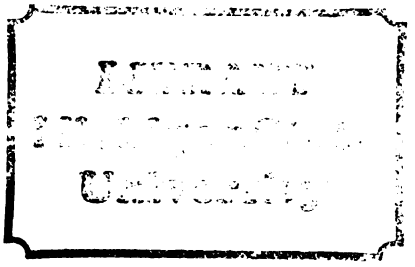
THESIS



This is to certify that the

thesis entitled

ISOLATION AND CHARACTERIZATION OF AN
APHIDICOLIN-RESISTANT MUTATOR MUTANT
OF CHINESE HAMSTER CELLS
presented by

Philip Kuocherng Liu

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Genetics

James E. Trosko

James E. Trosko, Ph.D.
Major professor

Date September 22, 1981

O-7639

ISOLATION AND CHARACTERIZATION OF AN APHIDICOLIN-RESISTANT MUTATOR MUTANT OF CHINESE HAMSTER CELLS

By

Philip Kuocherng Liu

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Genetics Interdepartmental Program

1981

ABSTRACT

ISOLATION AND CHARACTERIZATION OF AN APHIDICOLIN-RESISTANT MUTATOR MUTANT OF CHINESE HAMSTER CELLS

By

Philip Kuocherng Liu

Studies in T4 bacteriophage, prokaryotes and lower eukaryotes have demonstrated that DNA polymerase is involved in spontaneous and induced mutagenesis. However, due to the lack of suitable polymerase mutants, the role of the DNA replicative enzyme, polymerase α, in mutagenesis is unknown in mammalian somatic cells.

A direct selection method using aphidicolin, a specific polymerase α inhibitor, has been used to isolate aphidicolin-resistant (aph^r) mutants (Chang et al., 1981, Somatic Cell Genet. 7:235-253) at a frequency of 5 per 10^7 clonable cells from a population of Chinese hamster V79 cells previously treated with bromodeoxyuridine/black light-ultraviolet light (UV). One of these aph^r mutants, i.e., aph^r-4, exhibits pleiotropic effects such as slow growth, thymidine (TdR) auxotrophy, a high sensitivity to UV, cytidine, TdR, deoxy-guanosine or -adenosine and a high frequency of site-specific bromodeoxyuridine-dependent chromosomal aberrations. Revertants which retain part of the mutant characteristics have been isolated. Further characterization of aph^r-4 mutants is reported here.

Subclones of aph^r-4 mutant used in the present studies were aph^r-4-2, aph^r-4-R2, aph^r-4-RP4 and aph^r-4-RP5. Aph^r-4-2 cell line had similar pleiotrophic phenotypes as the aph^r-4 cells. Of the revertants isolated, aph^r-4-R2 is a fast growing TdR auxotroph and has similar deoxycytidine triphosphate (dCTP) levels as aph^r-4-2 cells; aph^r-4-RP4 and -RP5 cells are TdR prototrophs. The mutagen-sensitivity was also determined in aph^r-4-2, aph^r-4-R2 and V79 cells. As compared to the V79 cells, aph^r-4-2 was sensitive to UV, N-acetoxy-2-acetylaminofluorene (NAcAAF) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), but not sensitive to X-ray and dimethyl sulfate (DMS), and aph^r-4-R2 was sensitive to NAcAAF and MNNG, but not to UV, X-ray and DMS.

The UV-induced mutability of aph^r-4-2, aph^r-4-R2 and V79 cells was determined in 3 genetic loci measuring forward mutations from sensitive to resistant to ouabain (oua), diphtheria toxin (DT) and 6-thioguanine (6TG). The results showed that the mutant was hypermutable for oua^r and DT^r mutations compared to V79 cells at the same UV dose or at the same survival level. The mutant exhibits a delayed expression of maximal frequencies of induced 6TG^r mutants. When maximal frequencies were compared at the same UV dose, the mutant also had higher 6TG^r mutation frequencies. The revertant was similar to the V79 in UV sensitivity and mutability.

The ability to repair UV damage, as measured by incorporation of ^{3}H-TdR, in aph^r-4-2, aph^r-4-R2, aph^r-4-RP4 and the V79 cells was the same. Furthermