the chromosomally amplified sequences stable; while extrachromosomally amplified sequences unstable during the growth under nonselective conditions? Why is the frequency of amplification enhanced by agents that either block DNA replication, damage DNA, or stimulate cell growth?

The diverse characteristics of amplified DNA raise an interesting question about whether there is a single mechanism or more than one mechanisms underlying the process of gene amplification. It is likely that the various forms of amplified DNA may be generated by different mechanisms, or that a single basic mechanism may lead to different outcome. Several models for the molecular mechanisms of gene amplification have been proposed (reviewed by Stark et al., 1989; Wahl, 1989; Windle and Wahl, 1992). The rereplication model (also known as disproportionate replication or onion skin replication model) proposed that multiple initiations of DNA synthesis from a specific DNA replication origins within a single cell cycle would generate a complex structure consisting of replication bubbles within bubbles. Resolution of such a structure by various recombination events would be flexible enough to generate diverse structures containing amplified DNA (Schimke, 1988). The concept that re-replication occurs within a single cell cycle under certain growth conditions such as transient depletion of DNA precursors has been challenged by experimental evidence (see Windle and Wahl, 1992 for more discussion). The unequal exchange model proposes that homologous or nonhomologouse recombinations between two misaligned chromosomes or chromatids would generate direct repeats on a chromosome. This model predicts that additional rounds of the similar type of recombination event occur in subsequent cell cycle in order to generate multple copies of a sequence and no inverted repeat could be produced (see Stark et al., 1989 for more discussion). The episome excision model was proposed to explain the observation that formation of

episomes that contain amplified gene is often associated with deletions of the target sequences in a chromosome (see Windle and Wahl, 1992 for more discussion). The double rolling circle model was proposed to account for how inverted repeats detected in many amplification intermediates are formed (Passananti et al., 1987; Hyrien et al., 1988). The breakage-fusion-bridge (BFB) model was proposed to explain the cytogenetic observations on the chromosomal structures which were thought to be the intermediates at the early stage of a gene amplification event (see Toledo et al., 1992b). The chromosome breakage model was proposed in attempting to account for the diverse amplified DNA structures observed so far in mammalian systems (Windle et al., 1991; Windle and Wahl, 1992). Further experimental evidence is needed to verify or modify the features of the models mentioned above.

II.D. Mechanisms that control the process of gene amplification

Several experimental approaches have been used in elucidation of the mechanism(s) that control gene amplification. One of these approaches is to analyze agents that modify the levels of gene amplification and by analysis of the mode of the action of these agents a conclusion is reached about the underlying process. In mammalian system, amplification frequency can be increased substantially by treating cells with agents such as UV light, hydroxyurea, or hypoxia (see Stark and Wahl, 1984; Schimke, 1988 for reviews). DNA damage and disturbance of nucleotide metabolism are the common effects of these agents. Thus, inhibition of DNA synthesis may induce the potentiation of the process leading to gene amplification. It is conceivable that the physiological conditions induced by DNA damage and/or disturbance of nucleotide metabolism facilitate the occurrence of amplification events. However, the specific signal(s) and the precise biochemical reactions responsible for this process are still not known. On the other hand, inhibition of DNA synthesis may produce substrates for gene amplification. Stalled replication forks or abortive replication bubbles, single strand nicks and double strand breaks, free ends of broken chromosomes, all of which can be resulted from inhibition of DNA synthesis, have been proposed to play important roles in the process of gene amplification (Windle and Wahl, 1992).

The second approach is to study the effects of chromosomal structure and specific sequence elements on gene amplification. One of the interesting questions to ask is whether there is any hot spot for gene amplification in the genome. Wahl et al (1984) analyzed the effect of various chromosomal locations on gene amplification and has found that there are up to 100-fold differences in the frequency of amplification

among different positions. The observation indicates that chromosomal structures may play a role in the development of gene amplification. The DNA breakage and joining reaction which form amplicons generate unique sequence combinations that are known as novel joints. Novel joints can be formed in the initial step of amplification, in which case they may directly reflect the molecular mechanisms involv l, or they can be generated in subsequent steps by incidental reactions not directly related to the amplification process. The information from analysis of novel joints has been thought to be informative about whether there are particular sequences involved in gene amplification and whether they are equivalent to recombination hot spot or to the termini of replicons. Analysis of the novel joints in mammalian systems has been reviewed (Stark et al., 1989). Many novel joints have been found in an inverted asymmetric palindromic structure. Analysis of jointed sequence has indicated that there is no absolute requirement for a particular type of sequence, although AT-rich elements seem to be involved frequently. The observations that amplified regions are not comprised of identical units and different sequences can be co-amplified with the selected gene in different independent events also suggested that novel joints are not likely to be defined by specific endpoints. The only specific sequence known to be involved in amplification is replication origins (Lavi, S. 1981; Carroll et al., 1987). Distinct initiation sites have also been found in the chromosomal amplicons (e.g. see Leu and Hamlin, 1989; Anachkova and Hamlin, 1989). The interspersed organization of early and late replication origins in the mammalian genome has also been suggested to have implications in the process of gene amplification (Windle and Wahl, 1992). It has been suggested that the rate of the progression from extrachromosomal to chromosomal amplicons is possibly dependent on the characteristics of the amplicon produced or the structure generated at the target site following the initiating events (Windle and Wahl, 1992).

The third approach is to identify the cellular process involved in gene amplification. In established mammalian cell lines, amplification arises spontaneously typically at the rates of 10^{-4} - 10^{-6} events/cell/cell generation at a single locus (e.g., see Johnston et al., 1983; Tlsty et al., 1989). However, amplification has been shown to be extremely rare in primary normal cell lines (Wright et al., 1990; Tlsty, 1990). Thus, established cell lines may carry genetic alterations and therefore changed physiological conditions that contribute to the relatively higher frequency of gene amplification. It is now reasonable to think that both replication and recombination machineries must be involved in the process of amplification. Does amplification result from differential expression of replication and recombination proteins under

conditions which induce stress? Two recent studies (Livingstone et al 1992; Yin et al., 1992) for the first time demonstrate that a cell cycle control protein plays an important role for the development of gene amplification. Cells with constitutively high frequencies of gene amplification have been isolated (Giulotto et al., 1987; 1991). These cells are very sensitive to UV radiation and mitomycin C, indicating that the DNA repair capacity of these cells is also alternated (Giulotto et al., 1987; 1991).

II.E. The significance of studies on gene amplification

Studies on gene amplification are of great significance to cancer treatment because gene amplification is implicated both in the development of resistance to anti-cancer drugs and in the development of cancer (Alitalo, 1984; Alitalo and Schwab, 1986; Schimke, 1984a; 1986; Stark, 1986). It is believed that understanding the mechanism of gene amplification would eventually lead to improved chemotherapeutic strategy. It is also important to understand how over-accumulation of a particular gene product, derived from amplified genes, contribute to particular phenomena during the development of cancer such as metastasis.

Gene amplification is a major genetic alteration within a cell. It seems likely that unprogrammed amplification is but one manifestation of more fundamental defects that lead to chromosomal abnormalities such as deletion, inversion, translocation, and chromosome loss. It is thus important to know about the mechanisms governing amplification events and to discover which cellular functions are responsible for gene amplification. Such studies may identify genes, the abnormal expression of which is responsible for underlying defects in the regulation of chromosomal integrity. Therefore, the phenomenon of gene amplification provides an excellent system for investigation of the mechanisms that control cell growth and genetic integrity (Stark et al., 1989; Hartwell, 1992).

One of the consequences of studies on gene amplification has been the utilization of amplification as a high-level expression system for overproduction of a specific gene product (Kaufman et al., 1985; Kaufman et al., 1986; Bebbington and Hentschel, 1987). Not only the genes selected by a drug but also flanking sequences are co-amplified. Thus, by linking a gene whose product is wanted in large quantity to one whose amplification can be selected with a drug, it is possible to amplify both genes and express both gene products at a high level. In addition, coamplification has been used as a system to increase the copy numbers of a regulatory element in studies on the mode of regulation of the passenger gene (Johnston and Kucey, 1988).

II.F. Studies on gene amplification in *S. cerevisiae*

The baker's yeast *S. cerevisiae* is readily amenable to both genetic and molecular manipulations and has been an excellent model system for a variety of studies in eukaryotic cells. However, studies on gene amplification in this model system have been very limited so far. The main reason for this limitation is that very few examples of gene amplification have been described in the nuclear genomes of this organism. In addition, all of the cases reported so far have not been particularly suitable for thorough studies on the mechanism of gene amplification because of the specific situations associated with the amplification events.

There are two cases that multiple copies of a gene or DNA sequence are located at a genetic locus in the genomes of the strains of *S. cerevisiae*. Up to 15 copies of the *CUP1* gene have been found at the $CUP1^R$ locus that is located on chromosome VIII and produces a copper-resistant phenotype (Fogel and Welch, 1982; Fogel et al., 1983; Welch et al., 1983). The repeated units have a size of about 2.0 kb and are organized as a tandemly repeated array (Fogel and Welch, 1982). Strains that contain a single copy of the *CUP1* gene are copper-sensitive. Efforts to amplify the *CUP1* gene from a single copy to more than one in such strains have not been successful (e.g. see Aladjem et al., 1988). Unequal sister chromatid exchange has been proposed as the mechanism that changes the *CUP1* gene copy number at various $CUP1^R$ alleles in strains with different copper-resistant levels (Fogel and Welch, 1982; Fogel et al., 1983). The *rDNA* locus is the other example in which multiple copies of a DNA fragment are present in the *S. cerevisiae* genome. This genetic locus is located in the chromosome XII and consists of approximately 200 copies of a 9 kb repeat per haploid genome (Schweizer et al., 1969; Cramer et al., 1976; Petes, 1979). Recombination events such as unequal sister-strand exchange and gene conversion have been demonstrated within this locus and they have been proposed as the mechanisms for maintenance of the copy number and sequence homogeneity of the rDNA repeats (Szostak and Wu, 1980; Petes, 1980; Klein and Petes, 1981).

Two other examples of gene amplification are found in the *ADH2* and *ADH4* genes in antimycin A-resistant mutants (Walton et al., 1986; Paquin et al., 1992). In the case of *ADH2* gene amplification, a DNA fragment containing the *ADH2* gene is found to be amplified within the *rDNA* locus, but not in the native *ADH2* locus (Paquin et al., 1992). Apparently, increases in the *ADH2* gene copy number is mediated by recombination events between the rDNA sequences, which follows the insertion of the *ADH2* gene into the rDNA locus (Paquin et al., 1992). Since the native *ADH2* is

conserved, the primary event, insertion the *ADH2* gene into the rDNA locus is most likely accounted for by a conservative transpositional event. Such an event has been shown to be extremely rare; the rate for such an event to occur has been estimated less than 5×10^{-12} events/cell/generation (Paquin et al., 1992). In the second example, the amplified *ADH4* gene is found in multiple copies of a extrachromosomal linear palindromic molecule which has telomeres at its ends (Walton et al., 1986). The *ADH4* locus is located at the extreme left arm of the chromosome VII. The amplified *ADH4* molecules are thought to result from a fusion of the two copies of the terminal 21-kb sequence of this chromosome (Walton et al., 1986; Dorsey et al., 1992). A chromosomal *ADH4* amplification event has been observed (Dorsey et al., 1992). However, the molecular nature of these intrachromosomal amplicons has not been characterized yet. Again, these *ADH4* amplification events are very rare with a rate of about 10^{-10} events/cell/generation (Walton et al., 1986).

Two examples of transpositional gene duplication have been demonstrated by genetic analysis. Duplication of the *ACP1* gene that codes for the acid phosphatase (aphtase) is found in mutants that were selected for *in vivo* hyper-aphtase activity (Hansche, 1975; Hansche and Beres, 1978). This type of duplication is rare at the rate between 10^{-11} and 10^{-12} (Hansche and Beres, 1978). Greer and Fink (1979) observed the duplication of the *his4C* gene that encodes the histidinol dehydrogenase in the histidine biosynthetic pathway. Again such duplication events occurs at the low frequency of less than 10^{-9} (Greer and Fink, 1979). In both cases, molecular evidence for the duplication events has yet to be obtained.

Because the yeast *S. cerevisiae* has the well-defined genetic system and the versatile molecular techniques are applicable to this simple eukaryotic organism, a suitable *de novo* amplification system has long been sought in *S. cerevisiae*. Thus, one of the primary goals of this research project is to develop the yeast *dhfr* gene system for gene amplification studies.

MATERIALS and METHODS

I. Growth, selection, and assay media

Standard media were used for routine growth of *S. cerevisiae* strains (Sherman et al., 1984). These media include: complex complete medium (**YEPD**): 1% Bacto-yeast extract, 2% Bacto-peptone, 2% dextrose; synthetic minimal medium (**SD**): 0.67% Bacto-yeast nitrogen base without amino acids, 2% dextrose; Mortimer's synthetic complete medium (**MC**): SD medium supplemented with 20 μg/ml each of adenine, uracil, arginine, histidine, lysine, methionine, and tryptophan, 30 μg of leucine and 350 μg of threonine; synthetic complete medium (**SC**): 20 μg/ml each of adenine, uracil, arginine, histidine, methionine, and tryptophan, 30 μg/ml each of isoleucine, leucine, lysine and tyrosine, 50 μg/ml of phenylalanine, 100 μg/ml each of glutamic acid and aspartic acid, 150 μg/ml of valine, 200 μg/ml of threonine and 400 μg/ml of serine; **omission** media: SC or MC medium without one or more of the base or amino acid supplements.

Non-fermentable carbon source media were used for assay of mitochondrial activity (Sherman et al., 1984). They are: **YPG** (1% Bacto-yeast extract, 2% Bacto-peptone, 3% glycerol); **SG** (0.67% Bacto-yeast nitrogen base without amino acids, 3% glycerol, with amino acid and base supplements same as for MC or SC).

Media used for induction of sporulation were **presporulation** medium (1% Bacto-yeast extract, 2% Bacto-peptone and 2% potassium acetate) and **sporulation** medium (F^+): 1% potassium acetate, 0.1% glucose, 0.25% yeast extract with supplements as for MC or SC.

Buffered **X-gal medium**: this medium was used for detection of β-galactosidase activity in yeast colonies. It is the synthetic selective medium which is buffered to pH 7 (Rose and Botstein, 1983). To prepare 1 liter of the medium, the salts (13.6g of KH_2PO_4, 2g of $(NH_4)_2SO_4$, 0.5 mg of $FeSO_4.7H_2O$) were prepared in 500 ml H_2O, while the agar and sugar were prepared in another solution. The two solutions were autoclaved separately. After cooling to 55°C, the two solutions were mixed, and the remaining supplements were added: 1 ml of an 1M solution of $MgSO_4.7H_2O$, amino acid and base supplements, and X-gal. The final concentration of X-gal in the medium is 200 μg/ml. X-gal stock solution was prepared as 20 mg/ml in dimethylformamide and stored frozen.

The media used for the analysis of the inhibitory effects of antifolate drugs and the selection of antifolate drugs-resistant mutants are described in "RESULTS".

All solid media were supplemented with 2% Bacto-agar. As required, folinic acid was supplemented at 250 μg/ml, and dTMP at 100 μg/ml, unless otherwise stated.

Preparation and composition of media (LB, M9 and M9CA) for *E. coli* were as described by Miller (1972). When required in selective procedures, appropriate amino acids were added to a final concentration of 50 µg/ml. Ampicillin was added to the media at 48°C after autoclaving, or spread on the LB plates. Trimethoprim was added at the final concentration of 2 µg/ml. Trimethoprim-contained M9CA medium was used for selection of *E. coli* transformants with plasmids that contain a cloned *DFR1* gene.

II. Yeast genetic methods

II.A. General procedures

Yeast mating, sporulation, random spore analysis and tetrad analysis were carried out by the standard yeast genetics methods (Sherman et al., 1984). Diploid strains were constructed by making crosses between haploid strains. Diploid cells were isolated either by micromanipulation of individual zygotes from a mixture of the parental haploids after mating at 30°C, or by selection of cells prototrophic for complementing auxotrophies in the parental haploids. To obtain asci, diploids were first grown in either YEPD or presporulation medium at 30°C and then transfered to sporulation F^+ medium. The cells were incubated at room temperature for five to six days to induce ascus formation. Prior to dissection, asci were transfered to 0.1 ml of 1:10 dilution of glusulase (Endo laboratories) in distilled water and incubated for 5 to 30 minutes at 37°C. The digestion solution was further diluted appropriately with distilled water, depending upon the initial concentration of asci. Samples of the suspension were then streaked onto appropriate plates for micromanipulation dissection. For selection of *dfr1* mutant spores, dTMP, or folinic acid, or both of them were added into the medium. The spores were allowed to grow at 30°C until colonies were formed, and then tested for genetic markers either by replica plating or streaking to appropriate selective media. Mating-type was determined by crossing the haploid spore clones to testers of defined mating-type and scoring mating either by visual inspection of zygote formation in the mating mixture or by the formation of cells prototrophic for complementing auxotrophies in the haploids. Yeast transformation was performed by the lithium acetate method of Ito et al. (1983).

II.B. Determination of the frequency of reversion of the *dfr1* mutant to prototrophy for dTMP

Cells from a single colony of TH5 were inoculated in dTMP-YEPD medium. After growth to stationary phase, cells were washed and plated on to 20 YEPD plates at

about 2 X 10^7 cells/plate. No colony was observed in any of the 20 plates. Reversion of the *dfr1* mutant to prototrophy for dTMP has not been observed during the course of this study.

II.C. Isolation of rho° strains

To isolate a rho° derivative (without mitochondrial DNA) from a rho$^+$ strain such as TH100, about 200 cells were inoculated in YEPD medium supplemented with 10 mg/ml ethidium bromide and the culture was grown for 2 days in the dark. Cells from the culture were washed and plated onto YEPD plate after dilutions. About 98% of the colonies were respiratory-deficient. Cells from a single respiratory-deficient colony were then treated with ethidium bromide for the second round. Cells after the second-round treatment with ethidium bromide were 100% respiratory-deficient and single cell clones from the second cultures were used in genetic crosses.

II.D. Fluctuation analysis of MTX-resistant mutants

Cells from a single fresh colony on a YEPD plate were resuspended in H_2O and about 500 cells from the suspension were inoculated in 3 ml YEPD medium. The number of cultures was indicated in the Table 17. The cultures were grown for 2 days and the cell concentrations were determined. About 1.8 x 10^7 cells of M1-2B were plated on each YEPD-mtx25 plate (YEPD supplemented with 25 µg/ml MTX and 5 mg/ml sulfanilamide. About 8.8 x 10^6 cell of M1-2B were plated on each MC-mtx2 plate (MC medium supplemented with 2 µg/ml MTX and 5 mg/ml sulfanilamide). About 1.4 x 10^7 cell of TH100 were plated on each YEPD-mtx25 plate (YEPD 25 µg/ml MTX and 5 mg/ml sulfanilamide). About 1.6 x 10^7 cell of TH100 were plated on each MC-mtx2 plate (MC supplemented with 2 µg/ml MTX and 5 mg/ml sulfanilamide). All visible resistant colonies were scored after 20 days incubation.

II.E. *S. cerevisiae* strains used in this study

The genotypes of haploid strains of *S. cerevisiae* used in this study are given in Table 2. The source and origin of these haploids and the diploids used in this study which were derived from these haploid strains are described as follows (strains are listed in an alphabetic order; MTX-resistant mutants are listed separately):

308-6C This *tmp1* strain was described previously (Barclay and Little, 1978).

BR414-4a This *fol2* strain was kindly provided by J. Game (University of California at Berkeley).

Table 2. The genotypes of *S. cerevisiae* haploid strains used in this study

Strain	genotype
308-6C	*MATa tmp1-6 rho tup1 his(1,7) ilv1-92 trp5 lys1-1*
BR414-4a	*MATα fol2-1*
ESLZ-150	*MATa ura3-52 leu2-3,112 trp1 tup* $mtx1^R$ (pESLZ=*URA3 dfr1-ES LEU2:lacZ*)
F33	*MATα met7 gal2 pet*
G1	*MATa ura3-52 leu2-3,112 trp1 tup DIR*
G2	*MATa ura3-52 leu2-3,112 trp1 tup DIR*
G3	*MATa ura3-52 leu2-3,112 trp1 tup DIR*
G4	*MATa ura3-52 leu2-3,112 trp1 tup DIR*
G5	*MATa ura3-52 leu2-3,112 trp1 tup DIR*
G6	*MATa ura3-52 leu2-3,112 trp1 tup DIR*
G1/rho°	*MATa ura3-52 leu2-3,112 trp1 tup DIR* rho°
G2/rho°	*MATa ura3-52 leu2-3,112 trp1 tup DIR* rho°
G3/rho°	*MATa ura3-52 leu2-3,112 trp1 tup DIR* rho°
G4/rho°	*MATa ura3-52 leu2-3,112 trp1 tup DIR* rho°
G5/rho°	*MATa ura3-52 leu2-3,112 trp1 tup DIR* rho°
G6/rho°	*MATa ura3-52 leu2-3,112 trp1 tup DIR* rho°
g610-5D	*MATα fol2-10 ade3*
g615-4B	*MATa fol1-6 trp*
g619-6a	*MATα fol1/3 ade4* rho^+
g887-1A	*MATa met7 ura3 leu2 ade2 trp* rho^-
g887-2a	*MATa met7 leu2 ura3 trp* rho^-
g877-4B	*met7 leu2 rad6-1 ura3 trp his? ade? rho+*

to be continued...

... continued from Table 2, page 27 of 2

HC4-2B	*MATα ura3-52 leu2-3,112 trp1*
M1-2B	*MATαura3-52 trp1-289*
M1-2B-pIUD1	*MATαura3-52 trp1-289* (pIUD1= URA3$^+$ DFR1$^+$)
S189-6d	*MATa met7 trp1 leu1 ade1 gal1 gal2 pet*
TH5	*MATa ura3-52 leu2-3,112 trp1 tup dfr1::URA3*
TH5/pBBL20	*MATa ura3-52 leu2-3,112 trp1 tup dfr1::URA3* (pBBL20=2μ LEU2$^+$ DFR1$^+$)
TH7	*MATa ura3-52 leu2-3,112 trp1 tup dfr1::URA3 fou*
TH20-4B	*MATα ura3-52 leu2-3,112 trp1 tup*
TH22-1B	*MATα ura3-52 leu2-3,112 trp1 tup dfr1::URA3*
TH23-2A	*MATα ura3-52 leu2-3,112 trp1 tup dfr1::URA3 fou*
TH100	*MATa ura3-52 leu2-3,112 trp1 tup*
TH100/ESLZ	*MATa ura3-52 leu2-3,112 trp1 tup* (pESLZ=*URA3 dfr1*-ES *LEU2:lacZ*)
TH100/rho°	*MATa ura3-52 leu2-3,112 trp1 tup* rho°
TH364-3D	*MATα ura3-52 leu2-3,112 trp1 tup*
UTL-7A	*MATa ura3-52 leu2-3,112 trp1*

F33 This *met7* strain was obtained from Berkeley Yeast Stock Center.

G1, G2, G3, G4, G5, and G6 These are the *DIR* mutants isolated from strain TH100 by their ability to grow in YPG medium supplemented with 1.5 mg/ml of KH_2PO_4, 100 µg/ml of methotrexate, 5 mg/ml of sulfanilamide and 200 µg/ml of dTMP at 30°C.

g610-5D This *fol2* strain was kindly provided by J. Game (University of California at Berkeley).

g615-4B This *fol1* strain was kindly provided by J. Game (University of California at Berkeley).

g877-2a This *met7* strain was kindly provided by J. Game (University of California at Berkeley).

g877-4b This *met7* strain was kindly provided by J. Game (University of California at Berkeley).

HC4-2B This haploid strain was obtained from Jürgen Heinisch (University of Alberta).

M1-2B This haploid strain was described by Casadaban et al. (1983).

S189-6d This *met7* strain was obtained from Berkeley Yeast Stock Center.

TH5 It was isolated as a transformant of TH100 with the 2.9-kb BamHI/SalI restriction fragment that contains a *dfr1::URA3* disruption mutation.

TH5/pBBL20 A transformant of TH5 with plasmid pBBL20.

TH7 This strain was isolated from *dfr1* strain TH5 by its ability to grow on a YEPD plate supplemented with 250 µg/ml folinic acid.

TH20 This diploid was constructed by a cross of *tup* mutant TH100 with haploid HC4-2B. Tetrad analysis revealed that the *tup* mutant marker segregated as a single genetic locus since the Tup^+ and tup^- markers segregated in a 2:2 pattern in a sample of 15 complete tetrads derived from this diploid.

TH20-4B This haploid strain is a *tup* mutant spore clone derived from diploid TH20.

TH22 This diploid was constructed by a cross of TH5 with TH20-4B. Therefore, it is heterozygous for the *dfr1* mutation, but homozygous for the *tup* mutation. As the zygotes could be isolated in YEPD medium without addition of dTMP, it suggests that the *dfr1* mutation was recessive. This heterozygous *dfr1* diploid was used for segregation analysis of the *dfr1* disruption mutation. Complete tetrads derived from this diploid could only be recovered in YEPD medium supplemented with 100 µg/ml dTMP.

TH22-1B This strain was a *dfr1* spore clone derived from diploid TH22.

TH23 This diploid was constructed by a cross of TH7 with TH20-4B. It is heterozygous for the *dfr1* disruption mutation and a *fou* mutant allele derived from the *dfr1 fou* mutant TH7. From this diploid, *dfr1 fou* spore clones were isolated that were used to construct homozygous and heterozygous *fou* mutant strains used in a test for dominance of the Fou phenotype.

TH23-2A This is a *dfr1 fou* spore clone derived from diploid TH23.

TH33 This diploid was constructed by a cross of the *tmp1* strain 308-6C with the *dfr1* strain TH22-1B. It is heterozygous for both *dfr1* and *tmp1* mutations.

TH55 This diploid strain was constructed by a cross of TH5 with TH22-1B. It is homozygous for both *dfr1* and *tup* mutations.

TH57 This diploid was constructed by a cross of TH5 with TH23-2A. It is homozygous for the *dfr1* mutation, but heterozygous for the *fou* mutant allele.

TH74 This diploid was constructed by a cross of MTX-resistant mutant ESLZ-150 with strain TH364-3D. It was used for segregation analysis of the stable MTX-resistant marker in strain ESLZ-150.

TH77 This diploid was constructed by a cross of TH7 with TH23-2A. It is homozygous for both *dfr1* and *fou* mutations.

TH100 This dTMP-uptake (*tup*) mutant strain was isolated from haploid UTL-7A on the selective medium that was YEPD medium plus 1.5 mg/ml KH_2PO_4, 100 µg/ml MTX, 5 mg/ml sulfanilamide and 100 µg/ml dTMP at 30°C (modified from Little and Haynes, 1979). It retains the respiratory-sufficient phenotype. A single mutation seemed to be responsible for the dTMP-uptake phenotype as the mutant phenotype segregated as 2 to 2 in a heterozygous diploid (see TH20). This haploid strain was used as the parental strain for the construction of *dfr1::URA3* disruption mutant by gene replacement.

TH100/ESLZ This strain was constructed by integration of plasmid pESLZ at the *DFR1* locus. The plasmid DNA used for the transformation was digested with restriction enzyme HindIII. The enzyme produced a linear plasmid by a single cut within the coding region of the *DFR1* gene.

TH100/rho° A respiratory-deficient derivative of TH100 after the treatment with ethidium bromide (see Section II.C. of this chapter).

TH119 This diploid was constructed by a cross of the *fol2* strain BR414-4a with the *dfr1* strain TH5. It was used for complementation analysis.

TH120 This diploid was constructed by a cross of *fol2* strain g610-5D with *dfr1* strain TH5. It was used for complementation analysis.

TH121 This diploid was constructed by a cross of *fol1* strain g615-4B with *dfr1* strain TH22-1B. It was used for complementation analysis.

TH122 This diploid was constructed by a cross of *met7* strain g877-4b with *dfr1* strain TH22-1B. It was used for complementation analysis.

TH123 This diploid was constructed by a cross of *met7* strain g877-2a with *dfr1* strain TH22-1B. It was used for complementation analysis.

TH177 This diploid was constructed by a cross of *met7* strain S189-6d with *dfr1* strain TH22-1B. It was used for complementation analysis.

TH178 This diploid was constructed by a cross of *met7* strain F33 with *dfr1* strain TH5. It was used for complementation analysis.

TH180 This diploid was constructed to test for the dominance of the *DIR* mutation. It was derived from a cross of *DIR* mutant G1 with strain TH20-4B.

TH181 This diploid was constructed to test for the dominance of the *DIR* mutation. It was derived from a cross of *DIR* mutant G2 with strain TH20-4B.

TH182 This diploid was constructed to test for the dominance of the *DIR* mutation. It was derived from a cross of *DIR* mutant G3 with strain TH20-4B.

TH183 This diploid was constructed to test for the dominance of the *DIR* mutation. It was derived from a cross of *DIR* mutant G4 with strain TH20-4B.

TH184 This diploid was constructed to test for the dominance of the *DIR* mutation. It was derived from a cross of *DIR* mutant G5 with strain TH20-4B.

TH185 This diploid was constructed to test for the dominance of the *DIR* mutation. It was derived from a cross of *DIR* mutant G6 with strain TH20-4B.

TH186 This diploid strain was constructed by a cross of TH100 with F33.

TH187 This diploid was constructed by a cross of *DIR* strain G1 with *met7* strain F33. From this diploid, respiratory-sufficient *DIR met7* double mutant spore clones were isolated.

TH188 This diploid was constructed by a cross of *DIR* strain G2 with *met7* strain F33. From this diploid, respiratory-sufficient *DIR met7* double mutant spore clones were isolated.

TH189 This diploid was constructed by a cross of *DIR* strain G3 with *met7* strain F33. From this diploid, respiratory-sufficient *DIR met7* double mutant spore clones were isolated.

TH190 This diploid was constructed by a cross of *DIR* strain G4 with *met7* strain F33. From this diploid, respiratory-sufficient *DIR met7* double mutant spore clones were isolated.

TH191 This diploid was constructed by a cross of *DIR* strain G5 with *met7* strain F33. From this diploid, respiratory-sufficient *DIR met7* double mutant spore clones were isolated.

TH192 This diploid was constructed by a cross of *DIR* strain G6 with *met7* strain F33. From this diploid, respiratory-sufficient *DIR met7* double mutant spore clones were isolated.

TH361 This diploid was constructed by a cross of G1 with TH22-1B. It is heterozygous for the *dfr1* disruption and a *DIR* mutation derived from a *DIR* strain G1, and homozygous for a *tup* mutation, which is required for the detection of the Dir phenotype.

TH362 This diploid was constructed by a cross of G2 with TH22-1B. It is heterozygous for the *dfr1* disruption and a *DIR* mutation derived from a *DIR* strain G2, and homozygous for a *tup* mutation, which is required for the detection of the Dir phenotype.

TH363 This diploid was constructed by a cross of G3 with TH22-1B. It is heterozygous for the *dfr1* disruption and a *DIR* mutation derived from a *DIR* strain G3, and homozygous for a *tup* mutation, which is required for the detection of the Dir phenotype.

TH364 This diploid was constructed by a cross of G4 with TH22-1B. It is heterozygous for the *dfr1* disruption and a *DIR* mutation derived from a *DIR* strain G4, and homozygous for a *tup* mutation, which is required for the detection of the Dir phenotype.

TH364-3D This is a *tup* mutant spore clone derived from diploid TH364. This haploid strain is respiratory-sufficient, but does not have a Dir phenotype.

TH365 This diploid was constructed by a cross of G5 with TH22-1B. It is heterozygous for the *dfr1* disruption and a *DIR* mutation derived from a *DIR* strain G5, and homozygous for a *tup* mutation, which is required for the detection of the Dir phenotype.

TH366 This diploid was constructed by a cross of G6 with TH22-1B. It is heterozygous for the *dfr1* disruption and a *DIR* mutation derived from a *DIR* strain G6, and homozygous for a *tup* mutation, which is required for the detection of the Dir phenotype.

TH367 This diploid was constructed to test for the genetic origin of the *DIR* mutation. It was derived from a cross of a *DIR* mutant derivative G1/rho° with strain TH364-3D.

TH368 This diploid was constructed to test for the genetic origin of the *DIR* mutation. It was derived from a cross of a *DIR* mutant derivative G2/rho° with strain TH364-3D.

TH369 This diploid was constructed to test for the genetic origin of the *DIR* mutation. It was derived from a cross of a *DIR* mutant derivative G3/rho° with strain TH364-3D.

TH370 This diploid was constructed to test for the genetic origin of the *DIR* mutation. It was derived from a cross of a *DIR* mutant derivative G4/rho° with strain TH364-3D.

TH371 This diploid was constructed to test for the genetic origin of the *DIR* mutation. It was derived from a cross of a *DIR* mutant derivative G5/rho° with strain TH364-3D.

TH372 This diploid was constructed to test for the genetic origin of the *DIR* mutation. It was derived from a cross of a *DIR* mutant derivative G6/rho° with strain TH364-3D.

UTL-7A This haploid strain was obtained from Jürgen Heinisch (University of Alberta).

The following are the MTX-resistant mutants described in this study:

R5 A MTX-resistant mutant isolated from M1-2B in YEPD medium supplemented with 25 µg/ml methotrexate and 5 mg/ml sulfanilamide.

R5p This is a secondary mutant which was derived from R5. It was isolated from a colony that produced a P phenotype on MC-mtx2 plate.

R14 A MTX-resistant mutant isolated from M1-2B on a YEPD plate supplemented with 25 µg/ml MTX and 5 mg/ml sulfanilamide.

R14p This is a secondary mutant which was derived from R14. It was isolated from a colony that produced a P phenotype on a MC plate supplemented with 2 µg/ml methotrexate and 5 mg/ml sulfanilamide.

R14p400 This is the secondary isolate that was selected from R14p for resistance up to 400 µg/ml MTX in MC medium.

R14L and R14S These two strains were originated from mutant R14. The R14 cells were plated on an MC plate supplemented with 2 µg/ml methotrexate and 5 mg/ml sulfanilamide for isolation of single colonies. Some resistant mutant cells form small colony; one of them was picked as R14S. One of the other large colonies was picked as R14L. It has been observed that R14L gave rise to small and large colonies again after plating out for single colonies, but R14S only gave rise to small colonies. However, it is not known whether all these small colonies have the simi .. novel structures observed for the strain R14S. R14S seems to be the secondary mutant of R14; whereas R14L is the same as of R14.

R15 A MTX-resistant mutant isolated from M1-2B in YEPD medium supplemented with 25 µg/ml MTX and 5 mg/ml sulfanilamide.

25-1 A MTX-resistant mutant isolated from M1-2B on a YEPD plate supplemented with 25 µg/ml MTX and 5 mg/ml sulfanilamide.

25-1/50 A secondary culture derived from 25-1 for resistance to 50 μg/ml MTX in SULF-MC medium.

25-1/200 A secondary culture derived from 25-1/50 for resistance to 200 μg/ml MTX in SULF-MC medium.

25-1/400 A secondary culture derived from 25-1/200 for resistance to 400 μg/ml MTX in SULF-MC medium.

25-1/800 A secondary culture derived from 25-1/400 for resistance to 800 μg/ml MTX in SULF-MC medium.

25-1/1200 A secondary culture derived from 25-1/800 for resistance to 1200 μg/ml MTX in SULF-MC medium.

25-2 A primary MTX-resistant mutant isolated from M1-2B in YEPD medium supplemented with 25 μg/ml MTX and 5 mg/ml sulfanilamide.

ESLZ-150 A primary MTX-resistant mutant isolated from TH100/ESLZ in YEPD medium supplemented with 25 μg/ml MTX and 5 mg/ml sulfanilamide.

III. Nucleic acid manipulations

III.A. Plasmid constructions

Standard recombinant DNA manipulations were performed by the methods of Maniatis et al. (1982) or by those recommended by the manufactures. *E. coli* strain DH5α was purchased from BRL and used for plasmid isolation and propagation. The following describes the origin of the plasmids used in this study.

pBBL20 This plasmid was constructed by cloning a 2-kb BamHI fragment, derived from pTH2, into plasmid pYF91 (a 2μ plasmid obtained from Dr. J. D. Freisen's laboratory). This plasmid has a *LEU2* gene as a selectable marker for yeast transformation. It was used to transform the *dfr1* mutant TH5 to demonstrate the complementation of the *dfr1* mutant by a cloned *DFR1* gene.

pDN21 This plasmid was obtained from Dr. B. J. Barclay's laboratory. It was constructed by insertion of a 2-kb BamHI fragment into vector pSV2-neo. The 1.8-kb BamHI/SalI genomic fragment that contains the *DFR1* gene is within this 2-kb fragment.

pDR73 This plasmid was described previously (Barclay et al., 1988). It carries a 9.0-kb BamHI fragment that contains the *DFR1* gene.

pESLZ A HindIII/SalI fragment that contains a *LEU2:lacZ* fusion was taken from the 9.5-kb SalI/SalI fragment that was constructed by J. M. Clement. This approximate 7.3-kb HindIII/SalI fragment was then changed to a SalI/SalI fragment by addition of a SalI linker and cloned into plasmid pTH26.

pIUD-1 This plasmid was constructed by cloning the 1.8-kb BamHI/SalI fragment, derived from plasmid pDR73, that contains the *DFR1* gene into vector Yip5 (Struhl et al., 1979).

pLG669-Z This plasmid (Guarrente and Ptashne, 1981) was obtained from Dr. L. Guarrente . The 1.1-kb *URA3* fragment used in disruption of the *DFR1* gene was taken from this plasmid.

pRA1, pRA8, pRA8, and pRA10 These plasmid s were isolated by selection for trimethoprim resistance from a Rose pool library A (Rose et al., 1987). They all carry a genomic fragment that includes the 9.0-kb fragment containing the *DFR1* gene (Barclay et al., 1988).

pRB2 and pRB3 These plasmids were isolated by selection for trimethoprim resistance from a Rose pool library B (Rose et al., 1987). They all carry a genomic fragment that includes the 9.0-kb fragment containing the *DFR1* gene (Barclay et al., 1988).

pTH1 This plasmid was derived from vector pSV2-neo (obtained from BRL). It was generated by filling-in the unique HindIII site of pSV2-neo.

pTH2 This plasmid was constructed by insertion of a 2-kb BamHI fragment that contains a cloned *DFR1* gene into the unique BamHI site of plasmid

pTH1. The BamHI fragment containing the *DFR1* gene was derived from plasmid pND21.

pTH3 This plasmid was constructed by cloning a HindIII fragment of *URA3* gene, derived from pLG669-Z (Guarrente and Ptashne, 1981), into the HindIII site within the coding region of the *DFR1* gene on plasmid pTH2. The insertion generated a *dfr1::URA3* disruption mutation. The BamHI/SalI restriction that contains the *dfr1::URA3* mutation, as shown in Figure 4, was isolated from this plasmid and used for the replacement of the *DFR1* allele by yeast transformation.

pTH8 This plasmid was constructed by cloning of the 1.8-kb BamHI/SalI *DFR1* fragment into yeast vector YRp7 (Struhl et al., 1979).

pTH14 This plasmid was constructed by inserting the 0.74-kb BamHI/HindIII fragment containing the 3' portion of the *DFR1* gene (see Figure 4), into vector pGEM-4Z.

pTH16 This plasmid was constructed by inserting the 1.1-kb HindIII fragment containing the yeast *URA3* gene, derived from plasmid pLG669-Z, into plasmid TH14.

pTH26 This plasmid was constructed by deletion of the 0.7-kb EcoRI fragment from plasmid pIUD-1. The EcoRI/SalI fragment, thus, contains only a portion of the *DFR1* gene. *E. coli* transformants with this plasmid are sensitive to the growth inhibition of trimethoprim.

pTH27 This plasmid was constructed by cloning the 1.8-kb BamHI/SalI *DFR1* fragment into yeast vector YRp17.

pTH31 This plasmid was constructed by cloning the 1.8-kb BamHI/SalI *DFR1* fragment into yeast vector pLG669-Z (Guarrente and Ptashne, 1981).

pTH32 This plasmid was constructed by cloning the 1.8-kb BamHI/SalI *DFR1* fragment into yeast vector YCp50 (Rose et al., 1987).

pTH33 This plasmid was constructed by inserting the 1.1-kb HindIII fragment containing the yeast *URA3* gene, derived from plasmid pLG669-Z, into plasmid TH26.

pTL221 This plasmid was generated by ligating a 2.4 kb BglII fragment that contains the *TMP1* gene into the BamHI site of pBR322 (B. J. Barclay). It was used as the *TMP1* probe for the experiment shown in Figure 12.

III.B. Isolation of total yeast DNAs and RNAs

The miniprep procedure of Sherman et al. (1984) was used for preparation of yeast genomic DNAs for Southern hybridization analysis. Preparation of yeast total RNAs for Northern hybridization analysis was carried out by the method of Carlson and Botstein (1982).

III.C. Gel electrophoresis and hybridization

The contour-clamped homogeneous electric field electrophoresis (CHEF) was used for separation of large chromosomal molecules in agarose gels (Chu et al., 1986). The BRL HEX·A·FIELD horizontal gel electrophoresis system and the BioradCHEF-DRII system were employed. DNA samples for the pulsed-field gel electrophoresis were prepared in agarose plugs essentially as described by Carle and Olson (1987). The specific running conditions for pulsed-field gel electrophoresis are given in the figure legends. Standard conditions were used for conventional agarose gel electrophoresis. Prior to transfer, DNAs were depurinated by incubating the gel in 0.25N HCl. RNAs or the acid-nicked DNAs in agarose gels were then transfered onto GeneScreen Plus membranes, and hybridized as recommended by the manufacturer (Du Pont Co.).

IV. Assay of DHFR enzyme activity

To assay DHFR enzyme activity, strain TH5 was grown in dTMP-YEPD medium, while M1-2B was grown in YEPD medium. Cells from 200 ml of mid-log-phase cultures were harvested and washed with extract buffer (0.01M Tris-HCl, 0.06M $MnCl_2$, 0.06 NaCl, glycerol 25%). Cells were disrupted by glass beads in the extract buffer and the crude extracts were collected by centrifugation. Protein concentration was determined by the Bradford method with bovine serum albumin as standard. Other details about the assay of DHFR activity in the crude extracts are described in Table 3.

V. Analysis of P compounds with azo dyes

The method described by Shane (1986), which is suitable for identification of folate compounds extracted from bacterial cells and mammalian tissues, was adapted to analyze the P compounds derived from MTX-resistant mutants with the P phenotype. The yellow-colored P compounds were collected from a culture of R14p400 in MC

medium supplemented with 2 μg/ml MTX and 5 mg/ml Sulfanilamide. The precipitates were dissolved in H_2O. The procedure used in this study started with the adjustment of the pH of the sample to 0.5. After Zinc reduction and reaction with azo dyes, the azo dye derivatives were purified by a BioGel P2 column.

RESULTS

I. Construction of a yeast *dfr1* mutant deficient in DHFR activity

In spite of previous attempts (e.g. see Little and Haynes, 1979), a *S. cerevisiae* mutant deficient in DHFR activity had not been described prior to this study. In the genome of the yeast *Saccharomyces cerevisiae*, a genetic locus designated as *DFR1* encodes DHFR activity. This conclusion arises from several observations. First, cloned genomic fragments containing the *DFR1* gene are able to confer resistance to DHFR inhibitors such as trimethoprim to *E. coli* cells, and methotrexate or aminopterin to yeast cells (Barclay et al., 1988; Fling et al., 1988). Second, the polypeptide sequence deduced from the DNA sequence data shows significant homology with DHFR proteins from both prokaryotic and eukaroytic sources (Huang, 1987; Barclay et al., 1988; Fling et al., 1988). Third, yeast cells with multicopy plasmids bearing the *DFR1* gene have increased DHFR activity (Fling et al., 1988). Fourth, a cloned *DFR1* gene complements an *E. coli* DHFR-deficient (*fol*) mutant (Barclay et al., 1988). Since a cloned *DFR1* gene was available (Barclay et al., 1988), the general strategy for gene replacement described by Rothstein (1983) was employed in this study to construct a *dfr1* mutant that was expected to be deficient in DHFR activity.

As a first step, a disruption mutation in the coding region of a cloned *DFR1* gene was constructed *in vitro*. It has been shown previously that the yeast *DFR1* gene has a coding region of 211 amino acid residues and there is a unique HindIII site between codons #81 and #83 (Huang, 1987; Barclay et. al., 1988). Insertion of a 1.1-kb HindIII fragment bearing the *URA3* gene into this unique HindIII site in a cloned *DFR1* gene, a *dfr1::URA3* disruption mutation was generated in a BamHI/SalI restriction fragment (Figure 4). *E. coli* transformants with a plasmid, such as pTH3, carrying this fragment that contains the *dfr1::URA3* disruption were sensitive to growth inhibition by the DHFR inhibitor trimethoprim. This indicates that the disrupted gene was defective in expression of DHFR activity. The second step was to replace the *DFR1* allele with the *dfr1::URA3* mutation by transformation. The DNA fragment used for transformation is shown in Figure 4. The selectable marker *URA3* is flanked by 1.0-kb and 0.8-kb sequences, homologous with sequences at the chromosomal *DFR1* locus. This transforming *dfr1::URA3* disruption construct was therefore expected to target the genomic *DFR1* locus by homologous recombination (Rothstein, 1983).

The strategy used in selection of a *dfr1::URA3* disruption transformant was based upon the assumption that a DHFR-deficient mutant would have auxotrophic requirements similar to wild-type strains grown in the presence of antifolate drugs.

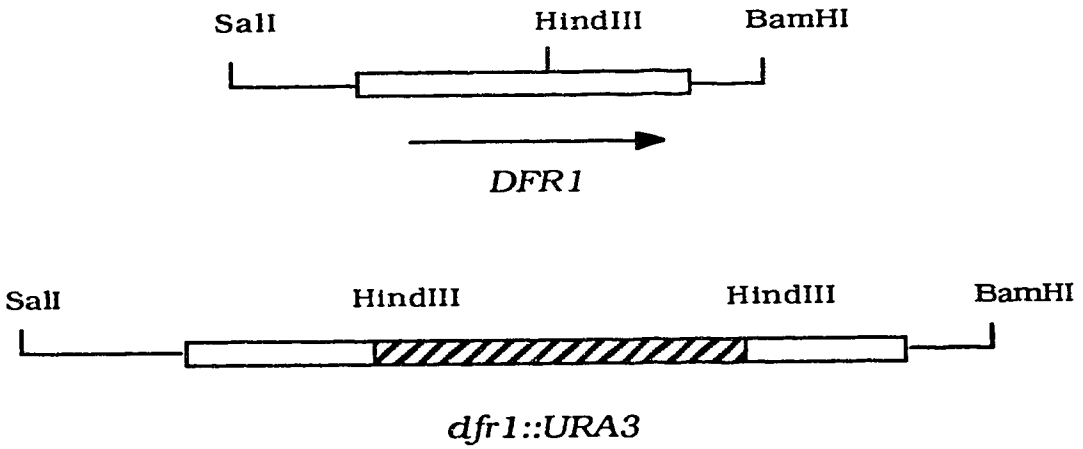


FIG. 4. A *dfr1::URA3* disruption mutation. Details about the *in vitro* construction of this mutation were described in the construction of plasmids pTH1, pTH2 and pTH3 (see "MATERIALS and METHODS"). Shown on the top is the *DFR1* gene. On the bottom is the *dfr1::URA3* disruption mutation. The open bar represents the coding region of the *DFR1* gene. The striped bar represents the fragment that contains the *URA3* gene.

such as methotrexate plus sulfanilamide. Addition of dTMP, adenine, histidine and methionine to growth medium supports the growth of yeast cells in the presence of these antifolate drugs (Barclay and Little, 1977; Little and Haynes, 1979). Based on the above conjecture, these four end-products of tetrahydrofolate-dependent one-carbon metabolism were included in the selective medium in order to recover *dfr1::URA3* transformants. To allow the use of this selection scheme, it was necessary that the parental strain for the disruption transformation was a dTMP-permeable (*tup*) mutant, as wild-type strains of the yeast *S. cerevisiae* are impermeable to exogenous dTMP (Jannsen et al., 1973; Laskowski and Lehmann-Brauns, 1973; Fäth et al., 1974; Wickner, 1974; Little and Haynes, 1979). A *tup* derivative of UTL-7A was therefore isolated. This strain, TH100, also carried a stable mutation at the *URA3* locus which allowed the positive selection of *dfr1::URA3* mutants as uracil-prototrophic transformants. Thus, to transform strain TH100, the 2.9-kb BamHI/SalI fragment containing the *dfr1::URA3* disruption mutation was cut from plasmid TH3 and purified from an agarose gel. About 1 µg DNA was used for the transformation by the lithium acetate method of Ito et al., (1983). In this disruption transformation experiment, several hundred uracil-prototrophic transformants were obtained in uracil-minus MC medium supplemented with 100 µg/ml dTMP.

The auxotrophic requirements of the uracil-prototrophic transformants were then examined to see whether they were as expected for a yeast [illegible] deficient in DHFR activity. Analysis of the growth requirements of several randomly picked transformants showed that all uracil-prototrophic transformants were concomitantly auxotrophic for dTMP, adenine, histidine, and methionine, in addition to leucine and tryptophan which are required by the parental strain (see Section II.A. of "RESULTS"). Thus, the auxotrophy for the four C1 metabolites and prototrophy for uracil were as predicted for a *dfr1::URA3* disruption transformant. Of the putative *dfr1* mutants, one strain designated as TH5 was chosen for further confirmation of the *dfr1::URA3* disruption mutation.

The *dfr1::URA3* disruption mutation should show the cosegregation of the *dfr1* mutant phenotype with the uracil-prototrophic phenotype in tetrads derived from a diploid heterozygous for the *dfr1* mutation. In a sample of 30 tetrads derived from diploid TH22 that was constructed by crossing an original *dfr1* mutant isolate with a wild-type strain, all four meiotic products of each tetrad were recovered in dTMP-YEPD medium, 2:2 segregation was observed for both dTMP and uracil requirements and the dTMP auxotrophy and the uracil prototrophy cosegregated. Thus, these data are consistent with the prediction of the segregation pattern of a *dfr1::URA3* mutation.

Southern hybridization was used to further confirm the *dfr1::URA3* mutant allele at the molecular level. As shown in Figure 5, when the total genomic DNA preparations were digested with the restriction enzyme EcoRI, the BamHI/HindIII *DFR1* DNA probe detected a 5.3-kb band (4.2-kb + 1.1-kb) for the *dfr1* strain, a 4.2-kb band for *DFR1* strains, and a 3.0-kb fragment for both wild-type and *dfr1* mutant strains, as predicted.

Deficiency in DHFR activity in the *dfr1::URA3* transformant is indicated by its auxotrophic phenotype for the end products of tetrahydrofolate-dependent C1 metabolites. Northern hybridization analysis and DHFR activity assay were used to confirm deficiency in the expression of DHFR activity. As shown in Figure 6, a Northern blot experiment revealed that the normal *DFR1*-specific transcripts (about 900 bases) were not detected in the *dfr1* mutant (TH5). The size of the *DFR1* transcript detected in the wild-type control strain is consistent with that reported previously (Lagosky et al., 1987). In addition, the results shown in Table 3 reveal that DHFR enzyme activity was not detectable in this strain. Taken together, the data described above indicate that a *S. cerevisiae* mutant deficient in DHFR activity had been constructed.

II. Characterization of the *dfr1* auxotrophic phenotype

II.A. Auxotrophy of the *dfr1* mutant for the end products of C1 metabolism

The *dfr1::URA3* transformants were recovered in MC minus uracil medium supplemented with dTMP. Further characterization of the auxotrophic phenotype of several individual transformants revealed that they required concomitantly dTMP, adenine, histidine and methionine for growth. No growth was observed if any one of these metabolites was omitted from the growth medium (Table 4). This auxotrophic phenotype mimics that of wild-type *S. cerevisiae* strains grown in the presence of methotrexate and sulfanilamide (Little and Haynes, 1979). Diploids homozygous for the *dfr1* mutation (TH55) had the same auxotrophic phenotype as haploid *dfr1* mutants; while heterozygous diploids (TH22) were prototrophic for the C1 metabolites. As mentioned above, the *dfr1* disruption mutation segregated in a 2:2 pattern in tetrads derived from heterozygous diploid (TH22). A multicopy plasmid that carries the *DFR1* gene was able to transform the *dfr1* mutant to wild-type (TH5/pBBL20). These observations indicate that the *dfr1* disruption mutation alone was responsible for the dTMP-auxotrophic phenotype of the originally isolated *dfr1* transformant. The implication of this conclusion is that a further mutation in the *TMP1* gene was not required by the *dfr1* mutant for viability. The disruption mutation was very stable as

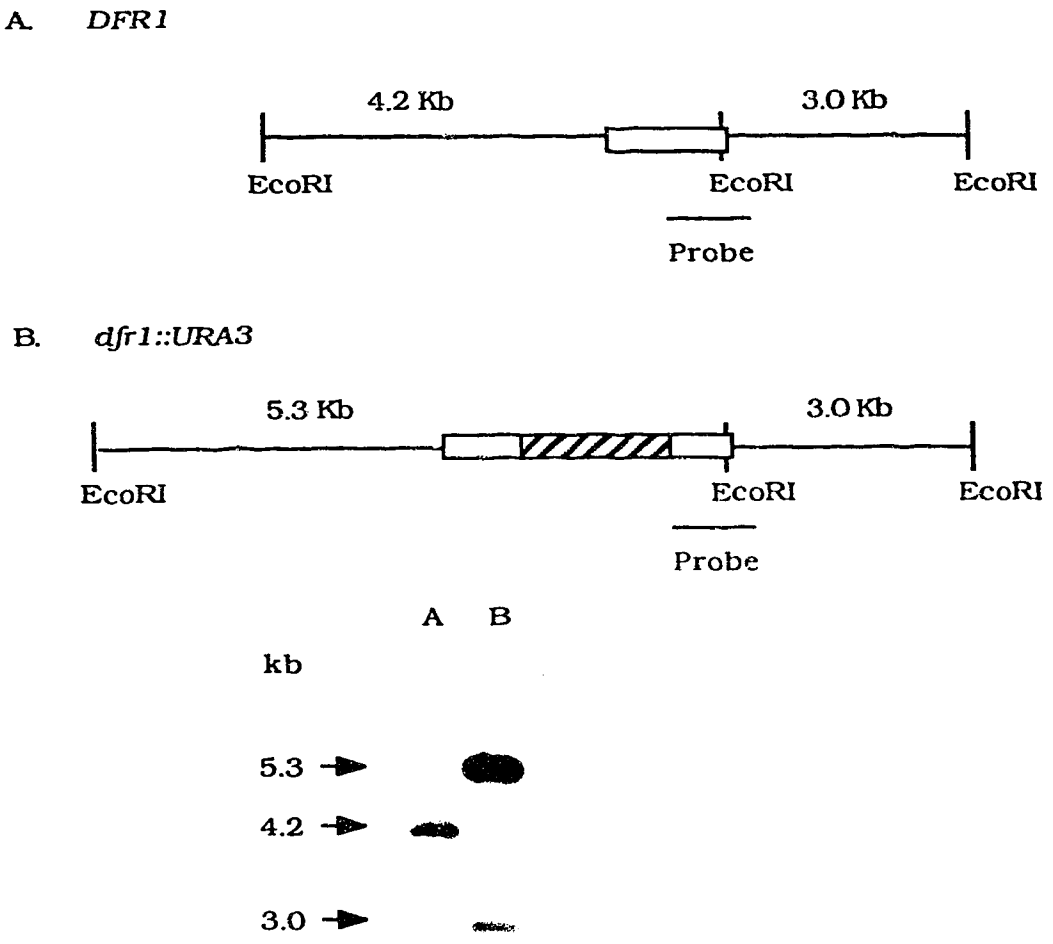


FIG. 5. Southern blot analysis of the *dfr1::URA3* disruption mutation. (A) TH100; (B) TH5. TH5 was grown in dTMP-YEPD medium. Total yeast genomic DNA was isolated by the mini prep procedure of Sherman et al. (1984). DNAs were digested with restriction enzyme EcoRI. The fragments were resolved on 1% agarose gel. Separated DNA fragments were alkali denatured, transferred to a GeneScreen Plus membrane. The Southern blot was hybridized as recommended by the manufacturer (Du Pont Co.) by using a gel-purified BamHI/HindIII fragment as a probe. This fragment was radiolabeled with [^{32}p] dCTP by the random oligonucleotide priming method (Feinberg and Vogelstein, 1983).

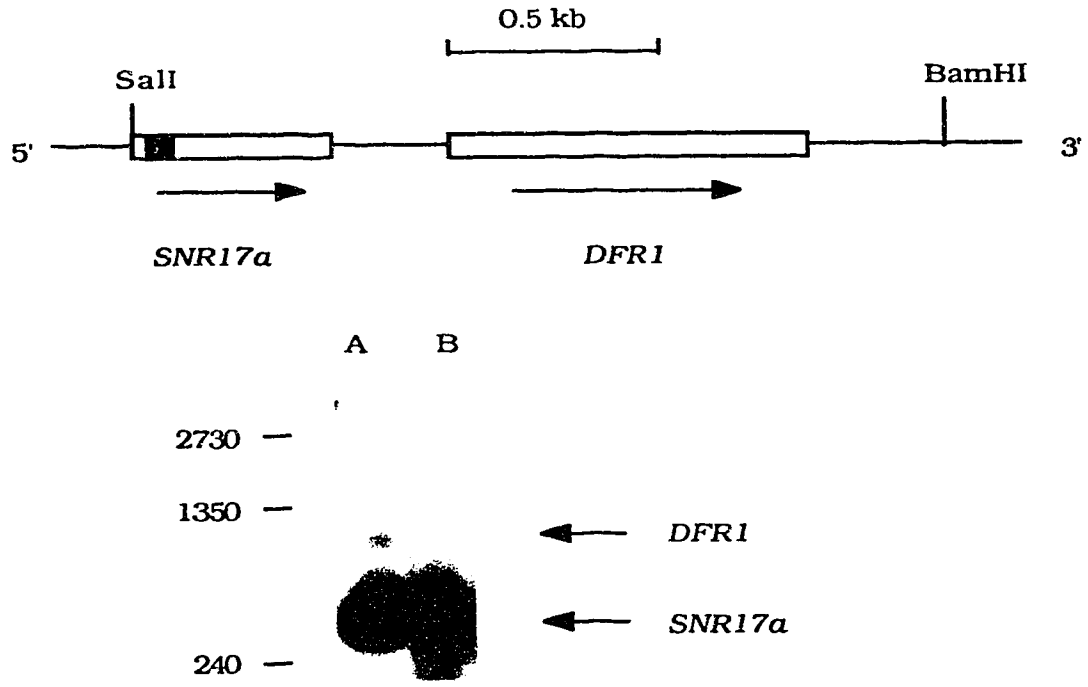


FIG. 6. Northern blot analysis of the *dfr1::URA3* transcript. (A) TH100 (*DFR1*); (B) TH5 (*dfr1::URA3*). Total yeast RNAs were isolated from 20 ml of mid-log-phase culture as described by Carlson and Botstein (1982). RNA samples were fractionated by electrophoresis on 1.2% agarose gel in the presence of 1M formaldehyde and transferred to a GeneScreen Plus membrane. The Northern blot was hybridized as recommended by the manufacturer (Du Pont Co.) by using a gel-purified BamHI/SalI fragment (shown on the top). This DNA fragment was radiolabeled with [^{32}p] dCTP by the random oligonucleotide priming method (Feinberg and Vogelstein, 1983). This probe also hybridized to the transcripts (328 nucleotides) of the *SNR17a* gene (Hughes et al., 1987). This serves as a internal control for the presence of RNAs in lane B. The size markers on the left are in bases. The open boxes represent the coding regions. The intron within the *SNR17a* gene is indicated by the black box.

Table 3. Assay of DHFR activity in a *dfr1* mutant

Strain	Genotype	DHFR activity (U/mg)*
TH100	*DFR1*	18.54
TH5	*dfr1::URA3*	< 0.01

* DHFR activity was assayed by the spectrophotometric method of Mathews et al. (1963). The standard assay was performed in 0.1M imidazole chloride buffer pH6.8 with 12 mM β-mercaptoethanol, 60 μM NADPH and about 50 μM dihydrofolate in a final volume of 1.0 ml at 20°C. Dihydrofolate was prepared just prior to use by the reduction of folic acid. The reaction was initiated by addition of the enzyme. The conversion of dihydrofolate to tetrahydrofolate and NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 mμ. The decrease in absorbancy at 340 mμ was measured for 5 minutes. The rate is corrected for a blank reaction in which dihydrofolate is omitted. One unit of activity is defined as the amount of enzyme that causes a decrease in absorbancy of 0.010 per minute under standard assay condition described above. Specific activity is defined as units of activity per milligram of protein in the crude extracts.

Table 4. The auxotrophic phenotype of a yeast *dfr1* mutant*

Growth condition**			Growth response
YEPD			-
YEPD	+	dTMP	+
SD			-
SD	+	C1 metabolites	+
SD	-	dTMP	-
SD	-	Adenine	-
SD	-	Histidine	-
SD	-	Methionine	-

* Several *dfr1::URA3* transformants were examined. All of them had the same growth requirements.

** The SD medium used in this analysis was supplemented with tryptophan and leucine which were required by the parental strain TH100.

no revertant of TH5 to dTMP-prototrophic was seen among approximate 4 x 10^8 cells of TH5 screened in YEPD plates. Thus, the frequency of revertants to dTMP prototrophy was found to be less than 2.5 X 10^{-9} revertant/cell plated. Since the *DFR1* locus was mapped to a location that is different from the loci of *fol1*, *fol2*, and *tmp1* mutation, complementation of the *dfr1* mutant with the *fol1*, *fol2*, and *tmp1* mutants was expected (see Table 5).

II.B. Growth response of the *dfr1* mutant to exogenous folinic acid

In the presence of methotrexate and sulfanilamide, growth of *S. cerevisiae* strains is supportable by exogenous folinic acid in both synthetic (SD) and complex (YEPD) media (Table 6). Thus, if the auxotrophy of the *dfr1* mutant for dTMP, adenine, methionine and histidine resulted from deficiency in tetrahydrofolate biosynthesis, substitution of these end products of C1 metabolism with exogenous folinic acid should satisfy the growth requirement of the *dfr1* strains. However, it was found that exogenous folinic acid at concentrations of 50 to 500 µg/ml did not support the growth of *dfr1* strain TH5 in complex medium (YEPD), although some slow growth was observed in synthetic medium supplemented (SD) with high concentrations of exogenous folinic acid (500 µg/ml). A similar growth response was observed when the *dfr1* mutant was grown in the presence of methotrexate and sulfanilamide, indicating that the inability to utilize exogenous folinic acid was due to the *dfr1* mutation. These observations suggest that there is a difference in the growth response to exogenous folinic acid between antifolate-induced DHFR-deficient cells and *dfr1* mutant cells (Table 6).

In spite of the failure in utilization of exogenous folinic acid by the primary *dfr1* mutants, spontaneous derivatives were readily isolated in YEPD medium supplemented with folinic acid (250 µg/ml). From these primary folinic acid-utilizing *dfr1* derivatives, it was also possible to isolate secondary isolates that could grow on low concentration of folinic acid (e.g. 50 µg/ml) in YEPD medium. These folinic acid-utilizing *dfr1* mutants may have resulted from mutations that are metabolic suppressors. Understanding the nature of these suppressor mutations may provide insights into the mechanism that is responsible for the difference in growth response to exogenous folinic acid between antifolate-induced DHFR-deficient cells and *dfr1* mutant cells. It was therefore of interest to characterize the genetic basis of this folinic acid utilization (Fou) phenotype. To address the question about whether mutations at a genetic locus other than *DFR1* were responsible for the Fou phenotype, diploid TH23 was constructed for segregation analysis. This diploid was generated by crossing a *dfr1*

Table 5. Complementation of the *dfr1* mutant with *fol1*, *fol2*, and *tmp1* mutants

Strain*	Ploidy	Growth response in YEPD medium
fol1 mutant	n	-
fol2 mutant	n	-
tmp1 mutant	n	-
dfr1 mutant	n	-
dfr1 x fol1	2n	+
dfr1 x fol2	2n	+
dfr1 x tmp1	2n	+

* The *fol1* strain used in this analysis is g615-4B. The *fol2* strains are g610-5D and BR414-4a. The *tmp1* strains is 308-6C. The *dfr1* x *fol1* diploids are TH121. The *dfr1* x *fol2* are TH119 and TH120. The *dfr1* x *tmp1* diploid is TH33.

Table 6. Difference in the growth response to exogenous folinic acid between antifolate-treated wild-type cells and *dfr1* mutant cells

	Growth response**	
Strain*	YEPD + dTMP	YEPD + Folinic acid
antifolate-treated wt cells	+	+
dfr1 mutant cells	+	-

* Wild-type *S. cerevisiae* strains such as TH100 were grown in the presence of 100 µg/ml methotrexate plus 5 mg/ml sulfanilamide.

** 100 µg/ml dTMP and folinic acid (from 50 up to 500 µg/ml) were supplemented.

mutant with the Fou phenotype, designated TH7, with haploid strain TH20-4B. In 30 tetrads derived from this diploid, all the meiotic spores were germinated on dTMP-supplemented YEPD medium. As expected, a 2:2 segregation was observed for the auxotrophy for dTMP. Among the 60 *dfr1* mutant spore clones obtained in this tetrad analysis, 16 had the Fou mutant phenotype, indicating that the mutation responsible for the Fou phenotype segregated from the *dfr1* disruption mutation. To perform a test for dominance, diploids TH57 and TH77 were constructed. TH57 was derived from a cross between two *dfr1* mutants (TH7 x TH22-1B), but only one of these two parents (i.e. TH7) has the Fou phenotype. Diploid TH77 was also derived from two *dfr1* mutants (TH7 x TH23-2A). However, both of the parental strains carried the same mutation responsible for the Fou phenotype since TH23-2A was a spore clone derived from diploid TH23 that was constructed by crossing TH7 with strain TH20-4B. It was found that diploid TH57 failed to grow on folinic acid (up to 500 µg/ml) in YEPD medium, but diploid TH77 was capable of growing on folinic acid in YEPD medium. These observations (see Table 7) suggest that a recessive nuclear mutation at a genetic locus other than the *DFR1* gene was responsible for the Fou phenotype. This mutant allele is thus designated as *fou* for *fo*linic acid utilization.

III. Characterization of the *dfr1* respiratory competence

III.A. The *dfr1* mutant is respiratory-deficient

In *S. cerevisiae*, respiratory competence can be conveniently assayed by the growth response of cells on media containing nonfermentable substrates such as glycerol as the sole carbon and energy source. Respiratory-sufficient strains can grow on these media; while respiratory-deficient mutants cannot. The strain TH100 used as the recipient for the disruption gene replacement is respiratory-sufficient. However, all *dfr1* disruption transformants were found to be respiratory-deficient (Table 8). It was possible that this respiratory-deficient phenotype of the *dfr1* transformants was a consequence of the selective method. Little and Haynes (1979) have shown that the primary isolates of *tmp1* mutants defective in thymidylate synthase activity, as well as those of *fol1* and *fol2* mutants deficient in folate metabolism, were all respiratory-deficient. From diploids constructed by crossing these primary mutant isolates with a wild-type haploid strain, respiratory-sufficient *tmp1*, *fol1* and *fol2* mutant strains were readily isolated in spore clones. Isolation of these respiratory-sufficient mutant strains required the presence of high concentrations of dTMP (100 µg/ml) for the *tmp1* mutant or high concentrations of folinic acid (250 µg/ml) for the *fol1* and *fol2* mutants

Table 7. Utilization of exogenous folinic acid by *dfr1 fou* mutants

	Growth response**	
Genotype*	YEPD + dTMP	YEPD + folinic acid
dfr1	+	-
dfr1 fou	+	+
dfr1/dfr1 FOU/fou	+	-
dfr1/dfr1 fou/fou	+	+

* The *dfr1* strain is TH5. The *dfr1 fou* mutants were isolated in YEPD plates supplemented with folinic acid at concentrations of 50 to 500 µg/ml. The strain TH7 was isolated with 250mg/ml folinic acid in YEPD and was used in the test for dominance and segregation analysis. The *dfr1/dfr1 FOU/fou* strain is TH57. The *dfr1/dfr1 fou/fou* strain is TH77.

** 100 µg/ml dTMP and 250 µg/ml folinic acid) were added as indicated, unless otherwise stated.

Table 8. Growth response of the *dfr1* mutant on nonfermentable carbon source

Growth condition	*dfr1*	*DFR1*
YEPD + dTMP	+	+
YPG + dTMP	-	+
YPG + folinic acid	-	+
YPG + dTMP + folinic acid	-	+

* As indicated, dTMP was added at 100 µg/ml in YEPD and 200 µg/ml in YPG; Concentratrions of folinic acid from 250 to 500 µg/ml have been used. Other details are described in the text.

in the spore regeneration media (Little and Haynes, 1979). Thus, in these cases, the respiratory-deficient phenotype appears to be a consequence of the growth conditions used during isolation of the mutants. To test whether this was also true for the *dfr1* transformants, the respiratory-competent diploid TH22 was constructed by crossing a *dfr1* mutant with a grande wild-type haploid TH20-4B. All *dfr1* mutant spores derived from this diploid were recovered on fermentable dTMP-YEPD selective plates, not on nonfermentable dTMP-YPG selective plates. Those recovered were all deficient in respiration even though various dTMP concentrations (from 100 µg/ml up to 500 µg/ml) were used during sporulation and spore germination. All *dfr1* spores stopped cell division within about six generations on dTMP-YPG medium. These observations indicate that the *dfr1* disruption mutation is responsible for the respiratory-deficient phenotype in these strains. Accordingly, respiratory deficiency must be an integral part of the *dfr1* mutant phenotype.

If the respiratory deficiency of the *dfr1* mutant is the result of depletion of tetrahydrofolate pools, one might expect that a *fou dfr1* double mutant should have a respiratory-sufficient phenotype. To test this, the diploid strain (TH23) heterozygous for both the *fou* and *dfr1* mutant alleles was constructed and various concentrations of folinic acid (from 250 µg/ml up to 500 µg/ml) and/or dTMP (from 100 µg/ml up to 200 µg/ml) were used during sporulation and spore germination. None of the *dfr1* mutant spores derived from TH23 formed colonies on nonfermentable YPG plates supplemented with folinic acid and/or dTMP. However, petite *dfr1* mutant spore clones were readily recovered when they were germinated on fermentable dTMP-YEPD selective media. All homozygous *dfr1* diploid strains (TH55, TH57, TH77) were deficient in sporulation, which is expected of a petite diploid strain. The results described above suggest that DHFR activity is essential for respiratory function in yeast.

Do the *dfr1* mutants still retain mitochondria? This was addressed by the following experiments. First, a rho° strain (without mitochondrial DNA) was isolated from TH100 by the ethidium bromide method and then crossed to a *dfr1* mutant TH22-1B. Similar to both parental haploids, this diploid did not grow on YPG medium. Second, a *dfr1* mutant strain TH5 was transformed with a multicopy plasmid (pBBL20) that carries a cloned *DFR1* gene and it was found that all transformants did not grow on nonfermentable dTMP-YPG medium. This result suggests that the *dfr1* mutants did not retain functional mitochondria.

III.B. Respiratory sufficiency of a *dfr1* mutant can be conferred by *DIR* mutations

There are at least two reasons that it was desirable to have a respiratory-sufficient *dfr1* strain. First, such a strain would be a useful experimental tool for tetrad analysis, since sporulation requires respiratory-sufficiency in diploids. Second, such a strain would be useful in dissection of the role of DHFR in mitochondrial function. The failure of experiments designed to isolate a respiratory-sufficient *dfr1* mutant strain from respiratory-sufficient diploid strains heterozygous for the *dfr1* disruption mutation required an alternative approach. As described later in Section IV. of "RESULTS", yeast *DIR* mutants, which retain respiratory activity when intracellular DHFR activity was inhibited by antifolates, were isolated. It was thought that these *DIR* mutations may also allow respiration in the *dfr1* mutant. To isolate *dfr1 DIR* double mutant strains that presumably would have a respiratory-sufficient phenotype, diploids (TH361, TH362, TH363, TH364, and TH366) were constructed by crossing a *dfr1* strain with the *DIR* strains. All these diploid strains were capable of sporulation under the standard sporulation conditions (without any supplementation of dTMP or folinic acid). In these crosses, respiratory-sufficient *dfr1* mutant clones were readily isolated as spore segregants from tetrads derived from these diploids (Table 9). It was possible to select respiratory-sufficient *dfr1* mutant segregants on YEPD supplemented with 100 µg/ml dTMP or directly on YPG medium supplemented with 200 µg/ml dTMP. Thus, these data indicate that respiratory-competent *dfr1* mutants can be isolated in a *DIR* mutant background.

IV. Isolation and characterization of yeast mutants with a DHFR-independent respiration (Dir) mutant phenotype

IV.A. The inhibitory effect of antifolate drugs on respiration

As described previously (e.g. see Little and Haynes, 1979), supplementation of the end products of tetrahydrofolate-dependent C1 metabolism (i.e. dTMP in YEPD or dTMP, adenine, histidine and methionine in SD) is sufficient for overcoming the inhibitory effect of methotrexate and sulfanilamide on the growth of *S. cerevisiae* strains in media using a fermentable carbon source such as glucose. However, even with the addition of these C1 metabolites, growth on a nonfermentable carbon source such as glycerol was not observed when intracellular DHFR activity was inhibited by these antifolate drugs (Table 10). Thus, the C1 metabolic end products can only overcome the antifolate-induced inhibition of biosynthetic pathways, but not the antifolate-induced respiratory deficiency. In considering that *dfr1* mutants were respiratory-deficient (see

Table 9. Growth response of *dfr1 DIR* double mutants on nonfermentable medium

Growth condition	*dfr1*	*dfr1 DIR*
YPG	-	-
YPG + dTMP	-	+

* Respiratory-sufficient *dfr1* strains have been isolated from diploids TH361, TH364 and TH366. Other details are described in the text.

Table 10. Inhibitory effect of antifolate drugs on respiratory activity in *S. cerevisiae*

Growth condition*	Growth response
YEPD + MTX/SULF	-
YEPD + MTX/SULF + dTMP	+
YPG + MTX/SULF	-
YPG + MTX/SULF + dTMP	-
YPG + MTX/SULF + folinic acid	-

* 100 µg/ml MTX, 5 mg/ml SULF, dTMP (up to 200 µg/ml) and folinic acid (250 or 500 µg/ml) were added in the growth medium as indicated.

Section IIIA of "RESULTS"), these observations suggest that DHFR must play an essential role for respiration in yeast cells.

IV.B. Exogenous folinic acid is not sufficient for yeast cells to retain a respiratory-sufficient phenotype when intracellular DHFR activity was depleted by antifolate drugs

If depletion of the reduced folate pools resulted in respiratory deficiency, exogenous folinic acid would be expected to be sufficient to overcome the inhibitory effects of antifolate drugs on both biosynthetic metabolism and mitochondrial activity. However, it was found that exogenous folinic acid was not able to allow yeast strains to retain respiratory activity when intracellular DHFR activity was depleted by antifolate drugs (Table 10). It is worth mentioning here that respiratory-sufficient *dfr1* spore clones could not be isolated from diploids heterozygous for the *fou* and *dfr1* mutant alleles with the use of folinic acid as a rescuing agent (see Section III.A. of "RESULTS"). These surprising observations raise the question about why exogenous folinic acid is not able to fulfil the requirement of reduced folates for mitochondrial activity.

IV.C. Isolation and characterization of yeast mutants with a Dir mutant phenotype

To find an alternative approach to obtain a respiratory-sufficient *dfr1* mutant, it was decided to isolate yeast mutants with a DHFR-independent respiration (Dir) phenotype. These mutants could be isolated under non-fermentable growth condition when intracellular DHFR activity was inhibited by antifolate drugs while the end products of C1 metabolism were supplemented in the medium in order to remove the inhibitory effect of the antifolates on C1-mediated biosynthetic pathways. Spontaneous mutants with a Dir phenotype were readily isolated from haploid strain TH100 (see Table 11). Characterization of the growth requirements of six individual isolates revealed that none of them were antifolate-resistant mutants since their growth on nonfermentable medium in the presence of antifolates depended on the presence of C1 end products. As these mutants had only the nutritional requirements of the parental strain, there was no auxotrophy associated with the Dir phenotype.

The following experiments were designed to address questions about the genetic basis underlying the Dir phenotype. First, diploids were constructed by crossing all six mutants with the Dir phenotype to a strain wild-type with respect to the Dir mutant phenotype. The dominance of the Dir phenotype was suggested as all resulting diploids

Table 11. Isolation of mutants with a DHFR-independent respiration (Dir) phenotype*

Strain	Growth response	
	YPG + MTX/SULF	YPG + MTX/SULF + dTMP
Wild-type	-	-
Dir mutant	-	+

* A single colony of TH100 from a YEPD plate was inoculated in 5 ml YEPD medium. The culture was grown for overnight. Approximate 10^7 cells were plated on the selective plates that were YPG medium supplemented with 200 μg/ml dTMP, 100 μg/ml methotrexate, and 5 mg/ml sulfanilamide. After incubation at 30°C for 10 days, six colonies were obtained in three plates. These six clones were named as G1, G2, G3, G4, G5 and G6.

grew on nonfermentable media supplemented with methotrexate and sulfanilamide plus the C1 end products . The dominant Dir phenotype could be accounted for by mutational events in either mitochondrial or nuclear genome. To distinguish these possibilities, rho° derivatives of the six mutants with the Dir mutant phenotype were isolated (see "MATERIALS AND METHODS") and crossed to a respiratory-sufficient wild-type strain. If the mutational events responsible for the Dir phenotype were of mitochondrial origin, no Dir mutant phenotype should be observed in the resulting diploids. However, if the mutations were nuclear, one would expect that these diploids have a Dir mutant phenotype. Analysis of the growth response of these diploids revealed that they all grew in non-fermentable growth media supplemented with methotrexate and sulfanilamide plus dTMP (Table 12). It is suggested that the mutational events giving rise to the Dir mutant phenotype occurred in the nuclear genome. These dominant mutant alleles were therefore designated as *DIR*.

IV.D. *DIR* mutations also confer a respiratory-sufficient phenotype on *met7* mutant strains

The *DIR* mutants provide a useful approach to study the role of DHFR activity or folate metabolism in respiratory function. One example of epistatic analysis between mutants in folate metabolism was illustrated by characterization of the *DIR dfr1* double mutant as described in Section III.B. of "RESULTS". Presented in this section is the analysis of *DIR met7* double mutants. The *met7* mutant is interesting because of its pleiotrophic phenotype. This mutant is respiratory-deficient and auxotrophic for adenine and methionine (Lowentein 1973; cited in Jones and Fink, 1982). Although the *MET7* gene product has not been identified, the phenotype is suggestive of a defect in folate metabolism.

Prior to this study, it had been thought that *met7* mutations might be allelic to *DFR1*. This was raised by two arguments. First, the *met7* mutant phenotype is similar, in several respects to the one expected for a DHFR-deficient mutant. Second, the *DFR1* locus was mapped indirectly by using a *URA3* marker inserted between two copies of the *DFR1* gene and the *MET7* locus and the integrated *URA3* gene were found to be very closely linked at a distance of the approximate 1.4 cM (Barclay et al. 1988). Among a sample of 108 tetrads analyzed, only two tetratype tetrads which showed reciprocal recombination between the *URA3* marker and the *met7* allele were observed. Therefore, the possibility existed that the *DFR1* and *met7* are allelic. To determine whether *met7* and *DFR1* were allelic, complementation analyses were performed. Diploids heterozygous for both *dfr1* and *met7* mutations had a wild-type phenotype with respect

Table 12. Characterization of the mutants with a Dir mutant phenotype

	Growth condition**		
Strain*	SD	YPG + MTX/SULF	YPG + MTX/SULF + dTMP
Dir^-	+	-	+
Dir^- x Dir^+	+	-	+
Dir^--rho° x Dir^+- rho^+	+	-	+

* All six isolates with the Dir^- phenotype had similar growth response. The diploids (Dir^- x Dir^+) are represented by TH180, TH181, TH182, TH183, TH184 and TH185 as well as TH361, TH362, TH363, TH364, TH365 and THTH366. The other diploids (Dir^--rho° x Dir^+- rho^+) are represented by TH367, TH368, TH369, TH370, TH371 and TH372. The rho° derivatives of the six *DIR* mutants were isolated as described in "MATERIALS AND METHODS".

** The SD medium used in this analysis was supplemented with tryptophan and leucine which were required by the parental strain TH100. 100 µg/ml MTX, 5 mg/ml SULF, and 200 µg/ml dTMP were added in the growth medium as indicated.

to both *dfr1* and *met7* (see Table 13). In addition, a *met7* strain (g877-2a) transformed with plasmids carrying a cloned *DFR1* gene retained the *met7* mutant phenotype. Similar results were produced by plasmids carrying either the 1.8-kb BamHI/SalI or the 9.0-kb BamHI genomic fragment that contains the *DFR1* gene (see Table 13). These observations indicate that *met7* is not allelic to *DFR1* and that a functional *MET7* gene is not located within the 9-kb BamHI restriction fragment.

One of the most likely biochemical mechanisms underlying the Dir phenotype is that the *DIR* mutants were able to bypass the dependence on DHFR activity for the production of a reduced folate (10-formyltetrahydrofolate) that is required for the initiation of mitochondrial protein synthesis. Thus, it was conceivable that if the pleiotrophic phenotype of the *met7* mutants resulted from defects in folate metabolism, *DIR* mutations should be able to confer a respiratory-sufficient phenotype to *met7* strains. To test this, the *DIR* mutants were crossed with a *met7* strain to generate diploids for isolation of *DIR met7* double mutants. As indicated in Table 14, from these diploids, respiratory-sufficient *met7* spore clones were readily isolated. These *met7* mutant spore clones did carry a *DIR* mutation since they grew on glycerol in the presence of methotrexate and sulfanilamide plus dTMP. Thus, this result suggests that *MET7* gene product is involved in some aspects of folate metabolism that are required for the synthesis of methionine and adenine as well as for mitochondrial activity.

V. Selection of MTX-resistant mutants from a wild-type strain

In *S. cerevisiae*, synergistic inhibition of both DHFR-catalyzed reduction of dihydrofolate to tetrahydrofolate and the *de novo* pathway for folate synthesis is required for effective growth inhibition (Barclay and Little, 1977; Little and Haynes, 1979). Methotrexate (MTX) is commonly used as DHFR inhibitor, while sulfanilamide (SULF) is employed to inhibit the enzyme dihydropteroate synthetase that catalyzes the condensation of pteridine and pABA to dihydropterate in the folate biosynthetic pathway. Usually, SULF is added at the concentration of 5 mg/ml, which is close to the limits of the solubility of this compound in yeast growth media. In spite of this high drug concentration, only a transient growth inhibition is observed when this drug is used alone. MTX concentrations are varied according to the selective requirements. The typical condition used to inhibit the growth of yeast cells in a number of studies on the effect of folate antagonism is addition of 100 µg/ml MTX plus 5 mg/ml SULF in YEPD medium (e.g. see Little and Haynes, 1979). However, as revealed by DNA hybridization analysis, there was no amplification of the *DFR1* gene in 37 MTX-resistant mutants analyzed. These resistant mutant strains were either isolated under

Table 13. *met7* is not allelic to *dfr1*

	Phenotype**		
Strain*	*met7*	*dfr1*	MTX resistance
met7 x *dfr1*	wild-type	wild-type	NA
met7 with a *DFR1* plasmid	mutant	NA	yes

* Four *met7* strains (g877-2a, g877-4b, F33, and S189-6d) were used in this analysis. The *met7* x *dfr1* diploids represent TH122, TH123, TH177, and TH178 that were constructed by crossing a *met7* strain with a *dfr1* strain. The *met7* strain transformed with a *DFR1* plasmid is strain g877-2a. Several plasmids were used for the transformation. Plasmids pTH8, pTH27, pTH30, and pTH31 all carry the 1.8-kb BamHI/SalI genomic fragment that contains the *DFR1* gene (see Figure 4). Plasmids pRA1, pRA8, pRA10, pRB2, pRB3, and pDR73 all carry the 9.0-kb BamHI genomic fragment containing the *DFR1* gene (see Barclay et al., 1988).

** All diploids were able to grow in the absence of dTMP, adenine, histidine and methionine. The transformants were auxotrophic for methionine. NA: not applicable.

Table 14. *DIR* mutations confer a respiratory-sufficient phenotype to *met7* mutants

Genotype*	Growth response in YPG medium
met7	-
met7 DIR	+

* A *met7* strain (F33) was crossed to all six *DIR* mutants described in this study. Respiratory-sufficient *met7* mutant strains were isolated as spore segregants from the resulting diploids TH187, TH188, TH189, TH190, TH191 and TH192 in either tetrad or random spore analysis. No grande *met7* spore clone was obtained from a control diploid TH186 (TH100 x F33).

this standard selective condition or selected for resistance to higher MTX concentrations (e.g. up to 500 µg/ml MTX in YEPD medium). I considered it possible that lack of any detectable amplification events might have been a consequence of the selective conditions that prevented the primary amplification mutants from undergoing sufficient cell divisions to form colonies in the selective media. It is likely that the mutant clones isolated resulted from other mutational alterations than *DFR1* gene amplification. These might include mutations that could induce any one of the potential biochemical mechanisms underlying the resistant phenotype (see Section IV of "DISCUSSION"). Since one of the primary goals of this research project was to develop the yeast system for gene amplification studies, an alternative selective condition for isolation of amplification mutants was sought.

It seemed conceivable that the primary amplification events may generate only one or a few extra copies of the *DFR1* gene in the initial mutant cell. In order for this primary mutant cell to form a colony on the selective plates, the growth inhibitory stringency imposed by the drug concentration must be low enough that this primary mutant cell and those derived from it will have sufficient intracellular folate pools to bypass the growth inhibitory effect of folate antagonism. Yeast strains with two or multiple copies of the *DFR1* gene per haploid cell can be constructed by transforming a wild-type strain with a integrating plasmid or an ARS plasmid that carries a cloned *DFR1* gene. The growth response of these strains to various MTX concentrations was examined in order to define conditions that favored the growth of cells with a single additional copy of the *DFR1* gene. Shown in Figure 7 is one of the experiments performed in liquid culture medium. The results of this experiment showed that a strain containing two copies of the *DFR1* gene had a growth advantage over the strain with a single copy of the *DFR1* gene only in the presence of low MTX concentrations (see Figure 7). These observations suggested that under such low drug selective conditions mutants with two copies of the *DFR1* gene had a slight growth advantage and could be positively selected. On agar plates, a similar drug concentration-dependent growth response was observed. Based on these experiments, the addition of 25 µg/ml MTX plus 5 mg/ml SULF in YEPD agar plates or 2 µg/ml MTX plus 5 mg/ml SULF in MC plates was used as the standard selective conditions for isolation of primary resistant mutants. Thus, cells that are able to form colonies under these conditions were defined as MTX-resistant. In this study, unless otherwise indicated, strains referred to as primary resistant mutants are those isolated under these conditions. Secondary resistant mutants are those derived from these primary clones.

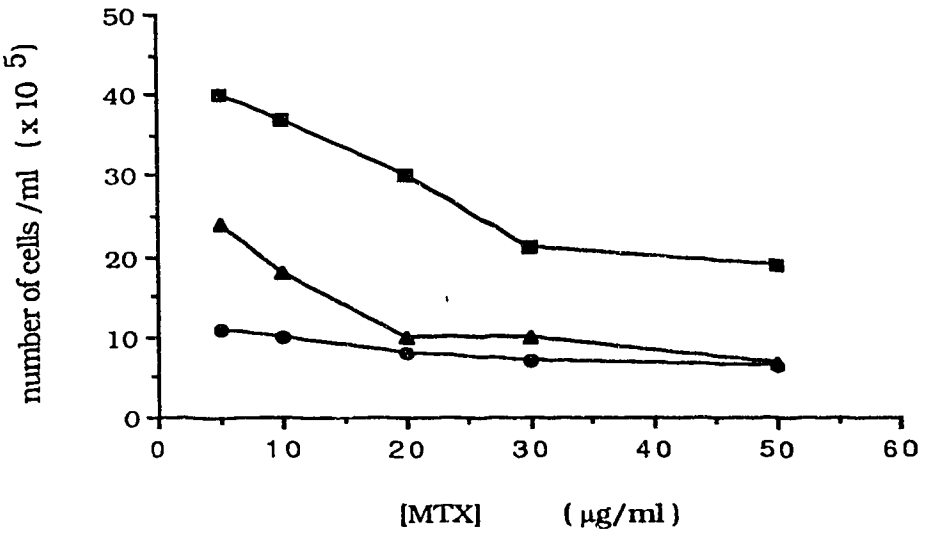


FIG. 7. Effect of the *DFR1* gene copy number on the growth response of *S. cerevisiae* to methotrexate. M1-2B (●) is a wild-type haploid strain with respect to the *DFR1* locus. M1-2B/pIUD-1 (▲) was derived from M1-2B by integration of plasmid pIUD-1 at the *DFR1* locus and therefore contains two copies of the *DFR1* gene. Strain M1-2B/pDSB1-Z (■) was a transformant of M1-2B with multi copy plasmid pDSB1-Z that carries a cloned *DFR1* gene. Cells were pre-grown in SD medium supplemented with tryptophan and uracil (uracil was omitted for strain M1-2B/pDSB1-Z for plasmid selection). Exponentially growing cells of each strains were harvested by centrifugation and resuspended at approximately 5 x 10^5 cell/ml in the SD medium supplemented with 5 mg/ml sulfanilamide and various methotrexate concentrations as indicated. After incubation for 24 hour at 30°C, the cell densities were determined in an electronic particle counter. In the absence of methotrexate, the cell density for each culture was about 10^7 cells/ml.

VI. Phenotypic characterization of the MTX-resistant mutants

Visible variation in the MTX-resistant phenotype was observed. For example, many of the primary drug-resistant isolates were phenotypically unstable. In addition, there were variations in the degree of drug resistance among the primary isolates. Most of them had a low-level MTX-resistant phenotype (i.e. only grew in the primary selective condition), while a few of them grew on higher MTX concentrations (e.g. 100 μg/ml in MC medium). In comparison with the primary selective MTX concentration (2 μg/ml in MC), this represents at least a 50-fold increase in MTX-resistant level. From the primary low-level MTX-resistant mutants, it was also possible to isolate higher-level MTX-resistant clones by step-wise increases in MTX concentrations. For instance, secondary mutants resistant to MTX concentrations up to 1200 μg/ml in MC medium were readily isolated from primary resistant isolate 25-1. This represents at least a 600-fold increase in MTX-resistant level. Thus, MTX resistance in yeast cells is a gradient rather than an 'all or nothing' phenotypic response.

In addition to these parameters (such as stability and resistance level) of the MTX-resistant phenotype described above, variations in other phenotypic characteristics of MTX-resistant mutants were also noted. For example, some resistant isolates did not grow in non-selective medium (YEPD) following the transfer from MTX-supplemented medium, but continue to growth in drug-contained medium. Another interesting observation associated with some resistant mutants was the formation of a precipitate during the growth in antifolate-contained medium. This phenomenon is referred to as P phenotype and the chemicals precipitated are called to P compounds (since the chemical nature of these compounds has yet to be identified).

VII. Analyses of the genetic and biochemical basis of MTX resistance

VII.A. MTX resistance can be mediated by *de novo* amplification of the *DFR1* gene

As shown previously (Huang, 1987), yeast strains transformed with a plasmid that carries a cloned *DFR1* gene are less sensitive to the growth inhibition by MTX (Figure 7). This suggested that MTX resistance in *S. cerevisiae* could be mediated by an increase in the copy number of the *DFR1* gene. It was therefore conceivable that *de novo* amplification of the *DFR1* locus could be a potential mechanism for MTX resistance in *S. cerevisiae*. As described in details in Section IX of "RESULTS", DNA hybridization analysis did reveal that the *DFR1* gene was amplified in some of the MTX-resistant mutants.

VII.B. Stable MTX resistance can also be mediated by a recessive mutation event that segregates from the *DFR1* locus

In order to help with preliminary identification of resistant mutants mediated by *DFR1* amplification from those resulting from other mutational events, haploid strain TH100/ESLZ was constructed by transformation with plasmid pESLZ. This plasmid contains a *LEU2:lacZ* fusion and a mutant *dfr1* gene with a 3' end deletion. Integration of this plasmid at the *DFR1* locus resulted in one complete copy of the *DFR1* gene and a repeat of the 5' *DFR1* sequence. Between the repeats is a *LEU2:lacZ* fusion construct and the *URA3* gene. The unique feature of this strain is that unequal sister-chromatid exchange between the repeats cannot generate extra copies of a functional *DFR1* gene and therefore such events cannot be selected as resistance to the antifolate drugs. The MTX-resistant mutants isolated from this strain could be a result of either *DFR1* gene amplification during which adjacent sequences such as the *LEU2:lacZ* fusion construct were likely co-amplified, or some mutational events other than *DFR1* gene amplification. Thus, over-expression of β-galactosidase activity in a resistant mutant (e.g. a dark blue drug-resistant colony) may indicate that an amplification event occurred at the *DFR1* locus. On the other hand, low level of β-galactosidase activity (e.g. a light blue resistant isolate) may indicate that the resistant phenotype resulted from a mutational event other than *DFR1* gene amplification.

The resistant mutants isolated from haploid strain TH100/ESLZ exhibited the typical phenotypic characteristics of MTX-resistant mutants as described above. The resistant isolate ESLZ-150 was identified as a large colony with light blue color on a X-gal MC plate that was supplemented with 2 μg/ml MTX and 5 mg/ml SULF. This primary resistant clone was able to grow on higher MTX concentrations (e.g 150 μg/ml MTX in YEPD medium) without secondary step-wise drug selection. The expression level of β-galactosidase in this resistant mutant was similar to that of the parental strain TH100/ESLZ as both strains developed light blue color on a X-gal plate that did not contain the antifolate drugs. The resistant mutant did not have an auxotrophic phenotype and the primary high-level resistant phenotype was mitotically stable (see Table 15). No P phenotype was found to be associated with this resistant mutant.

The stable resistant phenotype has greatly facilitated the analysis of its underlying genetic basis. Diploids such as TH74, constructed by crossing resistant mutant ESLZ-150 with a wild-type strain, were unable to grow on the primary selective medium even through the haploid parental resistant mutant grew on at least 50-fold higher MTX concentrations (100 μg/ml MTX in MC medium). This suggests that a recessive mutation is responsible for the stable MTX-resistant phenotype. Shown in

Table 15. Stability of the MTX-resistant phenotype of mutant strain ESLZ-150*

Number of colony grown on YEPD plate	Number of colony grown on mtx60-YEPD	Number of colony grown on mtx150-YEPD
688 (4)	688/688	688/688

* Cells of ESLZ-150 were picked from the colony grown on a mtx150-YEPD plate after replica-plating from the original selective plate and resuspended in water. The cell suspension was used to inoculate 20 ml of YEPD to give rise to approximate 10^3 cells/ml. The culture was grown for overnight which reached about 1.46 x 10^8 cells/ml (about 17 generations). Cells from the overnight culture were washed with water, then diluted in water and finally plated onto 4 YEPD plates, as indicated by the number within the blank in column 1. Colonies were seen after 4-5 days. The colonies on YEPD plates were then replica-plated onto mtx60-YEPD and mtx150-YEPD plates. In contrast to the colonies on the original selective plates, almost all colonies formed on these selective plates were uniform in size.

Table 16 are the results of tetrad analysis of the heterozygous diploid TH74. It can be seen that the resistant marker segregated from both the *URA3* and *LEU2:lacZ* markers. As the mutational event responsible for the MTX-resistant phenotype is recessive, it is then reasonable to conclude that the resistant phenotype cannot be associated with the transpositional *DFR1* gene amplification since additional copies of *DFR1* gene should result in a dominant or at least semidominant resistant phenotype. Taken together, the observations described above demonstrated that a recessive mutational event that occured in a genetic locus other than the *DFR1* gene is responsible for the stable MTX-resistant phenotype in strain ESLZ-150. This mutant allele is thus designated as $mtx1^R$ for MTX resistance.

VII.C. Characterization of the P phenotype that was seen in some of the MTX-resistant mutants

As mentioned previously, some of the MTX-resistant mutants generated a precipitate when grown in the antifolate drug-supplemented medium. A number of experiments were designed to examine the relationship between MTX resistance and the P phenotype. P compounds were seen as yellow or orange- colored precipitates in MC liquid medium supplemented with MTX and SULF. When dissolved in H_2O, the saturated solutions had pH values at about 4.5 to 5, similar to those of the medium used for growth of the resistant mutants. When the pH of the solution was adjusted to above neutral, they became soluble. P compounds were also soluble in boiling water, but precipitated again when the solutions were cooled. The color and the soluble behavior in higher pH and temperatures resemble the characteristics of folate derivatives such as methotrexate and folinic acid. A method described by Shane (1986) for identification of folate compounds extracted from bacterial cells and mammalian tissues was adapted to test whether P compounds contain *p*-aminobenzoylpoly(γ-glutamates). After a number cleavage procedures, folate compounds in cell-free extracts could be converted into *p*-aminobenzoylpolyglutamates (see Figure 2 for structure). The products could react with a color-less azo dye solution to produce purple-colored azo dye derivatives. It was found that strong purple-colored azo dye derivatives were produced by the P compounds. This observation is, therefore, consistent with the idea that P compounds may contain a structure similar to *p*-aminobenzoylglutamate.

There is a possibility that P compounds were derived directly from the medium. For example, changes in the medium pH during the growth of the resistant mutants might contribute to the precipitation. However, there was no significant change in the pH of the medium before and after the growth of the resistant cells. In addition, on agar

Table 16. Segregation analysis of the MTX-resistant marker in mutant strain ESLZ-150*

Diploid	Haploid parent	Relevant Phenotype	# of asci dissected	# of complete tetrad	# of complete tetrad with 2:2 segregation for Ura^+ and Mtx^R	# of PD tetrad	# of TT tetrad	# of NPD tetrad
TH74	a: ESLZ-150 α: TH364-3D	Ura^+ Mtx^R Ura^- Mtx^S	38	32	30	3	26	1

* MTX resistance was scored for growth on YEPD supplemented with 5 mg/ml SULF and two different MTX concentrations (60 μg/ml and 150 μg/ml). Expression of the β-galactosidase in spore clones co-segregated with the Ura^+ marker. Standard condition was used for sporulation and spore germination.

plates, the P phenotype was seen as a turbid ring around resistant colonies during their initial growth and eventually seen in the entire plates, presumably due to the diffusion of the precipitated P compounds. These observations seemed to suggest that either the P compounds were secreted from the mutant cells during the selective growth or something was secreted from the mutant cells that in turn caused the precipitation of the P compounds.

Thus, if the P compounds were folate-related chemicals and were secreted out of the resistant cells, could they be the products of altered folate metabolism that led to the resistant phenotype? This was tested by examining whether the P compounds could support the growth of yeast folate-requiring mutants such as the *fol1*, *fol2* (Little and Haynes, 1979) and the *dfr1* described in this study. P compounds obtained from a mixture of strain R14p in antifolate-contained MC medium were dissolved in H_2O by pH adjustment and then supplemented into YEPD or MC growth medium. However, none of the folate-requiring mutants was able to grow under these growth conditions. Thus, P compounds did not function as physiological folate substrates that could be utilized by a yeast cell. Could they, then, be the chemically modified methotrexate or sulfanilamide that were supplemented in the selective medium. This was tested by growing a resistant mutant that has a P phenotype (R14p) in medium supplemented with either MTX or SULF alone. It was found that P compounds were produced only in the medium supplemented with sulfanilamide, but not methotrexate. Thus, the production of the P compounds and the resistant phenotype may be related to intracellular metabolism of sulfanilamide that detoxifies the drug.

The P phenotype was seen in heterozygous constructed by a cross between a resistant mutant that has the P phenotype and a wild-type strain. Thus, the mutational event underlying the P phenotype appears to be dominant. Since resistant mutants with a high number of copies of the *DFR1* gene do not necessarily to have the P phenotype, it seems reasonable to suggest that the P phenotype is caused by a dominant mutation event other than an increase in the copy number of the *DFR1* gene.

VIII. Frequency and rate of MTX-resistant mutants

To investigate how often the resistant mutations occur, distribution of the resistant mutants in independent cultures was studied by fluctuation tests. The frequency of MTX-resistant mutations under the primary selective condition was found in a range of approximate 1.0×10^{-6} to 6.0×10^{-8} mutational event/cell plated (Table 17). If all the resistant mutants were assumed to arise before plating on selective medium, the rate of arising of resistant mutants was estimated to be about 3.0×10^{-7} to

Table 17. Estimation of the frequency and rate of MTX-resistant mutants*

Strain	Growth condition	No. of independent cultures	No. of cultures with resistant colonies	No. of resistant colonies scored	Frequency of resistant mutants	Rate of resistant mutants	
M1-2B	YEPD-mtx25	40	39	832	1.1×10^{-6}	3.6×10^{-7}	NA
M1-2B	MC-mtx2	40	23	55	1.6×10^{-7}	8.2×10^{-8}	1.0×10^{-8}
TH100	YEPD-mtx25	65	65	806	8.8×10^{-7}	2.9×10^{-7}	NA
TH100	MC-mtx2	50	24	45	5.6×10^{-8}	NA	4.0×10^{-8}

* The rates reported in the last column were calculated by the P_0 method. The rates reported in the second last column were calculated by the median method because all or most of the cultures had resistant mutants. The number of colonies in the median culture was 20 colonies for the experiment with M1-2B in YEPD-mtx25 and 11 colonies for the experiment with TH100 in YEPD-mtx25. The general procedures described for the use in yeast cells were employed (von Borstel, 1978). Other details are given in "MATERIAL and METHODS".

1.0×10^{-8} mutational event/cell/generation (Table 17). However, as discussed in Section V of "DISCUSSION", the antifolate drug-resistant mutants on the selective plates may actually arise after the cells were plated on to the drug-containing medium. For this reason, the rates of MTX-resistant mutants shown in Table 17, thus, may not be valid.

IX. Molecular characterization of the *de novo DFR1* gene amplification events in MTX-resistant mutants derived a wild-type strain

IX.A. *De novo* amplification of the *DFR1* gene

An increase in the copy number of the *DFR1* gene within a cell could result from either an increase in the number of the entire chromosome that carries the *DFR1* gene or amplification of a DNA segment containing the *DFR1* gene. In order to demonstrate *DFR1* amplification unambiguously in MTX-resistant mutants by DNA hybridization, it was necessary to know about the chromosomal location of the *DFR1* gene. Genetic mapping analysis revealed that the *DFR1* locus is about 1.4 cM proximal to a *met7* mutant allele on the right arm of chromosome XV (Barclay et al., 1988). Based on this map location, two DNA probes were used as controls for the copy number of the *DFR1* gene. One of them is a DNA fragment that contains the *URA3* gene which is mapped on chromosome V. Hybridization with this probe and a *DFR1* probe would demonstrate whether there was an increase in the copy number of the *DFR1* gene in the resistant mutants analyzed. However, these two probes would not distinguish whether an increase in the copy number of the *DFR1* gene resulted from gene amplification or from extra copies of the entire chromosome XV. The other probe is a DNA fragment at the *ADH4* region on the left arm of chromosome XV. Hybridizations with this *ADH4* probe specific for the left arm of chromosome XV and a *DFR1* probe specific for the right arm of chromosome XV would demonstrate whether the *DFR1* gene was amplified. An increase in the copy number of entire chromosome XV would not be detected by these two probes. With these strategies, primary and secondary MTX-resistant mutants derived from a wild-type haploid strain M1-2B that has a single copy of the *DFR1* gene were analyzed by Southern hybridization in order to determine whether the resistant phenotype was mediated by *de novo* amplification of the *DFR1* gene.

Shown in Figure 8 is the result of a conventional Southern hybridization analysis of two individual MTX-resistant mutants (R14 and R15) selected from M1-2B for resistance to 25 mg/ml MTX in SULF-YEPD medium. In this hybridization experiment, the *URA3*-specific probe detected an 11-kb (upper band); whereas the *DFR1*-specific probe revealed a 9-kb band (lower band). By using the 11-kb band as the

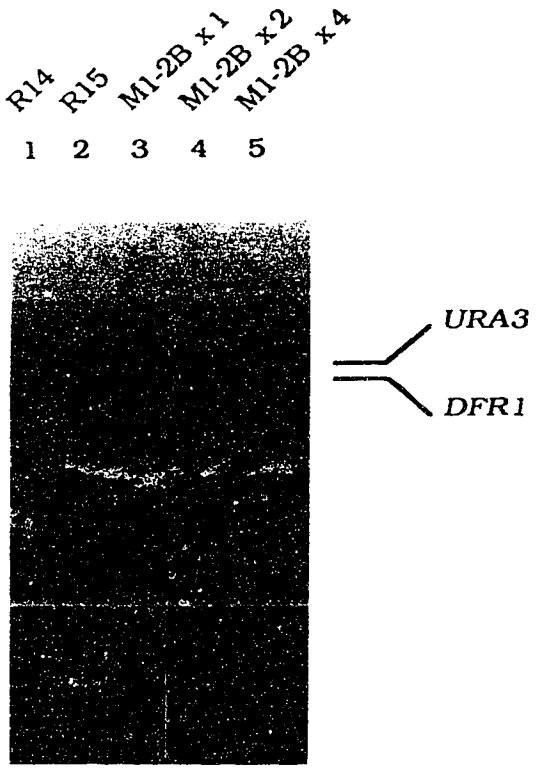


FIG. 8. Southern hybridization analysis of the MTX-resistant mutants R14 and R15. The MTX-resistant mutants were isolated from M1-2B for resistance to 25 µg/ml MTX in YEPD medium in the presence of 5 mg/ml of sulfanilamide. For R14 and R15, total genomic DNAs were isolated from cells grown in YEPD medium supplemented with 25 µg/ml MTX and 5 mg/ml sulfanilamide. The genomic DNA samples were digested with the restriction enzyme BamHI. The resulting fragments were separated on a 1% agarose gel and then transferred to a GeneScreen Plus membrane. The membrane was hybridized as recommended by the manufactures. Plasmic TH33 was used as the probe for this experiment. It contains two yeast genomic fragments specific for the *URA3* gene and the *DFR1* gene. The *URA3* fragment detected the upper band, while the *DFR1* probe hybridized the lower band.

internal control for the copy number of the *DFR1* gene, it can be seen that there are extra copies of the *DFR1* gene in resistant mutants R14 and R15 (lane 1 and lane 2). In addition to R14 and R15, such conventional Southern hybridization also reveals that there are extra copies of the *DFR1* gene in several other MTX-resistant mutants such as R1 and R5, which were isolated under the primary selective condition (data not shown). To demonstrate whether the extra *DFR1* gene copies results from gene amplification, these MTX-resistant mutants were further analyzed by slot blot Southern hybridization with probes specific for the *ADH4* and *DFR1* genes. The results from these experiments demonstrate that the extra copies of the *DFR1* gene did result from gene amplification. Shown in Figure 9 are the results for MTX-resistant mutants 25-1, R14, R14S (a secondary isolate of R14). There are about 6 copies of the *DFR1* gene per cell in MTX-resistant mutant 25-1; while at least 2 copies of the *DFR1* gene are present in mutant R14. There is apparently no extra copy of the *DFR1* gene in mutant R14S, when the *ADH4* probe was used as a control for copy number (see Figure 9). However, in a conventional Southern hybridization experiment (see Figure 10), in addition to the 9-kb BamHI fragment, an extra band was detected by the *DFR1* probe, indicating that an extra copy of the *DFR1* gene is present in this mutant strain. The observations described above indicate that the MTX-resistant phenotype of these primary mutants was mediated by *de novo* amplification of the *DFR1* gene. Of 32 primary resistant mutants analyzed by Southern hybridization, 7 had extra copies of the *DFR1* gene. Thus, about 22% of the MTX-resistant mutants isolated under the primary selective condition resulted from *DFR1* amplification. If the frequency of MTX-resistant mutant is about 1.1×10^{-6} (see Table 17 for strain M1-2B in the standard primary selective condition: 25 mg/ml MTX in SULF-YEPD medium), the estimated frequency for *DFR1* gene amplification are about 4×10^{-7} amplification event/cell plated. These results indicate that *DFR1* gene amplification is a relatively common molecular mechanism for MTX resistance under the primary selective condition used in this study.

IX.B. Chromosomal localization of the *DFR1* amplicons by pulsed-field gel electrophoresis

To localize the chromosomal position of the amplified *DFR1* gene in the MTX-resistant mutants, pulsed-field gel electrophoresis was employed. The chromosomal molecules of MTX-resistant mutants along with wild-type control strains were first separated by pulsed-field gel electrophoresis on agarose gels. The separated chromosomal molecules were visualized by staining with ethidium bromide and then blotted onto a membrane for Southern hybridization analysis. Shown in Figure 11 are

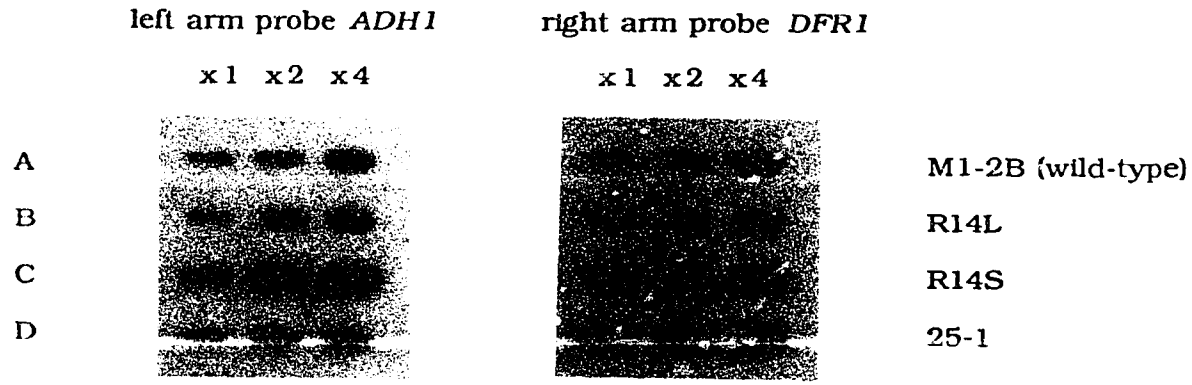


FIG. 9. Slot Southern hybridization analysis of MTX-resistant mutants. The method described by Costanzi and Gillespie (1987), with modification for yeast cells, was used to prepare the slot blot membrane for hybridization. For MTX-resistant mutants, DNAs were extracted from cells grown in YEPD medium supplemented with 25 μg/ml methotrexate and 5 mg/ml sulfanilamide. The DNA samples were blotted onto a GeneScreen Plus membrane and the relative concentrations in each slot were indicated by the numbers shown on the top. The membrane was first hybridized with the *ADH1* probe that was the 3-kb HindIII fragment derived from a yeast genomic clone (ATCC78877). The *DFR1* probe that was the 0.7-kb BamHI/HindIII fragment (see FIG. 4) was used for the second hybridization. These probes were radiolabeled with [^{32}p] dCTP by the random oligonucleotide priming method (Feinberg and Vogelstein, 1983).

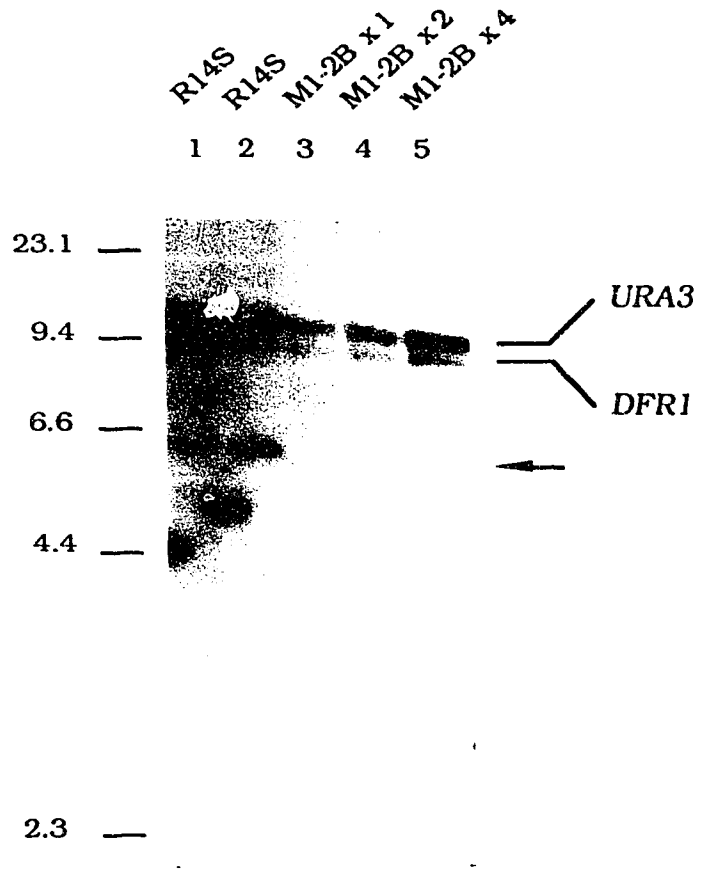
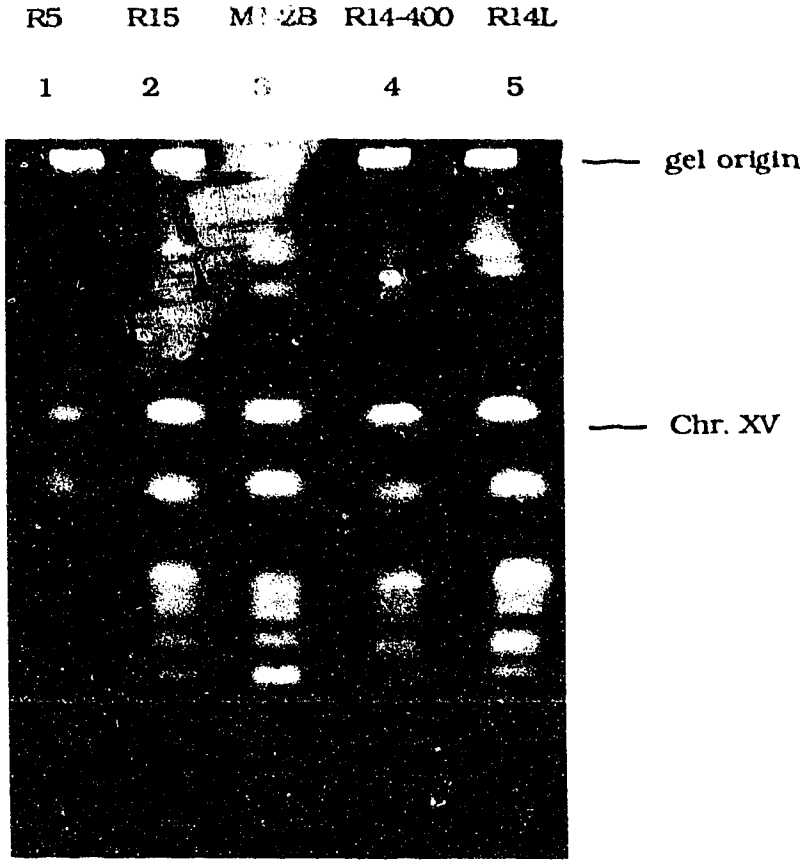


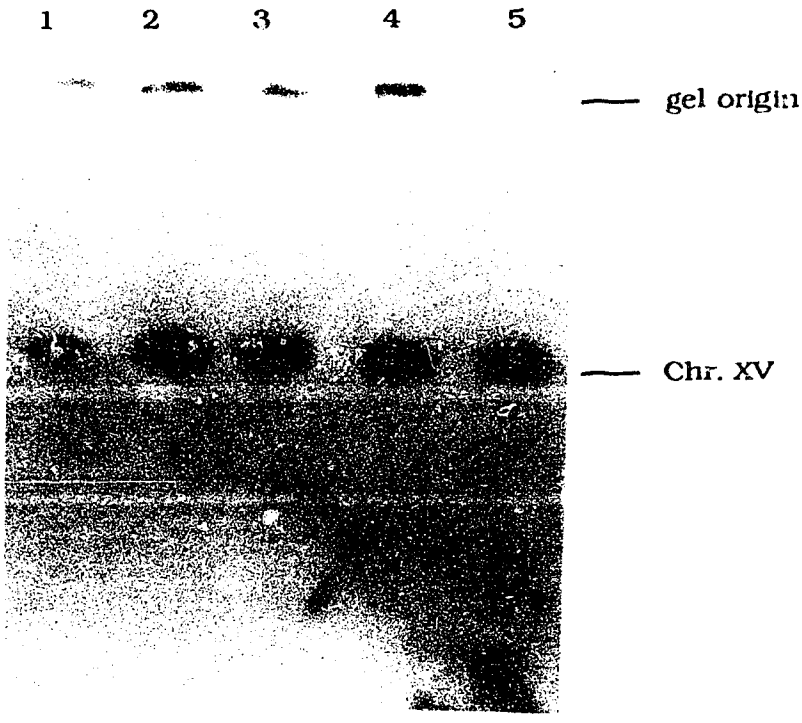
FIG. 10. Southern hybridization analysis of the MTX-resistant mutant R14S. For R14S samples, total genomic DNAs were isolated from cells grown in YEPD medium supplemented with 25 μg/ml MTX and 5 mg/ml sulfanilamide. The genomic DNA samples were digested with the restriction enzyme BamHI. The DNA fragments were separated on a 1% agarose gel and then blotted onto a GeneScreen Plus membrane. The membrane was hybridized as recommended by the manufactures. Plasmid TH16 was used as the probe for this experiment. It contains two yeast genomic fragments specific for the *URA3* gene and the *DFR1* gene, respectively. In lane 1 and lane 2, both R14S clones were derived from the same original R14S isolate. The difference in the *DFR1* band between these samples probably indicates the changes in the copy nmber of the *DFR1* sequence during the subsequent growth of the original R14S in the selective medium. The arrow indicates the novel bands detected by the *DFR1* probe.

FIG. 11. Chromosomal localization of the *DFR1* amplicons by pulsed-field gel electrophoresis and Southern hybridization. DNA samples of the MTX-resistant mutants were prepared from cells grown in YEPD medium supplemented with 25 mg/ml methotrexate and 5 mg/ml sulfanilamide. (A). The ethidium bromide-stained pulsed-field gel. The Biorad CHEF-DR II system was used for pulsed-field gel electrophoresis. Switch time was 70 seconds for 15 hours followed by a 120 second switch time for 11 hours. Gel was 1.0% agarose in 0.5X TBE. Electrophoresis was at 200 volts at 14°C. DNAs were visualized by staining with ethidium bromide. (B). Southern hybridization of the pulsed-field gel shown in (A). DNAs were transferred to a GeneScreen Plus membrane. The membrane was hybridized as recommended by the manufacturer (Du Pont Co.) with a *DFR1*-specific BamHI/HindIII fragment as a probe (see Figure 4). This fragment was radiolabeled with [^{32}P] dCTP by the random oligonucleotide priming method (Feinberg and Vogelstein, 1983). M1-2B is the parental strain of the MTX-resistant mutants.

A.


R5
R15
M 28
R14-400
R14L
1
2
3
4
5
gel origin
Chr. XV

B.


1
2
3
4
5
gel origin
Chr. XV

the results of a hybridization analysis on resistant mutants R5, R14L, R14-400, and R15 with a *DFR1* probe. This probe hybridized to the chromosomes in these mutants that corresponds to the chromosome XV of the parental strain M1-2B. As only chromosome XV was hybridized by the *DFR1* probe, this suggests that the amplified *DFR1* gene in these resistant mutants is retained on this chromosome. As shown previously (see Figure 9), the 9-kb BamHI fragment was amplified in the MTX-resistant mutants R14 and R15. This indicates that the *DFR1* amplicon must be as large as 9-kb in these resistant mutants. Since there is only 1 or possibly 2 extra copies of the *DFR1* gene in these resistant mutants (see Figure 8 and 9), migration of the *DFR1*-hybridized chromosome into the position corresponding to the wild-type chromosome XV was therefore expected only if it was assumed that the amplicon size in these mutants is small so that, under the conditions for this experiment, it was not possible to resolve the size difference as a result of gene amplification (e.g. within the range of 50 kb).

The MTX-resistant mutant R14S was isolated as a small colony on a SULF-MC plate supplemented with 2 mg/ml MTX among the colonies derived from a R14 cell population. Interestingly, as shown in Figure 12, a novel chromosome was detected by a *DFR1* probe, but not the normal chromosome XV, in this R14S mutant strain (lane 5 of Figure 12). As only this novel chromosome was hybridized by the *DFR1* probe, this suggests that it carries all copies of the *DFR1* gene which were detected in the conventional Southern blot hybridization (Figure 10). This new chromosome is larger than the normal chromosome XV; it migrated to a position corresponding to that close to chromosome IV (1640-kb) of a size-marker strain (Figure 12). This gives an estimated size of about 1500-kb for the novel chromosome. This novel chromosome was also hybridized by a probe specific to the *TMP1* gene that is over 50 map unit proximal to the *DFR1* locus on chromosome XV (see Figure 12). The observations described above indicate that this novel chromosome is likely an aberrant chromosome XV.

In resistant mutant 25-1, in addition to the normal chromosome XV, an extra band was detected by a *DFR1* probe, which migrated faster than the normal chromosome XV (see Figure 13). At this hybridization position, however, there was no visible ethidium bromide staining (indicated by the arrow in Figure 13). Therefore, the DNA content was very small at this position. Lack of an ethidium bromide-stained band was not expected if the *DFR1* sequence were present within a chromosome as such a chromosome would provide sufficient DNA content for visible ethidium bromide staining. Thus, the observations suggest that the amplified *DFR1* genes were likely present on a small extrachromosomal element.

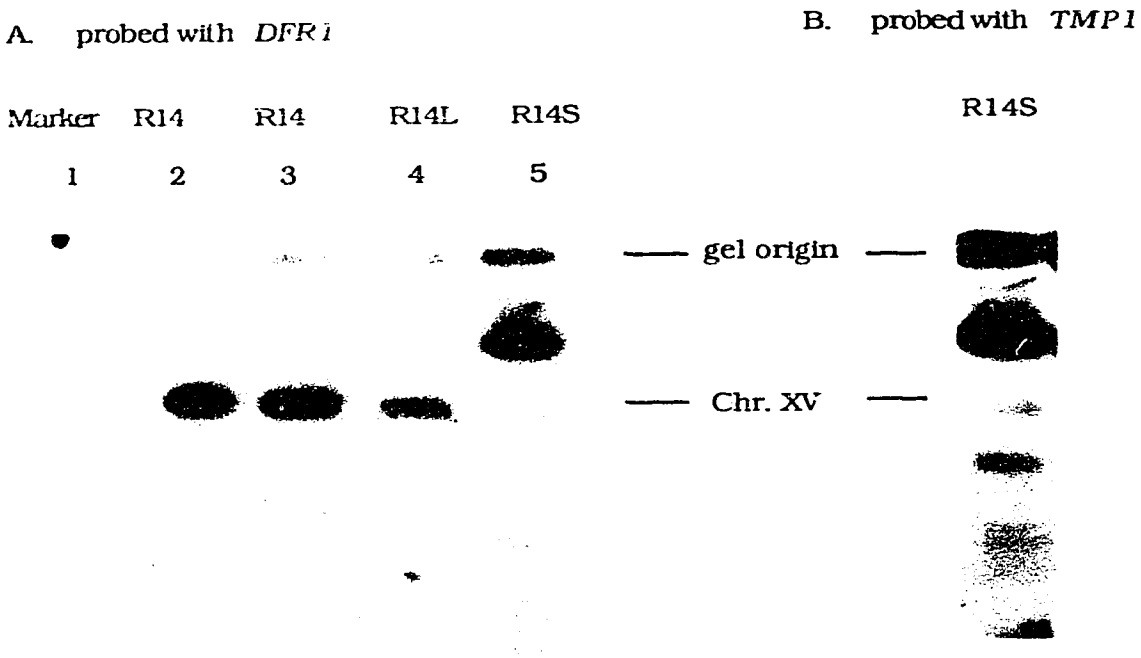


FIG. 12. Analysis of the MTX-resistant mutants R14, R14L, and R14S by pulsed-field gel electrophoresis and Southern hybridization. DNA samples of the MTX-resistant mutants were prepared from cells grown in YEPD medium supplemented with 25 µg/ml methotrexate and 5 mg/ml sulfanilamide. The pulsed-field gel was run in the Biorad CHEF-DR II system. Switch time was 90 seconds for 24 hours. Gel was 1.0% agarose in 0.5X TBE. Electrophoresis was at 200 volts at 14°C. (A). Southern hybridization of the pulsed-field gel membrane with a probe which is the 1.8-kb BamHI/SalI fragment containing the *DFR1* gene (see Figure 4). (B). Hybridization of the same membrane used in (A) with plasmid pTL221 which contains fragment derived from the *TMP1* locus. This probe also hybridized to several other chromosomes, but very weak hybridization occurred in the position corresponding to chromosome XV. This weak band may result from hybridization to chromosome VII which comigrated with chromosome XV. The first lane is the yeast chromosome PFG marker purchased from New England BioLabs. Chromosome XV of M1-2B migrated to the position corresponding to Chromosome XV of the marker strain.

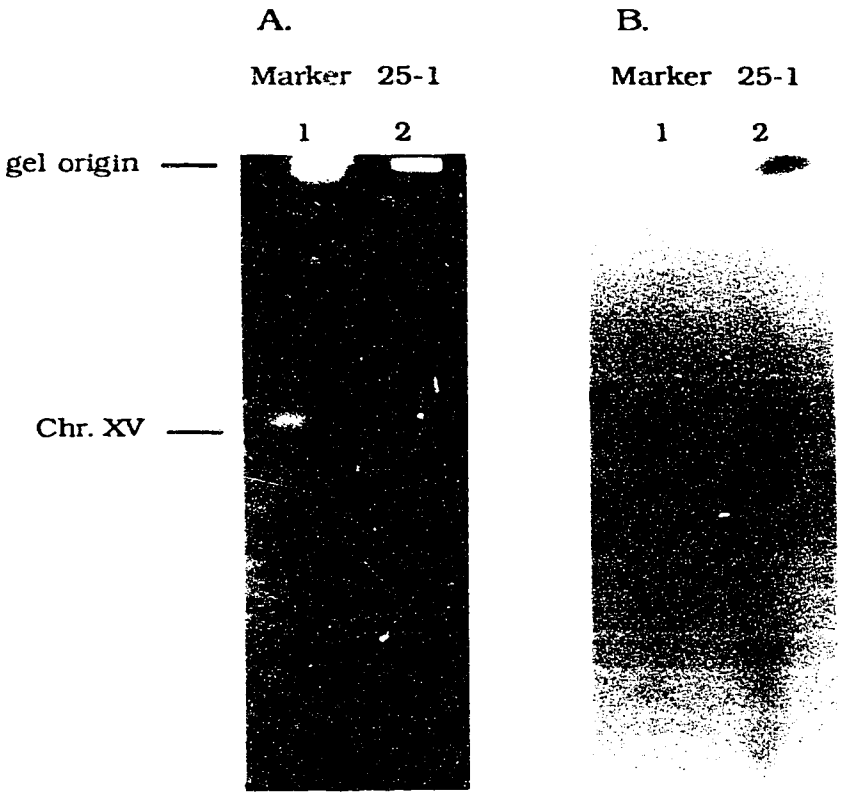


FIG. 13. Chromosomal localization of the *DFR1* amplicons in the MTX-resistant mutant 25-1 by pulsed-field gel electrophoresis and Southern hybridization. The DNA sample of the MTX-resistant mutant 25-1 were prepared from cells grown in YEPD medium supplemented with 25 µg/ml methotrexate and 5 mg/ml sulfanilamide. (A). The ethidium bromide-stained pulsed-field gel. The running condition was the same as in Figure 11. (B). Southern hybridization of the pulsed-field gel shown in (A). DNAs were transferred to a GeneScreen Plus membrane. The membrane was hybridized as recommended by the manufacturer (Du Pont Co.) with a *DFR1*-specific BamHI/HindIII fragment as a probe (see Figure 4). This fragment was radiolabeled with [^{32}p] dCTP by the random oligonucleotide priming method (Feinberg and Vogelstein, 1983). The first lane is the yeast chromosome PFG marker purchased from New England BioLabs. Chromosome XV of M1-2B migrated to the position corresponding to Chromosome XV of the marker strain.

IX.C. Increased MTX-resistant levels correlate with the number copies of the *DFR1* amplicons

From the primary drug-resistant mutants that result from *DFR1* amplification, secondary isolates resistant to higher levels of MTX could be isolated by step-wise selections for growth on higher MTX concentrations. It was easier to do this with some resistant mutants such as 25-1 than the others such as R14. For example, a culture of about 10^4 cell/ml of primary resistant mutant R14 in MC medium supplemented with 20 mg/ml MTX required at least 5 days to reach cell density of about 5 x 10^7 cells/ml. Step-wise selection of secondary mutants from primary resistant mutants such as R14 that carry the amplified *DFR1* gene on the native chromosome XV for resistance to higher MTX concentrations (e.g. 10, 20, 50, 100, 200, 400, and up to 600 mg/ml MTX in MC medium) was possible but each step required a long period of cultivation (from 4 to 7 days). However, about two days were normally required by a culture of about 10^4 cells/ml of resistant mutant 25-1 in MC medium supplemented with various MTX concentrations (20, 50, 200, 400, 800, and up to 1200 mg/ml) to reach the cell density of about 5 x 10^7 cells/ml. The rapid progression of the primary mutant 25-1 to high-level resistant isolates may be attributed to the unequal segregation or over-replication of the extrachromosomal element that carries the *DFR1* genes. There are at least 6 copies of the *DFR1* gene per cell when resistant mutant 25-1 was grown in the primary selective MC medium supplemented with 2 mg/ml MTX (see Figure 9). As shown in Figure 14 and 15, more copies of the *DFR1* gene were detected in cells grown in the presence of higher MTX concentrations. For example, there are probably at least 100 copies of the *DFR1* gene per cell in the secondary mutant cells that were grown in the presence of 800 mg/ml MTX in MC medium (see Figure 14). These observations suggest that there is a correlation between the levels of MTX resistance and the copy numbers of the *DFR1* gene.

IX.D. Further characterization of the extrachromosomal elements that contain *DFR1* amplicons by gel electrophoresis

As mentioned above, the observations described in Section B of this chapter have been interpreted as that a small extrachromosomal element carries amplified *DFR1* gene in resistant mutant 25-1. Under the experimental conditions for the results shown in Figure 13, the DNA molecule that contains the amplified *DFR1* gene would correspond to a linear molecule of at least 950-kb. If this were true, one would expect that this 950-kb molecule should form a ethidium bromide-staining band as the other chromosome molecules did. Since there is no visible ethidium bromide-staining band

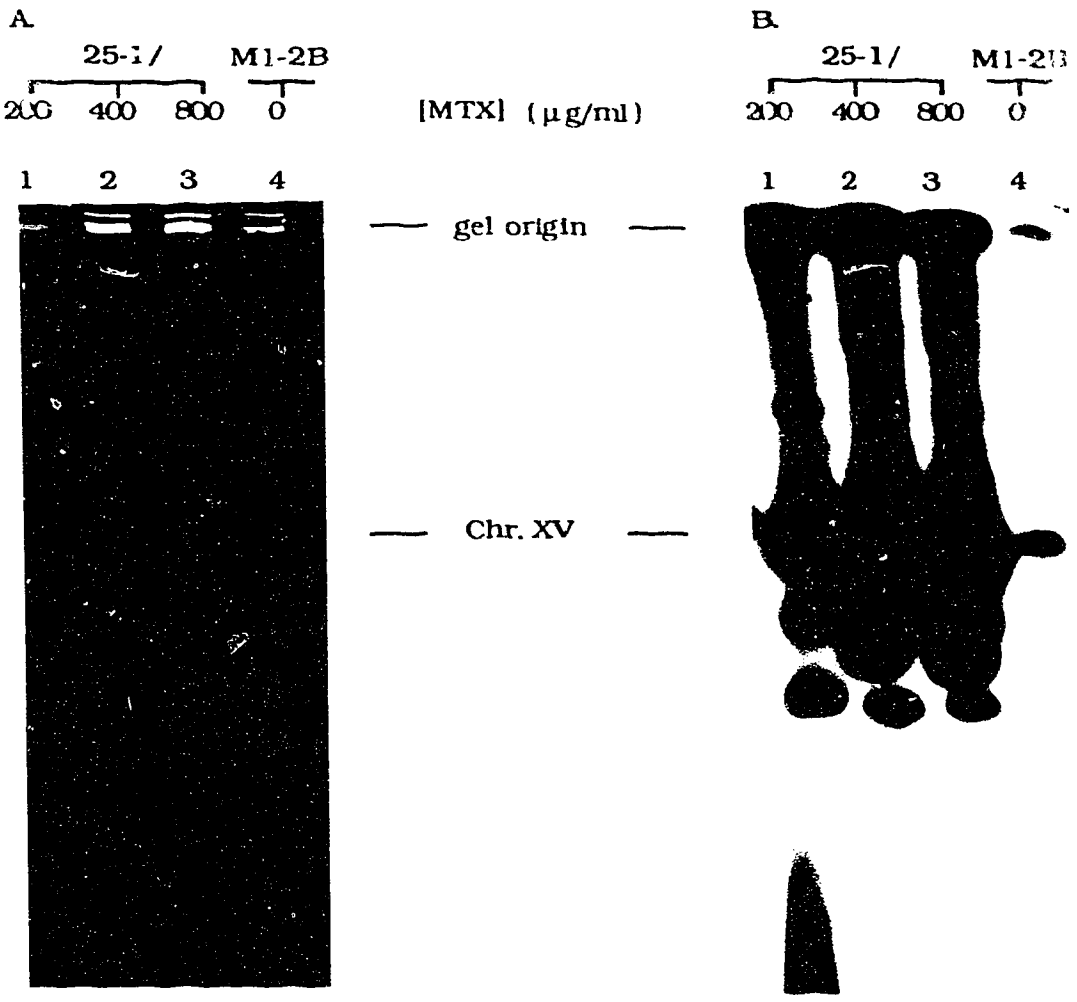


FIG. 14. Pulsed-field gel electrophoresis and Southern hybridization analyses of the *DFR1* amplicons in secondary MTX-resistant mutant cells isolated from 25-1 for resistance to higher MTX concentrations. DNA samples of the MTX-resistant mutants were prepared from cells grown in MC medium supplemented with methotrexate concentrations indicated on the top. (A). The ethidium bromide-stained pulsed-field gel. The BRL CHEF system was used for this pulsed-field gel electrophoresis. Switch time was 70 seconds for 15 hours followed by a 120 second switch time for 12 hours. Electrophoresis was at 150 volts at 8°C. (B). Southern hybridization of the pulsed-field gel shown in (A). DNAs were transferred to a GeneScreen Plus membrane. The membrane was hybridized as recommended by the manufacturer (Du Pont Co.) with a *DFR1*-specific BamHI/HindIII fragment as a probe (see Figure 4). The Southern photograph was resulted from an exposure of about 3 hours in order to show the hybridization signal for the wild-type control M1-2B. Shorter exposure (e.g., 45 minutes) could not reveal any hybridization band for the wild-type control.

at the position corresponding to the hybridization band (see Figure 13), the molecule that carries the amplified *DFR1* gene has to be relatively small. However, a small linear molecule should run out of the gel, but not migrate into this position under the running condition for this pulsed-field experiment. Thus, the mig ing position in the pulsed-field gel and the small size could not be explained if the extrachromosomal element was assumed to be linear. The observations, however, were expected if the extrachromosomal element was a circular molecule. The migration patterns of circular DNAs are known to be different from those of linear DNA molecules in pulsed-field gel electrophoresis. For example, in contrast to linear molecules, migration of small supercoiled circular DNAs in pulse-field electrophoresis is not size- and pulse time-dependent (Hightower et al., 1987). Thus, as observed (Figure 13), even though small in size, the circular DNAs were still migrating within the gel. This behavior was also observed in the secondary cultures selected from the primary resistant mutants for resistance to higher MTX concentrations (see Figure 14). Interestingly, as shown in lane 1 of Figure 14A, most of the chromosomal DNAs were degraded probably due to exonuclease activity. But strong hybridizations were still observed at several positions within the lane (lane 1 of Figure 14B). This suggests that the extrachromosomal molecules seemed to be much more resistant to the degradation. This observation again is consistent with idea that the extrachromosomal element is circular.

The migration patterns of circular DNAs are also different from those of large linear DNA molecules in conventional agarose gel electrophoresis (Mickel et al., 1977). For example, linear molecules larger than 50 kb cannot be resolved by conventional electrophoresis. They usually migrate as a single composite band. On the other hand, the migration patterns of circular DNAs in conventional electrophoresis are size-, topological state- (e.g. relaxed or suppercoiled), and voltage-dependent. Such difference in the migration patterns could be used to distinguish whether the DNA samples were linear or circular. Therefore, the same agarose plugs that were used for the experiment by pulsed-field gel electrophoresis (shown in Figure 14) were also analyzed by conventional gel electrophoresis. As shown in Figure 15A, the only strong distinct ethidium bromide-stained band is the composite chromosomal DNAs that migrated to a position coressponding to the size of about 21-kb linear fragment. However, when this gel was blotted onto a membrane and hybridized with a *DFR1* probe, as shown in Figure 15B and Figure 15C, the strongest hybridization bands were detected at the positions where there was almost no visible ethidium bromide straining. These observations indicate that a small amount of the DNA was present in these positions.

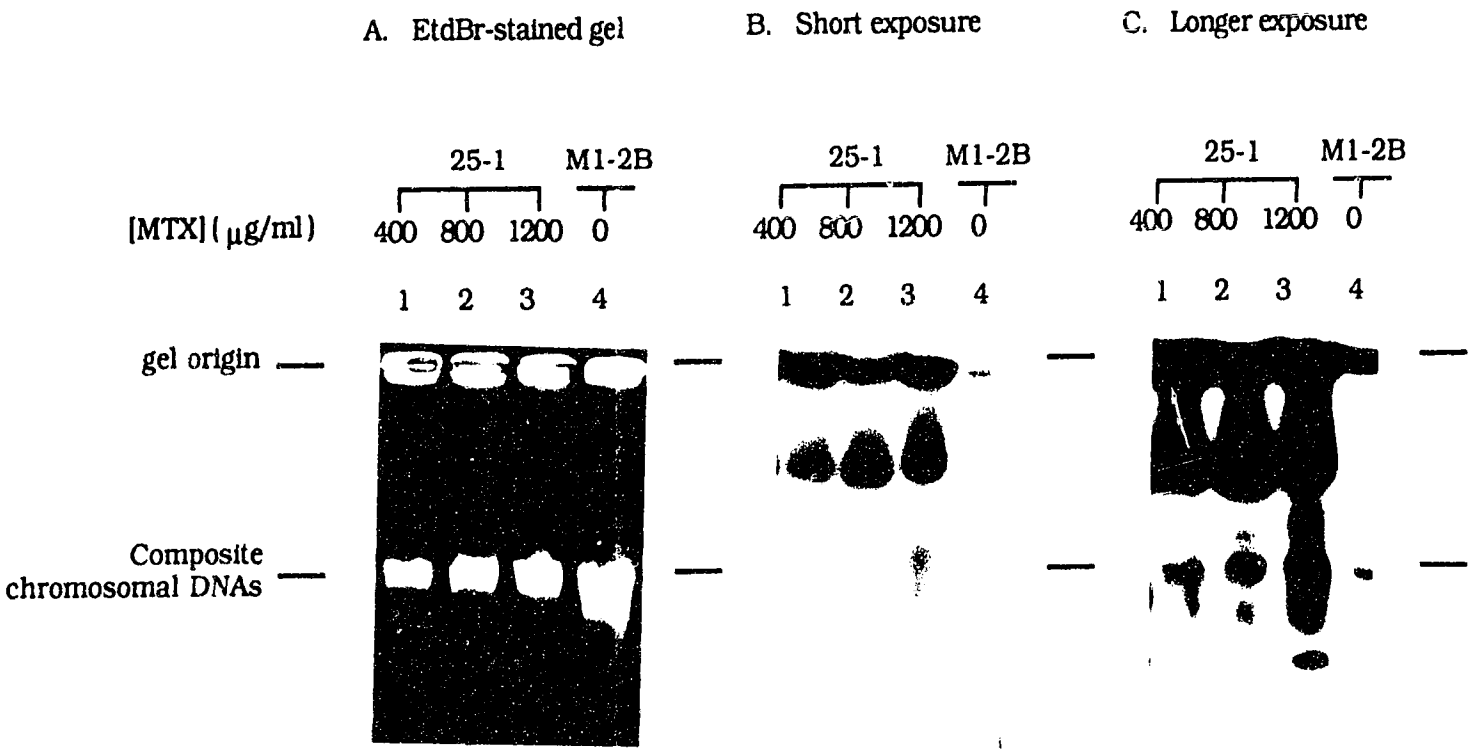


FIG. 15. Analysis by conventional gel electrophoresis of the *DFR1* amplicons in secondary MTX-resistant mutants isolated from 25-1 for resistance to high MTX concentrations. The agarose plugs prepared for the pulsed-field gel electrophoresis shown in Figure 14 were used in this experiment. The gel was 1% agarose which was run at a high voltage of approximate 13 volts/cM for 3 hours, followed by a lower voltage of approximate 2 volt/cM for about 16 hours. The probe used for this experiment was the BamHI/HindIII *DFR1* fragment (Figure 4).

but there was a high concentration of the *DFR1* gene, suggesting that the *DFR1* amplicons seem to present in a small extrachromosomal circular molecule.

IX.E. Isolation and restriction mapping analysis of the extrachromosomal elements that contain the *DFR1* amplicons

If the extrachromosomal elements were indeed circular, one would expect that it should be possible to purify them by methods used for isolation of circular DNAs such as plasmids. The alkaline lysis method described by Ruiz and Wahl (1988) was adapted for the isolation of the extrachromosomal element that contains the *DFR1* gene. The DNA samples prepared by this method from several cultures derived from 25-1 were run in a conventional agarose gel and the ethidium bromide-staining result is shown in Figure 16. In addition to the traces of the composite large chromosome DNAs, three distinct ethidium bromide-stained bands were observed (lane 2 of Figure 16A). One of them migrated slower than that of the composite chromosomal DNAs; while the other one migrated faster. These two bands were strongly hybridized by a *DFR1* probe (Figure 16B and C). They probably represent the different topological states of the same molecule. These observations are expected for a small circular molecule, again, suggesting that the amplified *DFR1* gene is located in a small extrachromosomal circular molecule.

To further determine the structure of the extrachromosomal element, the DNA samples prepared by the alkaline lysis method were analyzed by BamHI restriction enzyme digestion. As shown in Figure 17A, three strong ethidium bromide-staining bands were observed. The digested chromosomal DNA traces were seen as the smear bands. Southern hybridization analysis revealed that two fragments were detected by a *DFR1* probe (see Figure 17B and 17C). From these experiments, it is difficult to identify unambiguously the relationship among these fragments. If the three strong bands were derived from one molecule, this would give a monomer unit size of approximate 9.5-kb (4 + 3.5 + 2) for the extrachromosomal element. The two BamHI fragments detected by the *DFR1* probe are shorter than the wild-type 9-kb BamHI fragment (Barclay et al., 1988). This indicates that two break points had occured within the 9-kb fragment either during the amplification events or during the subsequent rearrangement events.

IX.F. Transformation of a *dfr1* mutant with the extrachromosomal elements that contain the *DFR1* amplicons

If the extrachromosomal elements did contain a functional *DFR1* gene, it would be expected that the extrachromosomal element should be able to transform a *dfr1*

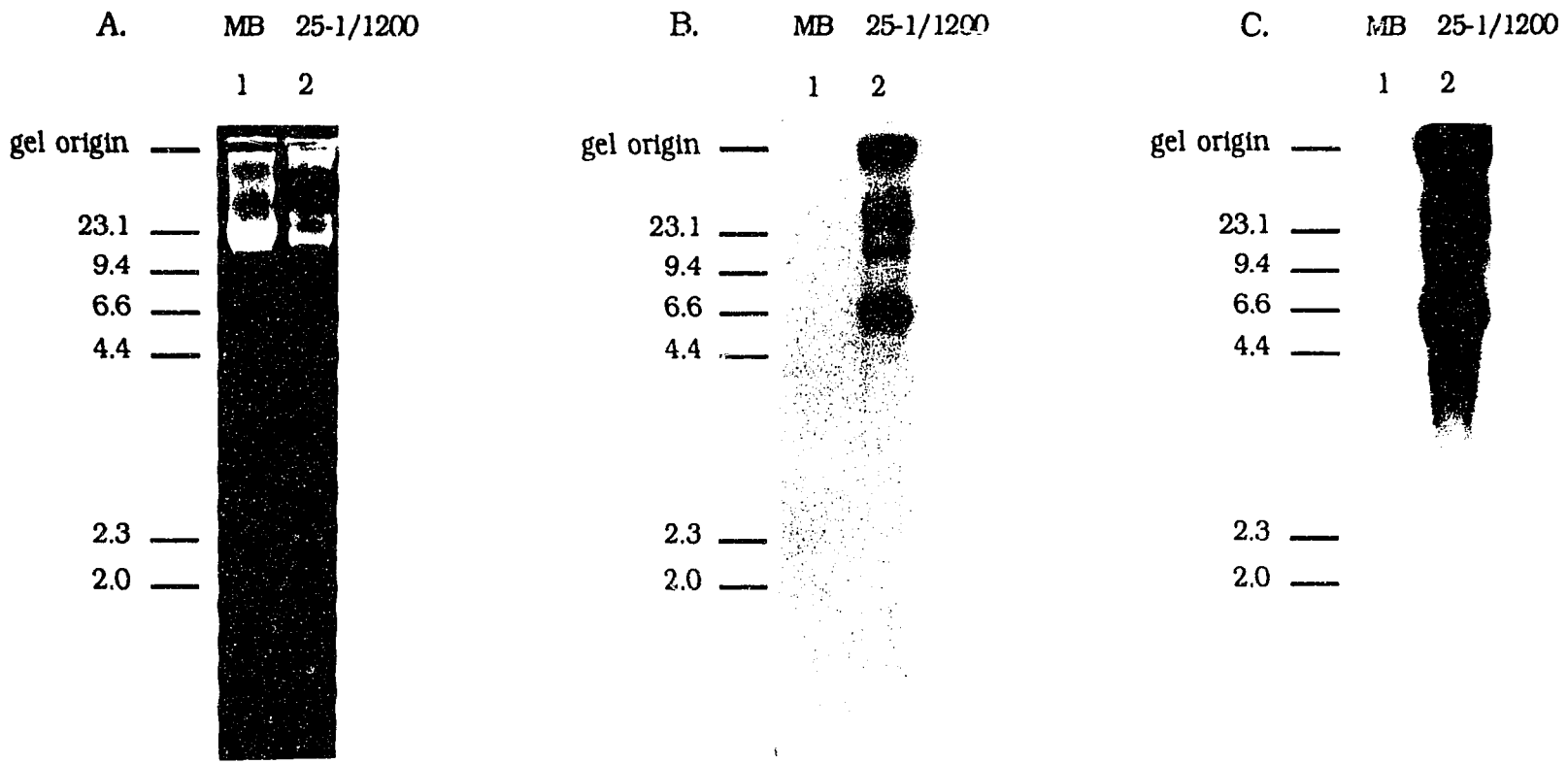


FIG. 16. Conventional gel electrophoresis and hybridization analyses of the DNAs prepared from 25-1/1200 by alkaline lysis method. The gel running condition was the same as in Figure 15.

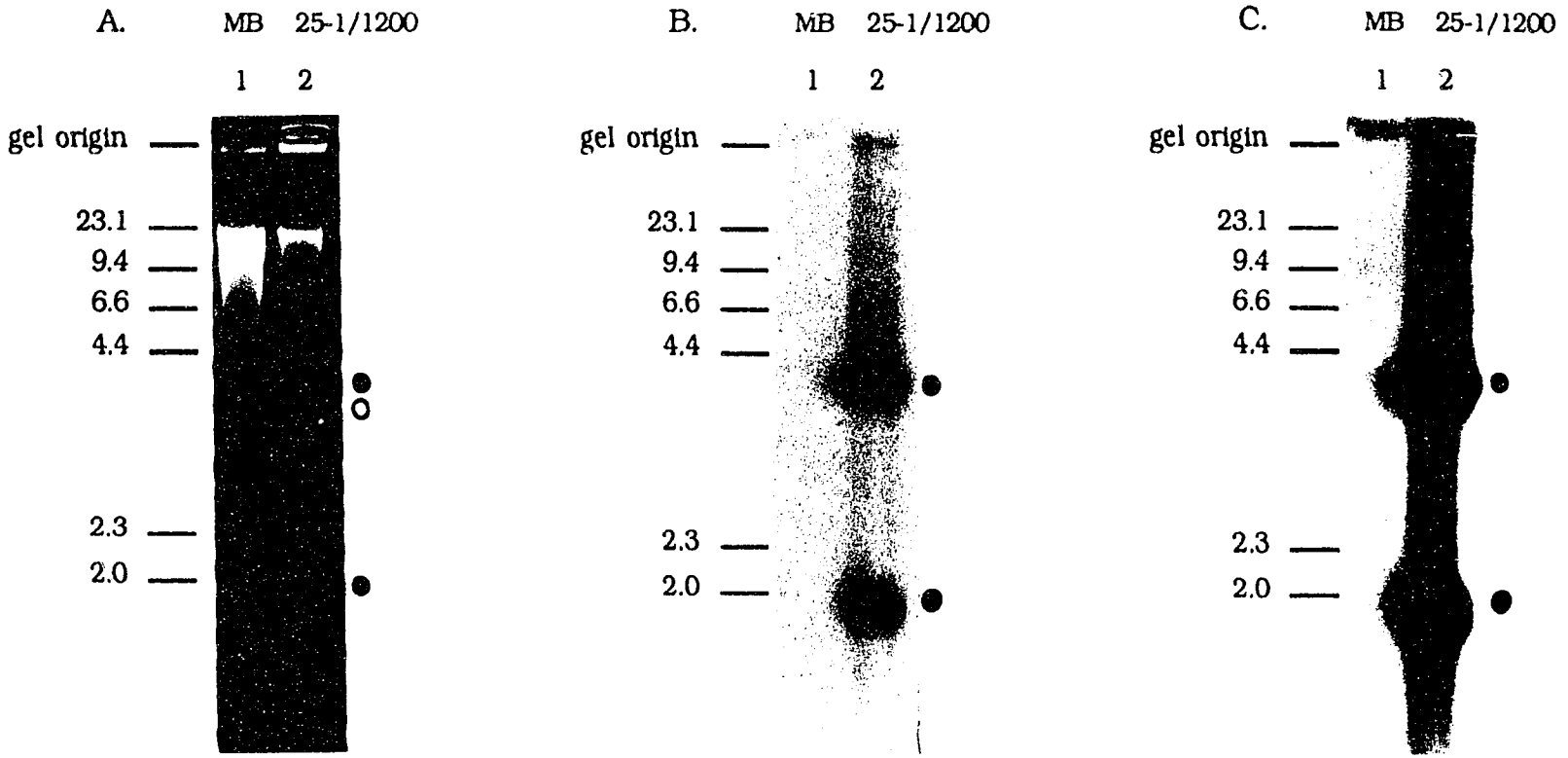


FIG. 17. Restriction mapping analysis of the extrachromosomal elements isolated by alkaline lysis method. The gel running condition was the same as in Figure 15. The three bands were indicated by the dots. The black dots indicate the bands hybridized by the *DFR1* probe. The open dot indicates the band which was not hybridized by the *DFR1* probe.

mutant to wild-type. As described in the previous section, the extrachromosomal element could be isolated by an alkaline lysis method from cultures derived from resistant mutant 25-1. A portion of this DNA preparation was then used to transform the *dfr1* mutant strain TH5 that was constructed in this study. In one transformation experiment with about 0.5 mg of the transforming DNA, 1058 transformants were obtained. These dTMP-independent transformants were recovered on both YEPD and uracil-minus MC plates. The transformation frequency was about 2.1×10^3 transformant/mg DNA. This frequency is compatible with transformation experiments with plasmids that carry an autonomously replicating sequence (ARS). Several conclusions could be made from this transformation experiment. First, the extrachromosomal element contains a functional *DFR1* gene. Second, it has an autonomously replicating sequence. Third, the extrachromosomal element appears to be circular.

IX.G. Chromosomal localization in MTX-resistant mutants as revealed by pulsed-field gel electrophoresis

The chromosomal profiles of MTX-resistant mutants could be obtained following pulsed-field gel electrophoresis on agarose gels. By comparison with the parental strain, it was possible to detect changes in the karyotype of the MTX-resistant mutants. Figure 18 showed the results of such an analysis on several MTX-resistant mutants that resulted from *DFR1* gene amplification. In this experiment, the 16 chromosomes from control strain M1-2B were resolved as 12 ethidium bromide-stained bands (see Figure 18). Obvious changes in the chromosomal migration pattern were observed in resistant mutant R5, R14L, and R14p (indicated in Figure 18A by arrows). An additional large novel chromosome was observed for strain R5 (see lane 1 and lane 5 of Figure 11). All these aberrant bands were consistently observed in separate experiments. None of these aberrant chromosome bands was hybridized by a *DFR1* probe. Changes in chromosomal pattern were not detected in resistant mutants R15, 25-1 and 25-2 under the experimental conditions described (Figure 11 and 18). Thus, chromosomal rearrangements seemed to be frequently observed in MTX-resistant strains.

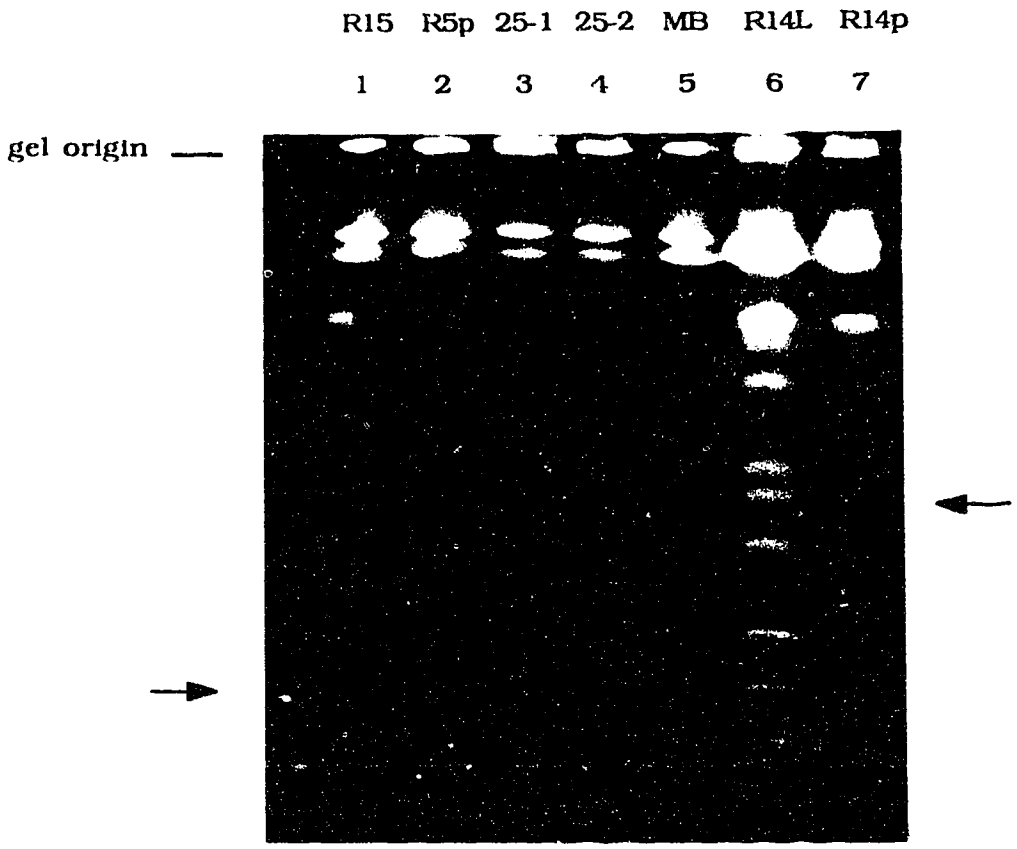


FIG. 18. Analysis of the chromosomal profile of MTX-resistant mutants by pulsed-field gel electrophoresis. DNA samples of the MTX-resistant mutants were prepared from cells grown in MC medium supplemented with 2 µg/ml methotrexate and 5 mg/ml sulfanilamide. Shown is the ethidium bromide-stained pulsed-field gel. The BRL CHEF system was used for this pulsed-field gel electrophoresis. Switch time was 70 seconds for 15 hours followed by a 120 second switch time for 12 hours. Electrophoresis was at 150 volts at 8° C. The aberrant chromosomes are indicated by the arrows. MB is the wild-type parental strain M1-2B. The arrows indicate the novel chromosomes.

DISCUSSION and CONCLUSIONS

I. A yeast *dfr1* mutant deficient in DHFR activity is viable

A mutant of *S. cerevisiae* deficient in DHFR activity has been constructed by gene replacement in this study. Successful isolation of a yeast DHFR-deficient mutant itself shows that a yeast *dfr1* mutant deficient in DHFR activity is viable. It had been speculated that DHFR might be essential for cell viability. Little and Haynes (1979) described a selective scheme by which yeast mutants deficient in either thymidylate synthase activity or dihydrofolate reductase activity could be isolated. In their study, four *tmp1* mutant strains deficient in thymidylate synthase were isolated, as expected. However, they failed to isolate any DHFR-deficient mutant though eleven *fol1* and *fol2* mutant strains were isolated that have a phenotype of folate deficiency. Lack of a DHFR-deficient mutant could be due to the small sample of the mutants isolated. However, there might be the possibility that null mutations leading to DHFR deficiency are lethal to yeast cells. One speculation was that DHFR protein might be an essential component involved in DNA replication. It has been shown both in prokaryotes and in eukaryotes that enzymes involved in synthesis of dNTPs are associated with DNA replication proteins to form a multi-enzyme complex during DNA replication. The function of the multi-enzyme complexes has been proposed to channel dNTPs for DNA replication (Mathews and Slabaugh, 1986). For example, the T4 phage DHFR protein is found as part of the T4 phage multienzyme complex required for dNTP synthesis (Allen, et al., 1983). Genetic evidence reveals that physical interaction of the T4 DHFR protein with replication proteins in the complex is required for normal DNA replication (MacDonald and Hall, 1984). Thus, in addition to its role in DNA precursor synthesis, the T4 phage DHFR protein has a structural role in the formation of a functional multi-enzyme complex involved in DNA replication. In eukaryotic cells, enzymes involved in DNA replication and synthesis of DNA precursors are also present as the multi-enzyme complex (Reddy and Pardee, 1980; Jazwinski and Edelman, 1984). The yeast *CDC8* gene product, dTMP kinase, is present in such a replication complex (Jazwinski and Edelman, 1984) and is an essential protein for DNA replication (Jong et al., 1984; Sclafani and Fangman, 1984). However, the yeast DHFR protein has not been found as part of the complex. Isolation of the viable *dfr1* mutant as described in this study indicates that the yeast DHFR cannot be an essential structural element in the complex required for DNA replication. If it were, a *dfr1* mutant would not be viable.

E. coli DHFR-deficient mutants cannot survive unless they also have a mutation at the *ThyA* gene encoding thymidylate synthase. It has been proposed that

the lethality results from cellular accumulation of dihydrofolate due to DHFR deficiency (Howell et. al.,1988; Ahrweiler and Frieden, 1988). In *S. cerevisiae*, both the *TMP1* gene encoding thymidylate synthase and the *DFR1* gene encoding dihydrofolate reductase were mapped on the right arm of chromosome XV (Barclay 1988; Mortimer et al. 1989). But the two loci are over 50 map units apart. If the *dfr1* transformants were selected as *tmp1 dfr1* double mutants, one would expect the segregation of the *tmp1* marker from the *dfr1* marker in tetrads derived from a diploid generated by a cross of the *dfr1::URA3* transformant with a wild-type haploid. However, segregation of a *tmp1* mutant allele (dTMP requirement) was not observed in the tetrads derived from such a diploid (see Section II.A. of "RESULTS"). Thus, unlike *E. coli* DHFR-deficient mutants, a yeast *dfr1* mutant is viable without a further mutation of the thymidylate synthase gene.

II. The biochemical basis of the *dfr1* auxotrophic phenotype

The auxotrophic phenotype of the *dfr1* mutant has been described in this study. The growth requirements of a *dfr1* mutant could be satisfied by the provision of C1 metabolic end-products (dTMP, adenine, histidine and methionine) in growth medium. In this respect, the mutation mimics the effects of enzymatic inhibition of DHFR activity by antifolate drugs. Thus, the *dfr1* auxotrophic phenotype was as expected for cells deficient in the biosynthesis of tetrahydrofolate due to the lack of DHFR activity. The lack of tetrahydrofolate in the *dfr1* mutant is consistent with the observation that this mutant is deficient in light-dependent DNA repair when its growth was supported by the C1 metabolic end products (Barclay et al., in preparation). A reduced folate has been shown to be required for light-dependent DNA repair (Sancar, 1990).

The dTMP requirement can be explained by the lack of 5,10-methenyl-THF that is required for the thymidylate synthase-catalyzed methylation of dUMP. The adenine requirement stems from the failure of the mutant to make 10-formyl THF that is required for 2 different steps in purine nucleotide biosynthesis. The methionine requirement can be explained by the need for 5-methyl THF for the biosynthesis of this amino acid. The histidine requirement of the *dfr1* mutant is more difficult to explain since none of the enzymes involved in the biosynthetic pathway of histidine requires reduced folate cofactors (Jones and Fink, 1982). This histidine requirement by the *dfr1* mutant seems to have a different cause from that shown by the yeast *ade3* mutant. Yeast *ade3* mutants require both histidine and adenine for growth (Lam and Jones, 1973). This auxotrophic phenotype results from mutations at the *ADE3* gene that encodes the cytoplasmic trifunctional C1-tetrahydrofolate synthase that is required

for interconversion among reduced folate derivatives (McKenzie and Jones, 1977; Staben and Rabinowitz, 1986). It has been shown that 5,10-methylene THF accumulates in *ade3* mutants (Luzzatti, 1975). This [illegible] results in the accumulation of BBMII (phosphoribosylformimino-5-aminoimidazolecarboxamide ribonucleotide), the substrate of the fourth enzyme (BBMII isomerase) in the histidine biosynthesis pathway (E. Jones; cited in Jones and Fink, 1982). Inhibition of the BBMII isomerae activity by 5,10-methylene THF has been confirmed by *in vitro* analysis (E. Jones; cited in Jones and Fink, 1982). However, the *dfr1* mutant should not accumulate any 5,10-methylene THF. One possible explanation for the histidine requirement of the *dfr1* mutant is that the precursors in the histidine biosynthetic pathway derive only from those in the pruine biosynthetic pathway that is tetrahydrofolate-dependent (see Jones and Fink, 1982 for the pathway).

The C1 metabolic end-products required by the yeast *dfr1* mutant differ from those required by the mammalian and *E. coli* mutants. This indicates the difference among these organisms in the pathways involved with folate-mediated one carbon metabolism. Urlaub and Chasin (1980) were the first to describe mutants deficient in DHFR activity. Their Chinese hamster DHFR-deficient mutants were auxotrophic for glycine, a purine and thymidine. This auxotrophic phenotype of the mammalian DHFR-deficient mutants indicates that glycine biosynthesis in mammalian cells is folate-dependent. It is now known that glycine is derived from the major pathway of serine catabolism (Schirch, 1984). Conversion of serine to glycine is catalyzed by the enzyme serine hydroxymethyltransferase (SHMT) with tetrahydrofolate as cofactor. Prototrophy of the yeast *dfr1* mutant for glycine was expected since this organism possesses an alternative folate-independent pathway for glycine biosynthesis (Ullane and Ogur, 1972).

Only thymine is required for the growth of *E. coli* DHFR-deficient (*fol*) mutants (Hamm-Alvarez et al., 1990). Lack of a requirement for other C1 end-products by *E. coli* mutants suggests that there is another pathway, in addition to the DHFR-catalyzed reaction, for the production of tetrahydrofolate in *E. coli* cells (Hamm-Alvarez et al., 1990). The requirement for thymine for growth of *E. coli* DHFR-deficient mutants could be explained by the fact that these viable *E. coli* mutants are double mutants deficient in both dihydrofolate reductase and thymidylate synthase. Lack of the alternate DHFR-independent pathway similar to that found in *E. coli* cells for the biosynthesis of reduced folate in yeast cells is suggested by the auxotrophic phenotype of the *dfr1* mutants.

The surprising finding in characterization of the auxotrophic phenotype of the *dfr1* mutant is the difference in growth response to exogenous folinic acid between a DHFR-deficient mutant and an antifolate drug-induced DHFR deficient strain. It is interesting to note that mammalian DHFR-deficient cells have a similar lack of growth response to exogenous folinic acid (Urlaub and Chasin, 1980; Chasin, personal communication). These observations show that, in this respect, DHFR-deficient mutants are not phenocopies of cells treated with DHFR inhibitors. Lack of the physical presence of DHFR protein in the mutant cells may play a role in this phenotypic difference. Several potential mechanisms underlying the difference can be postulated. For example, antifolate drug-treated wild-type cells may take up exogenous folinic acid normally, but not the mutant cells. This simple explanation would be possible only if the physical presence of a DHFR protein plays a role in folate transport. If folinic acid does get into the cell, it has to be converted into active folate cofactors for biosynthesis of the cellular components that are essential for cell growth. Accordingly, the mutants could be deficient in this ability. This could happen in several different ways. One possibility is that, in addition to its enzymatic activity for reduction of dihydrofolate to tetrahydrofolate, the DHFR protein may have another activity required for the metabolism of exogenous folinic acid. This activity could be retained in spite of the binding of antifolate drug, but absent in DHFR-deficient mutant cells. However, so far there is no evidence suggesting that DHFR has this enzyme activity. Alternatively, intracellular DHFR proteins may be associated with a multi-enzyme complex that consists of enzymes that metabolize folinic acid and perhaps other folates. The function of such an enzyme complex may be affected by the absence of a DHFR protein. Also, inactivation of the enzyme activities responsible for the conversion of folinic acid to active cofactors could result from more efficient accumulation of their inhibitors due to the deficiency in DHFR activity. For example, accumulated dihydrofolate may have such a effect. Finally, it is interesting to consider the possibility that respiratory activity may play a role in the difference. Preliminary observations from this study reveal that some respiratory-sufficient spore clones, as conferred by the *DIR* mutations, are capable of utilizing folinic acid in YEPD medium. Currently, it is not know whether the *DIR* mutations or proficiency in respiration is responsible for the Fou phenotype in *DIR dfr1* mutant strains. In any case, it is of interest to know about the biochemical mechanism underlying the difference in growth response to folinic acid between the DHFR-deficient mutants and an antifolate-induced DHFR-deficient strains. The *fou* suppressor mutation that confers the folinic acid-utilization phenotype on a *dfr1* mutant may represent the further alterations that

allow the *dfr1* mutant cells to utilize exogenous folinic acid. Thus, it is likely that understanding the precise biochemical and genetic mechanisms that underlie the folate-utilization phenotype of the *dfr1* mutant may provide insights into the mechanism of the difference in growth response to folinic acid. In addition, these *four dfr1* mutants would be useful because growth of a *dfr1* mutant in YEPD medium could now be manipulated under different growth conditions.

III. DHFR activity is essential for respiration in yeast

The experimental results from this study and the others (Wintersberger and Hirsch, 1973a; 1973b) have demonstrated that respiratory deficiency was produced by a lack of DHFR activity, either as a result of mutation in the *DFR1* gene or as a result of antifolate treatment. Thus, DHFR activity is essential for respiration in yeast cells. Based on this conclusion, both the DHFR activities found in the mitochondria and in the cytoplasm (Zelikson and Luzzati, 1977) are probably encoded by the nuclear *DFR1* gene. However, there was a possibility that the two compartmentalized DHFR activities are encoded by two different genes. Three mitochondrial enzymatic activities which include the dihydrofolate reductase, thymidylate synthase, and serine transhydroxymethylase were thought to be absent in the yeast *tmp3* mutant (Luzzati, 1975; Zelikson and Luzzati, 1977). It has been shown by complementation tests that a *tmp3* mutation is allelic to a *fol1* mutation (J. Game, cited in Zelikson and Luzzati, 1982; Zelikson and Luzzati, 1982). Genetic mapping data (Mortimer, 1989) and complementation analysis described in this study both indicate that the *DFR1* locus is not allelic to the *FOL1* locus. These observations suggest the possibility that the *DFR1* and *TMP3/FOL1* encode the two DHFR activities in cytosol and mitochondria, respectively. However, no other evidence suggests that the *TMP3/FOL1* gene product encodes all three of the mitochondrial enzyme activities including DHFR activity that were thought to be deficient in the *tmp3* mutant. Lack of the three mitochondrial enzyme activities in *tmp3* mutants can be explained by the fact that the *tmp3* mutant cells used for the enzymatic activity assays (Zelikson and Luzzati, 1977) had become petite. As further demonstrated later by the same authors (Zelikson and Luzzati, 1982), *tmp3* mutant strains were indeed respiratory-deficient when grown in C1 metabolic end products (e.g. dTMP-supplemented YEPD medium).

The conclusion that DHFR activity is essential for respiration in yeast cells raises other interesting questions. For example, why does a lack of DHFR activity results in deficiency in respiration? What role does DHFR play in mitochondrial activity? The DHFR-catalyzed reaction is considered to be the sole pathway for

biogenesis of tetrahydrofolate, which was supported by the observed *dfr1* auxotrophic phenotype (see Section II of "Discussion"). Thus, the respiratory-deficient phenotype of the *dfr1* mutant strains must be intimately related to the folate depletion. There are two potential mechanisms by which folate depletion could lead to respiratory deficiency. First, it may result from inhibition of dTMP biosynthesis. Second, it may result from blockage of mitochondrial protein synthesis. The ultimate consequences of these mechanisms would be the inhibition of respiratory reactions, degradation of mitochondrial genomes, and cessation of mitochondrial biogenesis.

It has been shown that inhibition of pyrimidine nucleotide biosynthesis in *S. cerevisiae* as a result of deficiency in either thymidylate synthase (*tmp1* or *cdc21*) or thymidylate kinase (*cdc8*) caused rapid formation of respiratory-deficient cells (Barclay and Little, 1978; Newlon et al., 1979). A rapid loss of mitochondrial DNA in a thymidylate-starved *tmp1* mutant has been demonstrated (Barclay and Little, 1978). Thus, the effect of a deficiency in the 5,10-CH_2-THF cofactor for the methylation of dUMP to dTMP on the integrity of mitochondrial genomes could account for the respiratory deficiency of the *dfr1* mutants. However, Little and Haynes (1979) have shown that stable respiratory-sufficient *tmp1* mutants were readily obtained as spore clones from respiratory-sufficient heterozygous diploids as long as sufficient dTMP is present in the growth media (e.g. 100 μg/ml in YEPD). This observation suggests that there should be no essential connection between the deficiency in dTMP biosynthesis and a respiratory-deficient phenotype in yeast cells, as long as sufficient dTMP is present in the growth media. The isolation of respiratory-sufficient *tmp1* mutant strains also suggests that uptake of exogenous dTMP, or its derivative, by mitochondria occurs and that it is sufficient to support the replication of mitochondrial genomes. However, as demonstrated in this study, no respiratory-sufficient *dfr1* mutant could be isolated by using a similar method. As sufficient dTMP is present in the growth media, the respiratory-deficient phenotype of the *dfr1* mutant, therefore, cannot be explained by the inhibitory effects of dTMP depletion on the integrity of mitochondrial genomes.

An alternative explanation is that DHFR deficiency affects the initiation step of mitochondrial protein synthesis. Smith and Marcker (1968) demonstrated that initiation of protein synthesis in yeast mitochondria requires formylmethionyl-$tRNA^{fmet}$. The formyl group is donated by 10-formyltetrahydrofolate. Depletion of cellular tetrahydrofolate pools caused by DHFR deficiency is therefore expected to block the initiation step of mitochondrial protein synthesis, which in turn causes respiratory deficiency by suppressing the respiratory reactions and mitochondrial biogenesis. Consistent with this conjecture is the observation that the C1 metabolic

end products cannot support the growth of wild-type yeast strains on non-fermentable carbon source such as glycerol in the presence of the antifolate drugs methotrexate and sulfanilamide.

Based on this simple model, one would predict that folate-deficient strains grown in the presence of folinic acid should have a respiratory-sufficient phenotype. However, as shown in this study, exogenous folinic acid does not support the growth of antifolate-treated cells on glycerol. In addition, the results described in this study reveals that no respiratory-sufficient *dfr1* spore clones were isolated from a respiratory-sufficient diploid heterozygous for the *dfr1* mutation by using folinic acid as a rescue agent. To account for these observations, it is proposed that reduced folates are compartmentalized between cytosol and mitochondria, and can not cross the mitochondrial membranes. Consequently, folinic acid transported into the cytosol would become available only for the biosynthetic reactions, but not for the protein synthesis within mitochondria. Evidence is available which demonstrates that methotrexate and oxidized folates such as dihydrofolate and folic acid are able to cross the mitochondrial membrane, but not fully reduced folates (reviewed in Appling, 1991).

With respect to the interrelationship between folate deficiency and respiratory deficiency, the phenotype of yeast *fol1* and *fol2* mutants isolated by Little and Haynes (1979) is interesting. Similar to the *dfr1* mutant, *fol1* and *fol2* mutants are auxotrophic for the four C1 metabolic end products (dTMP, adenine, histidine and methionine). However, unlike the *dfr1* mutant, these C1 metabolites can be replaced with exogenous folinic acid for the growth requirements. These observations suggest that the *fol1* and *fol2* mutants are defective in folate metabolism. Interestingly, these *fol1* and *fol2* mutants are respiratory-deficient when their growth was supported by the C1 metabolites, but respire normally if they are grown on medium containing folinic acid. This observation suggests that exogenous folinic acid is sufficient to support respiratory function. This implies that exogenous folinic acid and most likely its derivative are available in mitochondria for mitochondrial protein synthesis. So how does a reduced folate become available within mitochondria in these mutants? This is possible if folinic acid is eventually converted into dihydrofolate by the action of the thymidylate synthase activity. Dihydrofolate is then transported into mitochondria and is then converted back to the reduced form by the mitochondrial DHFR activity. This sort of folate interconversion would not expected to occur if there is no DHFR activity within mitochondria as in the case of the *dfr1* mutant cells. These explanations are testable by using *tmp1 fol1* or *tmp1 fol2* double mutants. The

prediction is that both these strains would be petite when grown in medium supplemented with folinic acid.

In addition to the compartmentation model described above, there is an alternative explanation for the failure of exogenous folinic acid to support a respiratory-sufficient phenotype. Because of deficiency in DHFR activity, thymidylate synthase activity would trap folates, derived from the exogenous folinic acid, in the form of dihydrofolate. High concentrations of accumulated dihydrofolates may then inhibit the uptake or metabolism of folinic acid, and ultimately cell growth. The inhibitory effects of dihydrofolate on cell growth and on the activities of some folate enzymes have been demonstrated (Allegra et al., 1986; Howell et. al.,1988; Ahrweiler and Frieden, 1988). However, these effects are not expected to happen in the *fol* mutants as they still have DHFR activity. Similarly, this explanation can be tested by using a *dfr1 tmp1* double mutant. The prediction is that such a double mutant should have a fou phenotype.

The *DIR* mutants isolated in this study would be useful in studying the interrelationship between folate metabolism and respiratory activity. According to the previous discussion, the respiratory-sufficient phenotype of the *DIR* mutants may result from the loss of the requirement for a reduced folate for mitochondrial protein synthesis. This seemed to be supported by the observation from this study that *DIR* mutations confer a respiratory-sufficient phenotype not only to *dfr1* mutant but also to *met7* mutants. How do the *DIR* mutations allow a cell to bypass the folate requirement for mitochondrial activity? In contrast to other bacteria such as *E. coli*, *Streptococcus faecalis* is capable of initiating protein synthesis in the absence of formylation of the $tRNA^{fmet}$ (reviewed in Staben and Rabinowitz, 1984). In addition, *E. coli* mutants have been isolated that use unformylated methionyl-$tRNA^{fmet}$ in the initiation of protein synthesis (reviewed in Staben and Rabinowitz, 1984). The ability to initiate protein synthesis without formylmethionyl-$tRNA^{fmet}$ has been attributed to a new tRNA species. The modified nucleoside ribothymidine is absent in the altered tRNA molecules, which bind more efficiently to ribosomes than the formylmethionyl-$tRNA^{fmet}$ does. These two bacteria have different basis for the altered tRNA. The lack of ribothymidine in the altered tRNA in *S. faecalis* resulted from the lack of folate which is required for the methylation of uridine in tRNA. Deficiency in ribothymidine in the *E. coli* mutant resulted from a decreased levels of a methylase that forms ribothymidine. In *S. cerevisiae*, formylmethionyl- $tRNA^{fmet}$ is encoded by the mitochondrial genome (Staben and Rabinowitz, 1984). As the *DIR* mutations described in this study are of nuclear origin, genetic alterations in the gene encoding the

formylmethionyl-tRNA^{fmet} could not be the reason for the *DIR* mutations. It seems likely that the *DIR* mutations may be the mutations in the genes whose mutant products allow the initiation step to bypass the folate requirement for mitochondrial protein synthesis. Thus, further characterization of the *DIR* mutants would lead to the identification of these genes.

Isolation of mammalian mutants deficient in DHFR activity (Urlaub and Chasin, 1980) raises questions similar to those discussed above. Initiation of mitochondrial protein synthesis in mammalian cells also requires formylmethionyl-tRNA^{fmet} as the initiator (Smith and Marcker, 1968). Thus, one specific aspect related to the mammalian system is that, if mitochondrial activity is considered to be essential for cell growth, why DHFR-deficient mutants are viable (Urlaub and Chasin, 1980) and why cells are able to grow in the presence of methotrexate if the end products of C1 metabolism are supplemented in the growth medium (Wallace and Freeman, 1974). These observations indicate that, in mammalian cells, either the initiation of mitochondrial protein synthesis does not necessarily require formylmethionyl-tRNA^{fmet} or there is an alternate source of 10-formyltetrahydrofolate for mitochondrial protein synthesis.

IV. The biochemical and genetic mechanisms underlying the MTX-resistant phenotype in *S. cerevisiae*

To produce an effect on cell growth, antifolate drugs such as methotrexate must be transported into the cell, converted into their physiologically active forms, and accumulate to an intracellular level high enough to bind to all available target protein. Accordingly, resistance to these antifolate drugs could arise through any mechanisms that alter these processes. The existence of various modes of methotrexate resistance has been demonstrated (reviewed in Albrecht and Biedler, 1984). For example, one major cause of resistance to methotrexate in mammalian cells has been the overproduction of dihydrofolate reductase. This prevents the blocking of all DHFR proteins by methotrexate. Other documented biochemical mechanisms of methotrexate resistance include diminished or abolished uptake of methotrexate that prevents the accumulation of the drug to a toxic level in the cytosol, defective polyglutamylation of methotrexate that decreases cellular retention of this drug, synthesis of an structurally altered DHFR with decreased affinity for methotrexate, and reduced *de novo* thymidylate synthesis with consequent reduction in need for DHFR.

In *S. cerevisiae*, sequential blockage of the tetrahydrofolate biosynthesis pathway by the combined action of methotrexate and sulfanilamide is needed for

effective growth inhibition. Consequently, MTX resistance in this organism could arise, in addition to the schemes described above, through reduction of the inhibitory effects by sulfanilamide. Characterization of the biochemical mechanisms that cause MTX resistance in yeast cells has been very limited so far. As described in this study, the results from preliminary characterization of the MTX-resistant mutants reveal that MTX resistance in yeast cells can be accounted for by more than one mechanism. Overproduction of DHFR could be one of the mechanisms, since the MTX-resistant phenotype could be mediated by *de novo* amplification of the *DFR1* gene. MTX-resistant mutants which do not show amplification of the *DFR1* gene suggest that other biochemical mechanisms are involved. Although it may not be conclusive, the P phenotype described in this study suggests that the drug resistant phenotype may result from detoxifying sulfanilamide through metabolic modification and then secreting it from the cells.

One of the basic questions concerning resistance to antifolate drugs is what genes were altered that allowed a cell to gain the resistant phenotype. If several different biochemical mechanisms could account for the MTX-resistant phenotype, it is conceivable that each biochemical mechanism may result from mutations at a single or more than one genetic locus; whereas different biochemical mechanisms most likely result from mutations at different genetic loci. The well-known genetic alteration underlying the MTX-resistant phenotype has been the amplification of dihydrofolate reductase gene that results in overproduction of dihydrofolate reductase (Ali et al., 1978). Overproduction of dihydrofolate reductase in *E. coli* cells has also been shown to result from mutations in the regulatory region of the dihydrofolate reductase gene (Sheldon and Brenner, 1976). Other than the DHFR gene, genetic loci or genes whose mutant products mediate the MTX-resistant phenotype have been poorly characterized. The results from this study clearly show that MTX resistance in *S. cerevisiae* could result from either amplification of the *DFR1* gene, or mutations at the genetic loci other than the *DFR1* gene. However, more systematical analysis has to be done in order to ascertain whether additional type of genetic events or genetic loci contribute to the antifolate-resistant phenotype. Such analysis should be of significance for studies on the molecular mechanisms of antifolate drug resistance as the yeast system is amendable for both genetic and molecular analysis.

V. The origin of the mutations responsible for the MTX-resistant phenotype

Another basic question concerning antifolate drug resistance is whether the genetic alterations, particularly gene amplification, which underlie the resistant

phenotype occur spontaneously or are induced by the drug selection pressure. This concern is raised partly because antifolate drugs can produce diverse effects on DNA metabolism that in turn could result in the mutations that underlie the MTX-resistant phenotype. This question was addressed in this study by the classical fluctuation method. However, this approach has been complicated by the fact that resistant colonies may arise after plating on the selective medium. For example, the appearance of resistant colonies required a long incubation period (e.g. at least two weeks) to have visible colonies. Such a long period required for the formation of visible primary resistant colonies raises the possibility that the resistant colonies may arise after exposure of cells to the drugs following plating. In addition, because of the low drug concentrations required by the selective scheme used in this study to isolate mutants with amplification mutations, limited residual growth was usually observed when cells were plated into the drug medium. Thus, it is likely that drug-resistant mutants could be induced during the residual growth. Furthermore, continued emergence of drug-resistant colonies has been observed after prolonged incubation. In particular, after incubation for a week to two weeks, cells from a plate on which there are a few visible colonies were replica-plated onto a fresh drug-contained plate, many more resistant colonies on the new plate could be seen after about one week incubation. This observation again seems to indicate that mutations underlying the resistant phenotype may be induced by the antifolate drugs used in selection. If these considerations are correct, calculation of rates for the MTX-resistant mutants based on the fluctuation experiments, as shown in Table 17, may not reflect the actual situation in which the resistant mutants had arisen. Thus, an alternative experimental approach that avoids these problems should be sought in order to address the question about the genetic origin of mutations underlying MTX resistance.

VI. Characteristics of the *DFR1* gene amplification events

Although many questions remain to be answered, the characteristics of *DFR1* amplification events can be described based on the observations obtained from this study as follows:

1). The observations described in this study demonstrate that it is important to use the selection conditions described (e.g. 25 μg/ml MTX in YEPD medium) in order to isolate yeast mutants that have undergone *DFR1* amplification. These observations suggest that primary amplification events generate only one or a few extra copies of the *DFR1* gene and such primary amplification events can only be selected within a narrow range of low methotrexate concentrations. It is, then, possible that lack of rapid

evolution from the primary low-level amplification events at the very early stage of an amplification process to subsequent higher-level amplification may prevent the selection of mutants that have undergone *DFR1* amplification with relatively high MTX concentrations (e.g., 100 μg/ml in YEPD medium).

2). *DFR1* amplicons have been found either within an extrachromosomal element, or within a chromosome (the native chromosome XV or its derivative). This characteristic is similar to that observed in mammalian systems. The interrelationship between chromosomal and extrachromosomal *DFR1* amplicons is still not so clear. However, it is known that mutants that carry chromosomal *DFR1* amplicons do not necessarily have extrachromosomal *DFR1* amplicons. This observation suggests that these chromosomal *DFR1* amplicons may be generated either without an extrachromosomal element or with a transient extrachromosomal intermediate. In addition, it is not certain whether the primary mutants that carry extrachromosomal *DFR1* amplicons also contain chromosomal *DFR1* amplicons. However, as shown in Figure 14 and 15, in positions corresponding to the chromosomal DNAs, there are stronger hybridizations in samples grown in higher MTX concentrations. If these hybridization signals did result from the chromosomal amplicons, they may result either from further amplification of the initial chromosomal amplicons or from integration of the extrachromosomal amplicons. Based on the results described in this thesis, direct evidence has not been obtained on whether the extrachromosomal element that contains *DFR1* amplicons was produced as a direct product of the initial amplification event, or as a subsequent product derived from the initially formed chromosomal *DFR1* amplicons. The extrachromosomal *DFR1* amplicons were, however, detected in the early passage cells of the original mutant colony. This observation, therefore, suggests that if the circular extrachromosomal element that contains the *DFR1* amplicon was not the first amplification product, it must be produced at the very early stage of the amplification event.

3). There is a general trend that an increase in resistant level is associated with an increase in the *DFR1* gene copy number. The number copies of *DFR1* amplicons have been observed in a range from one extra copy to over one hundred copies. The high number copies of the *DFR1* gene were observed in secondary resistant mutants selected for higher MTX resistance from the primary mutant that carries extrachromosomal *DFR1* amplicons. Such high levels of amplification have not been observed in mutants that contain only chromosomal amplicons. Even though the observations obtained so far are still limited, they seem to suggest, however, that the nature of an amplification

event (such as extrachromosomal amplification vs intrachromosomal amplification) may have strong influences on how rapidly the primary amplification mutant can progress into higher levels of amplification.

4). The extrachromosomal element that carries *DFR1* amplicons seems most likely a circular molecule. This conclusion was reached based on several observations. First, its behavior on a pulsed-field gel electrophoresis was as expected for a small circular molecule. Second, its behavior on a conventional gel electrophoresis mimics that of a circular molecule. Third, it could be isolated by methods used for plasmid isolation such as an alkaline lysis protocol. Fourth, it was more resistant to degradation (probably due to exonuclease activity). Fifth, it transformed a *dfr1* mutant to wild-type and produced a transformation efficiency similar to that produced by an autonomously replicating plasmid.

5). Transformation of a *dfr1* mutant by the extrachromosomal elements suggests that, in addition to a functional *DFR1* gene, there must be an autonomously replicating sequence (ARS) within the molecules. Two different *DFR1* amplicons have been identified in the extrachromosomal elements in cells that were selected for higher-levels of MTX resistance. However, it is still not certain whether they are located on a single circular molecule or two different circular molecules. Presently, other than the *DFR1* sequence, it is not known whether the additional sequences in the extrachromosomal element were derived from the *DFR1* region or from somewhere else in the genome. Nevertheless, the ultimate source for amplified *DFR1* sequences found in the extrachromosomal element must be derived from the chromosomal *DFR1* region.

6). The structure of chromosomal *DFR1* amplicons remains to be fully characterized. Chromosomal *DFR1* amplicons with different sizes have also been observed. The size of the chromosomal *DFR1* amplicons in some mutants seem to be at least of 9-kb (see Figure 8). Currently, it is still not known about how the chromosomal *DFR1* amplicons in resistant mutant R14S are organized. Since the mutant R14S was derived from R14, the novel structures detected in R14S (see Figure 10 and Figure 12) must be the consequence of secondary events after the initial *DFR1* amplification in the primary mutant R14. Detection of the smaller BamHI fragment that contains a *DFR1* gene in conventional Southern hybridization experiment (see Figure 10) may indicate that the break point of a rearrangement event must be close to the *DFR1* gene. The nature of the larger size of the novel chromosome (lane 5 of Figure 12) is not clear. The possibility of being a dicentric chromosome derived from chromosome XV was suggested by the fact that there is no extra copy of the *DFR1* gene could be detected by using the *ADH4* probe as a control for the copy number of the *DFR1* gene (Figure 9).

Additional probes specific for the different regions of the chromosome XV are needed for the further characterization of this novel chromosome in mutant R14S.

7). When compared with other amplification events observed in *S. cerevisiae* (see Section II.F. of "LITERATURE REVIEW"), the frequency of *DFR1* amplification event (approximately 3×10^{-7}) is relatively common at least in the presence of methotrexate. Many of the characteristics summarized above have yet been described in the yeast system.

8). Based on the observations obtained so far, it is still premature to formulate any detailed molecular mechanisms that underlie the process of *DFR1* amplification. It is, however, important to remember that the mutants that have undergone *DFR1* amplification were derived from a haploid strain which is wild-type with respect to the *DFR1* locus. Thus, the *de novo DFR1* amplification events described in this study begin with a single copy of the *DFR1* gene before replication. The chromosome breakage model for gene amplification proposed by Windle and Wahl (1992) accommodates the results obtained so far.

VIII. Future prospects

Taken together, the *dfr1*, *fou*, *DIR* and mtx^R mutants described in this study extend significantly the list of *S. cerevisiae* mutants which have an altered phenotype with respect to folate metabolism. Importantly, this study demonstrates that a combined genetic and molecular approach is powerful for studies on folate metabolism. Further characterization of these mutants holds great prospects for understanding the role of folate metabolism in various cellular activities (such as respiration and DNA repair), and the molecular mechanism of antifolate drug resistance.

The other significant contribution of this study is the demonstration of *de novo* amplification of dihydrofolate reductase gene in yeast MTX-resistant mutants. This observation has important implications for research in several different areas (see Section II.E. of "LITERATURE REVIEW"). First of all, this is one of the few systems available for studies on the mechanism of gene amplification in *S. cerevisiae*. As demonstrated in this study, the characteristics of the *DFR1* amplification event in many respects mimic those observed in mammalian cells. Thus, the phenomenon of gene amplification, a major type of genetic alterations within a cell, can now be investigated in *S. cerevisiae*, an excellent eukaryotic model system. As indicated in the previous discussion, to better understand the molecular mechanism(s) of gene amplification, it would be essential to know more about the molecular structures of the *DFR1* amplicons. In addition, by taking the advantages of the well defined genetic

system of the yeast system, it should be possible now to identify and characterize gene products that participate in the process of gene amplification.

Bibliography

Ahrweiler, P. M., and C. Frieden. 1988. Construction of a *fol* mutant strain of *Escherichia coli* for use in dihydrofolate reductase mutagenesis experiments. J. Bacteriol. 170: 3301-3304.

Aladjem, M., Kollin, Y., and S. Lavi. 1988. Enhancement of copper resistance and *Cup1* amplification in carcinogene-treated yeast cells. Mol. Gen. Genet. 211:88-94.

Albrecht, A. M., and J. L. Biedler. 1984. Acquired resistance of tumor cells to folate antagonists, p. 317-353. In Sirotnak, F. M., Burchall, J. J., Ensminger, W. B., and J. A. Montgomery (ed.), Folate antagonists as therapeutic agents, Vol1. Academic Press.

Allen, J. R., Lasser, G. W., Goldman, D. A., Booth, J. W., and C. K. Mathews. 1983. T4 phase deoxyribonucleotide-synthezing enzyme complex: further studies on enzyme composition and regulation. J. Biol. Chem. 258: 5746-5753.

Alitalo, K., 1984. Amplification of cellular oncogenes in cancer cells. Medical Biology 62: 304-317.

Alitalo, K., and M. Schwab, 1986. Oncogene amplification in tumor cells. Adv. Cancer Res. 47:235-281.

Allegra, C. J., Baram, J., and B. A. Chabner. 1986. Evidence for direct inhibition of metabolic pathways as a mechanism of action of methotrexate, p. 981-984. In B. A. Cooper and V. M. Whitehead (ed.), Chemistry and biology of pteridines. Walter de Gruyter, New York.

Alt, F. W., Kellems, R. E., Bertino, J. R., 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.

Amler, L. C., and M. Schwab. 1989. Amplified N-myc in human neuroblastoma cells is often arranged as clustered tandem repeats of differently recombined DNA. Mol. Cell. Biol. 9:4903-4913.

Anachkova, B., and J. L. Hamlin, 1989 Replication in the amplified dihydrofolate reductase domain in CHO cells may initiate at two distinct sites, one of which is a repetitive sequence element. Mol. Cell. Biol. 9: 532-540.

Anderson, R. P.; and J. R. Roth. 1977. Tandem genetic duplications in phage and bacteria. Annu. Rev. Microbiol. 31:473-505.

Appling, D. R. 1991. Compartmentation of folate-mediated one-carbon metabolism in eukaryotes. The FASEB Journal 5:2645-2651.

Ardeshir, F., Giulotto, E., Zieg, J., Brison, O., Liao, W. S. L., and G. R. Stark, 1983. Structure of amplified DNA in different Syrian hamster cell lines resistant to N-(phosphonacetyl)-L-aspartate. Mol. Cell. Biol. 3: 2076-2088.

Barclay, B. J., and J. G. Little. 1977. Selection of yeast auxotrophs by thymidylate starvation. J. Bacterial. 132:1036-1037.

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., Huang, T., Nagel, M. G., Misener, V. L., Game, J. C., and G. M. Wahl. 1988. Mapping and sequencing of the dihydrofolate reductase gene (*DFR1*) of *Saccharomyces cerevisiae*. Gene 63:175-185.

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

Bebbington, G. R., and C. C. G. Hentschel. 1987. The use of vectors based on gene amplification for the expression of cloned genes in mammalian cells. p. 163-188. In D. M. Glover (Ed.), DNA cloning, Vol III, A practical approach. IRL Press, Oxford, U.K.

Biedler, J. L., Melera, P. W., and B. A. Spengler. 1980. Specifically altered metaphase chromosomes in antifolate-resistant Chinese hamster cells that overproduce dihydrofolate reductase. Cancer Genet. Cytogenet. 2:27-60.

Blakley, R. L. (ed.) 1969. The biochemistry of folic acid and related pterdines. North-Holland Publishing Co., Amsterdam.

Blakley, R. L., and S. J. Benkovic. (ed.) 1984. Folates and pterins. Vol. 1. A Wiley-Interscience Publication, John Wiley & Sons.

Carle, G. F., and M. V. Olson, 1987. Orthogonal-field-alternation gel electrophoresis. Methods Enzymol. 155:468-482.

Carlson, M., and D. Botstein. 1982. Two differentially regulated mRNAs with different 5' ends encode secreted and intracellular forms of yeast invertase. Cell 28:145-154.

Carroll, S. M., Gaudray, P., De Rose, M. L., Emery, J. F., Meikoth, J. L., Nakkim, E., Subler, M., Von Hoff, D. D., and G. M. Wahl. 1987. Characterization of an episome produced in hamster cells that amplify a transfected CAD gene at high frequency: functional evidence for a mammalian replication origin. Mol. Cell Biol. 7:1740-1750.

Carroll, S. M., De Rose, M. L., Gaudray, P., Moore, C. M., Needham-VanDevanter, D. R., Von Hoff, D. D., and G. M. Wahl. 1988. Double minute chromosomes can be producted from precursors derived from a chromosomal deletion. Mol. Cell Biol. 8:1525-1533.

Casadaban, M. J., Martinez-Arias, A., Shapira, S. K., and J. Chou. 1983. Beta-galactosidase gene fusion for analyzing gene expression in Escherichia coli and yeast. Methods in Enzymol. 100:293-308.

Chasin, L. A., Graf, L., Ellis, N., Landzberg, M., and G. Urlaub. 1982. Gene amplification in dihydrofolate reductase-deficient mutants. p. 161-166. *In* R. T. Schemke (ed.), Gene Amplification. Cold Spring Harbor Laboratories, Cold Spring Harbor, New York.

Chu, G., Vollrath, D., and R. W. Davis. 1986. Separation of large DNA molecules by contour-clamped homogeneous electric fields. Science 234:1582-1585.

Cossins, E. A. 1984. Folates in biological materials. p. 1-59. In Blakley, R. L., and S. J. Benkovic (ed.), Folates and pterins, Vol1. A Wiley-Interscience Publication.

Costanzi, C. and D. Gillespie. 1987. Fast blots: immobilization of DNA and RNA from cells. Methods Enz. 152:582-587.

Cowell, J. K., 1982. Double minutes and homogeneously staining regions: gene amplification in mammalian cells. Annu. Rev. Genet. 16: 21-59.

Cramer, J. H., Farrelly, F. W., and R. H. Rownd. 1976. Restriction endonuclease analysis of ribosomal DNA from *S. cerevisiae*. Mol. Gen. Genet. 148:233-241.

Dorsey, M., Peterson, C., Bray, K., and C. E. Paquin. 1992. Spontaneous amplification of the *ADH4* gene in *Saccharomyces cerevisiae*. Genetics 132:943-950.

Fäth, W. W., M. Brendel, W. Laskowski, E. Lehmann-Brauns. 1974. Economizing DNA-specific labelling by deoxythymidine-5'-monophosphate in *Saccharomyces cerevisiae*. Mol. Gen. Genet. 132:335-345.

Federspiel, N. A., Beverley, S. M., Schilling, J. W., and R. T. Schimke, 1984. Novel DNA rearrangements are associated with dihydrofolate reductase gene amplification. J. Biol. Chem. 259: 9127-9140.

Feinberg, A. P., and B. Vogelstein. 1983. Labelling DNA fragments with Klenow and hexadeoxyribonucleotides. Anal. Biochem. 132:6-13.

Fling, M. E., Kopf, J., and C. A. Richards. 1988. Nucleotide sequence of the dihydrofolate reductase gene of *Saccharomyces cerevisiae*. Gene 63:165-174.

Fogel, S., and J. W. Welch. 1982. Tandem gene amplification mediates copper resistance in yeast. Proc. Natl. Acad. Sci. USA 79:5342-5346.

Fogel, S., Welch, J. W., Cathala, G., and M. Karin. 1983. Gene amplification in yeast: *CUP1* copy number regulates copper resistance. Curr. Genet. 7:347-355.

Ford, M., and M. Fried. 1986. Large inverted duplications are associated with gene amplification. Cell 45:425-430.

Giulotto, E., Saito, I., and G. R. Stark. 1986. Structure of DNA formed in the first step of CAD gene amplification. EMBO J. 5:2115-2121.

Giulotto, E. Knight, C., and G. R. Stark. 1987. Hamster cells with increased rates of DNA amplification, a new phenotype. Cell 48:837-845.

Giulotto, E. Bertoni, L. Attolini, C., Ranaldi, G., and M. Anglana. 1991. BHK cell lines with increased rates of gene amplification are hypersensitive to ultraviolet light. Proc. Natl. Acad. Sci. USA 88:3484-3488.

Greer, H., and G. R. Fink. 1979. Unstable transposition of *his4* in yeast. Proc. Natl. Acad. Sci. USA 76:4006-4010.

Grivell, A. R., and J. F. Jackson. 1968. Thymidine kinase: evidence for its absence from *Neurospora crassa* and some other microorganisms and the relevance of this to the specific labelling of DNA. J. Gen. Microbiol. 54:307-317.

Guarente, L., and M. Ptashne. 1981. Fusion of *Escherichia coli lacZ* to the cytochrome a gene of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA 78:2199-2203.

Gudkov, A. V., and B. P. Kopnin, 1987. Gene amplification and multidrug resistance. Sov. Sci. Rev. Sect. D Physiochem. Biol. 7: 95-136.

Hamlin, J. L., Milbrandt, J. D., Heintz, N. H., and J. C. Azizkhan, 1984. DNA sequence amplification in mammalian cells. Int. Rev. Cytol. 90: 31-82.

Hamm-Alvarez, S., Sancar, A., and K. V. Rajagopalan. 1990. The presence and distribution of reduced folates in *Escherichia coli* dihydrofolate reductase mutants. J. Biol. Chem. 265:9850-9856.

Hahn, P., Morgan, W. F., and R. B. Painter. 1987. The role of acentric chromosome fragments in gene amplification. Somatic Cell. Molec. Genetics 13:597-608.

Hahn, P. Nevaldine, B., and W. F. Morgan. 1990. X-ray induction of methotrexate resistance due to *dhfr* gene amplification. Somatic Cell. Molec. Genetics 16:413-423.

Hansche, P. E. 1975. Gene duplication as a mechanism of genetic adaptation in *Saccharomyces cerevisiae*. Genetics 79:661-674.

Hansche, P. E., and V. Beres. 1978. Gene duplication in *Saccharomyces cerevisiae*. Genetics 33:673-687.

Hartwell, L. 1992. Defects in a cell cycle checkpoint may be responsible for the genomic instability of cancer cells. Cell 71:543-546.

Hightower, R. C., Metge, D. W., and D. V. Santi. 1987. Plasmid migration using orthogonal-field-alternation gel electrophoresis. Nuc. Acids Res. 15:8387-8398.

Howell, E. E., Foster, P. G., and L. M. Foster. 1988. Construction of a dihydrofolate reductase-deficient mutant of *Escherichia coli* by gene replacement. J. Bacteriol. 170:3040-3045.

Huang, T. 1987. Primary structure and expression studies of the dihydrofolate reductase gene (*DFR1*) of *Saccharomyces cerevisiae*. M.Sc. thesis. Department of Biological Sciences, Brock University, St. Catharines, Ont. Canada.

Hughes, J. M. X., Konings, D. A. M., and G. Cesareni. 1987. The yeast homologue of U3 snRNA. EMBO 6:2145-2155.

Hunt, J. D., Valentine, M., and A. Tereba. 1990. Excision of N-myc from chromosome 2 in human neuroblastoma cells containing amplified N-myc sequences. Mol. Cell. Biol. 10:823-829.

Hyrien, O., Debatisse, M., Buttin, G., and B. R. de Saint Vincent. 1988. The multicopy appearance of a large inverted duplication and the sequence at the inversion joint suggest a new model for gene amplification. EMBO J. 7:407-417.

Ito, H., Fukuda, Y., Murata, K., and A. Kimura. 1983. Transformation of intact yeast cells treated with alkalai cations. J Bacteriol 153:163-168.

Jannsen, S., Witte, I., and R. Megnet. 1973. Mutants for the specific labelling of DNA in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta. 299:681-685.

Jazwinski, S. M., and G. M. Edelman. 1984. Evidence for participation of a multiprotein complex in yeast DNA replication *in vitro*. J. Biol. Chem. 259:6852-6857.

Johnston, R. N., and B. Kucey. 1988. Competitive inhibition of hsp70 gene expression causes thermosensitivity. Science 242:1551-1554.

Johnston, R. N., Beverley, S. M., and R. T. Schimke. 1983. Rapid spontaneous dihydrofolate reductase gene amplification shown by fluorescence-activated cell sorting. Proc. Natl. Acad. Sci. USA 80:3711-3715.

Jong, A. Y. S., Kuo, C. L., and J. L. Campbell. 1984. The *CDC8* gene of yeast encoding thymidylate kinase. J. Biol. Chem. 259:11052-11059.

Jones, E. W., and G. R. Fink. 1982. Regulation of amino acid and nucleotide biosynthesis in yeast. p. 181-299. *In* J. Strathern, E. Jone and J. Broucl (ed.). The molecular biology of the yeast Saccharomyces metabolism and gene expression. Cold Spring Harbor Laboratory Press. NY.

Kaufman, R. J., Wasley, L. C., Spiliotes, A. J., Gossels, S. D., Latt, S. A., Larsen, G. R., and R. M. Kay. 1985. Coamplification and coexpression of human tissue-type plasminogen activator and murine dihydrofolate reductase sequences in Chinese hamster ovary cells. Mol. Cell. Biol. 5:1750-1759.

Kaufman, R. J., Murtha, P., Ingolia, D. E., Yeung, C-Y., and R. E. Kellems. 1986. Selection and amplification of heterologous genes encoding adenosine deaminase in mammalian cells. Proc. Natl. Acad. Sci. USA 80:3136-3140.

Klein, H. L., and T. D. Petes. 1981. Intrachromosomal gene conversion in yeast. Nature 289:144-148.

Kunz, B. A., Barclay, B. J., Game, J. C., Little, J. G., 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. A., Taylor, G. R., Konforti, B., Glickman, B. W., and R. H. Haynes. 1984. Inhibition of thymidylate biosynthesis induces mitotic unequal sister chromatid recombination in Saccharomyces cerevisiae. Curr. Genet. 8:211-217.

Kunz, B. A., Taylor, G. R., and R. H. Haynes. 1986. Intrachromosomal recombination is induced in yeast by inhibition of thymidylate biosynthesis. Genetics 114:375-392.

Lagosky, P. A., Taylor, G. R., and R. H. Haynes. 1987. Molecular characterization of the *Saccharomyces cerevisiae* dihydrofolate reductase gene (*DFR1*). Nucl. Acids Res. 15:10355-10371.

Lam, K. B., and E. W. Jones. 1973. Mutations affecting levels of tetrahydrofolate interconversion enzymes in *Saccharomyces cerevisiae*. I. Enzyme levels in *ade3-41* and *ADE15*, a dominant adenine auxotroph. Mol. Gen. Genet. 123:199-208.

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.

Lavi, S. 1981. Carcinogen mediated amplification of viral DNA sequences in SV40 transformed Chinese hamster embryo cells. Proc. Natl. Acad. Sci. USA 78:6144-6148.

Leu, T.-H., and J. L. Hamlin. 1989. High-resolution mapping of replication fork movement through the amplified dihydrofolate reductase domain in CHO cells by in-gel renaturation analysis. Mol. Cell. Biol. 9:523-531.

Little, J. G., and R. H. Haynes. 1979. Isolation and characterization of yeast mutant auxotrophic for 2' deoxythymidine 5'-monophosphate. Mol. Gen. Genet. 168:141-151.

Livingstone, L. R., White, A. Sprouse, J., Livanos, E., Jacks, T., and T. D. Tlsty 1992. Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. Cell 70:923-935.

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

Ma, C., Looney, J. E., Leu, T.-H., and J. L. Hamlin, 1988. Organization and genesis of dihydrofolate reductase amplicons in the genome of a methotrexate-resistant Chinese hamster ovary cell line. Mol. Cell. Biol. 8: 2316-2327.

MacDonald, P. M., and D. H. Hall. 1984. Genetic evidence for physical interactions between enzymes of nucleotide synthesis and proteins involved in DNA replication in bacteriophage T4. Genetics 107:343-353.

MacPhee, D. G., Haynes, R. H., Kunz, B. A., and D. Anderson. (ed.) 1988. Genetic aspects of deoxyribonucleotide metabolism. Mutat. Res. vol. 200.

Maeda, N., and O. Smithies. 1989. The evolution of multigene families: human haptoglobin genes. Annu. Rev. Genet. 20:81-108.

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

Mathews, C. K., Scrimgeour, K. G., and F. M. Huennekens. 1963. Dihydrofolate reductase. Methods in Enzymol. 6:364-368.

Mathews, C. K., and M. B. Slabaugh. 1986. Eukaryotic DNA metabolism: are deoxyribonucleotides channeled to replication sites? Exp. Cell. Res. 162:285-295.

McBurney, M. W., and G. F. Whitmore. 1974a. Isolation and biochemical characterization of folate-deficient mutants of Chinese hamster cells. Cell 2:173-182.

McBurney, M. W., and G. F. Whitmore. 1974b. Characterization of a Chinese hamster cell with a temperature-sensitive mutation in folate metabolism. Cell 2:183-188.

McGuire, J. J., and J. K. Coward. 1984. Pteroylpolyglutamates: biosynthesis, degradation, and function, p. 135-190. In Blakley, R. L., and S. J. Benkovic (ed.), Folates and pterins, Vol1. A Wiley-Interscience Publication.

McKenzie, K. Q., and E. W. Jones. 1977. Mutants of the formyltetrahydrofolate interconversion pathway of *Saccharomyces cerevisiae*. Genetics 86:85-102.

Mickel, S., Arena, Jr. ,V., and W. Bauer. 1977. Physical properties and gel electrophoresis of R12-derived plasmid DNAs. Nuc. Acids Res. 4:1465-1482.

Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Mortimer, R. K., Schild, D., Contopoulou, C. R., and J. A. Kans. 1989. Genetic and physical maps of *Saccharomyces cerevisiae*, Edition 10. Yeast 5:321-403.

Newlon, C. S., Ludescher, R. D., and S. K. Walter. 1979. Production of petites by cell cycle mutants of *Saccharomyces cerevisiae* defective in DNA synthesis. Mol. Gen. Genet. 169:189-194.

Passananti, C. B., Davis, B., Ford, M., and M. Fried. 1987. Structure of an inverted duplication formed as a first step in a gene amplification event, implications for a model of gene amplification. EMBO J. 6:1697-1703.

Paquin, C. E., Dorsey, M., Crable, S., Sprinkel, K., Sondej, M., and V. M. Williamson. 1992. A spontaneous chromosomal amplification of the *ADH2* gene in *Saccharomyces cerevisiae*. Genetics 130:263-271.

Pauletti, C., Lai, E., and G. Attardi. 1990. Early appearance and long-term persistence of the submicroscopic extrachromosomal elements (amplisomes) containing the amplified DHFR genes in human cell lines. Proc. Natl. Acad. Sci. USA 87:2955-2959.

Petes, T. D. 1979. Yeast ribosomal DNA genes are located on chromosome XII. Proc. Nat. Acad. Sci. USA 76:410-414.

Petes, T. D. 1980. Unequal meiotic recombination within tandem arrays of yeast ribosomal DNA genes. Cell 19:765-774.

Reddy, G. P. V., and A. B. Pardee. 1980. Multienzyme complex for metabolic channeling in mammalian DNA replication. Proc. Natl. Acad. Sci. USA 77:3312-3316.

Rose, M., and D. Botstein. 1983. Construction and use of gene fusion to lacZ (β-galactosidase) that are expressed in yeast. Methods in Enzymol. 101:167-180.

Rose, M. et al., 1987. A Saccharomyces cerevisiae genomic plasmid band based on a centromere-containing shuttle vector. Gene 60:237-243.

Rothstein, R. J. 1983. One-step gene disruption in yeast. Meth. Enzymol. 101:202-210.

Ruiz, J. C., and G. M. Wahl. 1988. Formation of an inverted duplication can be an initial step in gene amplification. Mol. Cell. Biol. 8:4302-4313.

Ruiz, J. C., and G. M. Wahl. 1990. Chromosomal destablization during gene amplification. Mol. Cell. Biol. 10:3056-3066.

Saito, I., Groves, R., Giulotto, E., Rolfe, M., and G. R. Stark. 1989. Evolution and stability of chromosomal DNA coamplified with the CAD gene. Mol. Cell. Biol. 9:2445-2452.

Sancar, G. B. 1990. DNA photolyases: Physical properties, action mechanism, and roles in dark repair. Mutation Res. 236:147-160.

Schimke, R. T. 1984a. Gene amplification, drug resistance, and cancer. Cancer Res. 44:1735-1742.

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

Schimke, R. T. 1986. Methotrexate resistance and gene amplification: mechanisms and implications. Cancer 57:1912-1917.

Schimke, R. T. 1988. Gene amplification in cultured cells. J. Biol. Chem. 263: 5989-5992.

Schirch, L. 1984. Folates in serine and glycine metabolism, p. 379-431. In Blakley, R. L., and S. J. Benkovic (ed.), Folates and pterins, Vol1. A Wiley-Interscience Publication.

Schweizer, E. MacKechnie, C., and H. O. Halvorson. 1969. The redundancy of ribosomal and transfer RNA genes in *S. cerevisiae*. J. Mol. Biol. 40:261-277.

Sclafani, R. A., and W. L. Fangman. 1984. Yeast gene *CDC8* encodes thymidylate kinase and is complemented by herpes thymidine kinase gene *TK*. Proc. Natl. Acad. Sci. USA 81:5821-5825.

Sen, S., Hittleman, W. N., and L. D. Teeter. 1989. A model for the formation of double minutes from prematurely condensed chromosomes of replicating micronuclei in drug treated cells undergoing DNA amplification. Cancer Res. 49:6731-6737.

Shane, B. 1986. Identification of folylpoly(γ-glutamate) chain length by cleavage to and separation of p-aminobenzoypoly(γ-glutamates). Methods in Enzymol. 122:323-330.

Shane, B. 1989. Folylpolyglutamate synthesis and role in the regulation of one-carbon metabolism. Vitam. Horm. 45:263-335.

Sheldon, R., and S. Brenner. 1976. Regulatory mutants of dihydrofolate reductase in E. coli K12. Mol. Gen. Genet. 147:91-97.

Sherman, F., Fink, G. R., and J. Hicks. 1984. Methods in yeast genetics. Cold spring Harbor Laboratory, Cold Spring Harbor, New York.

Singer, S., Ferone, R., Walton, L., and L. Elwell. 1985. Isolation of dihydrofolate reductase-deficient mutant of *Escherichia coli*. J. Bacteriol. 164:470-472.

Smith, A. E., and K. A. Marcker. 1968. N-formylmethionyl transfer RNA in mitochondria from yeast and rat liver. J. Mol. Biol. 38:241-243.

Smith, K. A., Gorman, P. A., Stark, M. B., Groves, R. P., and G. R. Stark. 1990. Distinctive chromosomal structures are formed very early in the amplification of CAD genes. Cell 63:1219-1227.

Staben, C., and J. C. Rabinowitz. 1984. Formation of formylmethionyl-tRNA and initiation of protein synthesis. p457-496. In Blakley, R. L., and S. J. Benkovic (ed.), Folates and pterins, Vol1. A Wiley-Interscience Publication.

Staben, C., and J. C. Rabinowitz. 1986. Nucleotide sequence of the *Saccharomyces cerevisiae ADE3* gene encoding C1-tetrahydrofolate synthase. J. Biol. Chem. 261:4629-4637.

Stark, G. R. 1986. DNA amplification in drug resistant cells and in tumors. Cancer Surv. 5:1-23.

Stark, G. R., and G. M. Wahl. 1984. Gene amplification. Ann. Rev. Biochem. 53: 447-491.

Stark, G. R., Debatisse, M., Giulotto, E., and G. M. Wahl. 1989. Recent progress in understanding mechanisms of mammalian DNA amplification. Cell 57: 901-908.

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

Szostak, J. W., and R. Wu. 1980. Unequal crossing over in the ribosomal DNA of Saccharomyces cerevisiae. Nature 284:426-430.

Taylor, R. T., and M. L. Hanna. 1977. Folate-dependent enzymes in cultured Chinese hamster ovary cells: Folylpolyglutamate synthetase and tis absence in mutants auxotrophic for glycine + adenosine + thymidine. Arch. Biochem. Biophys. 181:331-344.

Tlsty, T. D., Margolin, B., and K. Lum. 1989. Differences in the rates of gene amplification in nontumorigenic and tumorigenic cell lines as measured by Luria-Delbrück fluctuation analysis. Proc. Natl. Acad. Sci. USA 86:9441-9445.

Tlsty, T. D. 1990. Normal diploid human and rodent cells lack a detectable frequency of gene amplification. Proc. Natl. Acad. Sci. USA 87:3132-3136.

Toledo, F., Smith, K. A., Buttin, G., and M. Debatisse. 1992a. The evolution of the amplified adenylate deaminase 2 domain in Chinese hamster cells suggests the sequential operation of different mechanisms of DNA amplification. Mutat. Res. 276:261-273.

Toledo, F., Roscouet, D. L., Buttin, G., and M. Debatisse. 1992b. Coamplified markers alternate in megabase long chromosomal inverted repeats and cluster independently in interphase nuclei at early steps of mammalian gene amplification. The EMBO J. 11:2665-2673.

Trask, B. J., and J. R. Hamlin. 1989. Early dihydrofolate reductase gene amplification events in CHO cells usually occur on the same chromosome arm as the original locus. Genes & Dev. 3:1913-1923.

Ulane, R, and M. Ogur. 1972. Genetic and physiological control of serine and glycine biosynthesis in Saccharomyces. J. Bacteriol. 109:34-43.

Urlaub, G., and L. A. Chasin. 1980. Isolation of Chinese hamster cell mutants deficient in dihydrofolate reductase activity. Proc. Natl. Acad Sci. USA 77:4216-4220.

Urlaub, G., Kas, E., Carothers, A. M., and L. A. Chasin. 1983. Deletion of the dihydrofolate reductase locus from cultured mammalian cells. Cell 33:405-412.

von Borstel, R. C. 1978. Measuring spontaneous mutation rates in yeast. Methods in Cell Biol. 20:124.

Wahl, G. M., de Saint Vincent, B. R., and M. DeRose. 1984. Effect of chromosomal position on amplification of transfected genes in animal cells. Nature 307:516-520.

Wahl, G. M., 1989. The importance of circular DNA in mammalian gene amplification. Cancer Res. 49:1333-1340.

Wallace, R. B., and K. B. Freeman, 1974. Inhibition of mammalian mitochondrial protein synthesis: The effect of methotrexate. Biochem. Biophys. Acta. 366:466-473.

Walton, J. D., Paquin, C. E., Kaneko, K., and V. M. Williamson. 1986. Resistance to antimycin A in yeast by amplification of *ADH4* on a linear, 42 kb palindromic plasmid. Cell 46:857-863.

Welch, J. W., Fogel, S., Cathala, G., and M. Karin, 1983. Industrial yeasts display tandem gene iteration at the CUP1 region. Mol. Cell. Biol. 3:1353-1361.

Wickner, R. B. 1974. Mutants of *Saccharomyces cerevisiae* that incorporate deoxythymidine 5'-monophosphate into DNA in *vivo*. Methods in cell Biol. 11:295-302.

Windle, B., and G. M. Wahl. 1992. Molecular dissection of mammalian gene amplification: new mechanistic insights revealed by analysis of very early events. Mutat. Res. 276:199-224.

Windle, B., Draper, B. W., Yin, Y., O'Gorman, S., and G. M. Wahl. 1991. A central role for chromosome breakage in gene amplification, deletion formation, and amplicon integration. Genes & Development 5:160-174.

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

Wintersberger, U., and J. Hirsch. 1973b. Induction of cytoplasmic respiratory deficient mutants in yeast by the folic acid analogue, methotrexate. II. genetic analysis of the methotexate-induced petites. Mol. Gen. Genet. 126:71-74.

Wright, J. A., Smith, H. S., Watt, F. M., Hancock, M. C., Hudson, D. L., and G. R. Stark. 1990. DNA amplification is rare in normal human cells. Proc. Natl. Acad. Sci. USA 87:1791-1795.

Yin, Y., Tainsky, M. A., Bischoff, F. Z., Strong, L. C., and G. M. Wahl. 1992. Wild-type p53 restores cell cycle control and inhibits gene amplification in cells with mutant p53 alleles. Cell 70:937-948.

Zelikson, R., and M. Luzzati. 1977. Mitochondrial and cytoplasmic distribution in *Saccharomyces cerevisiae* of enzymes involved in folate coenzyme mediated one carbon group transfer: a genetic and biochemical study of the enzyme deficiencies in mutant *tmp3* and *ade3*. Eur J. Biochem. 79:285-292.

Zelikson, R., and M. Luzzati. 1982. Influence of the nuclear gene *tmp3* on the loss of mitochondrial genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 2:457-466.

Apppendix: Distribution of MTX-resistant mutants in individual cultures*

Culture number	Total number of colony in each individual culture			
	M1-2B in YEPD-mtx25	M1-2B in MC-mtx2	TH100 in YEPD-mtx25	TH100 in MC-mtx2
1	4	2	9	1
2	39	2	24	0
3	15	10	7	1
4	17	0	11	0
5	22	2	14	0
6	2	0	11	0
7	41	2	5	0
8	19	0	15	0
9	42	0	6	0
10	19	5	10	2
11	17	0	7	0
12	30	0	1	0
13	33	0	5	2
14	29	2	6	1
15	38	0	10	3
16	26	0	6	1
17	7	3	11	0
18	26	1	29	0
19	5	1	23	0
20	34	5	27	0
21	21	1	29	1
22	16	0	14	1
23	2	0	12	2
24	43	0	15	0
25	0	0	17	7
26	38	0	7	0
27	9	0	16	3
28	29	1	12	2
29	27	1	10	0

Apppendix continued

Culture number	Total number of colony in each individual culture			
	M1-2B in YEPD-mtx25	M1-2B in MC-mtx2	TH100 in YEPD-mtx25	TH100 in MC-mtx2
30	15	2	11	2
31	12	0	28	3
32	11	0	4	1
33	22	1	16	0
34	1	1	17	1
35	24	1	17	0
36	3	1	29	1
37	18	1	1	1
38	34	3	11	1
39	25	1	17	0
40	17	6	13	3
41			8	0
42			15	0
43			26	1
44			16	0
45			16	0
46			7	2
47			9	2
48			15	0
49			3	0
50			6	0
51			5	
52			18	
53			22	
54			14	
55			11	
56			10	
57			1	
58			1	

Apppendix continued

Culture number	Total number of colony in each individual culture			
	M1-2B in YEPD-mtx25	M1-2B in MC-mtx2	TH100 in YEPD-mtx25	TH100 in MC-mtx2
59			7	
60			2	
61			7	
62			23	
63			22	
64			6	
65			3	

* These are the data used for the analysis summarized in Table 17.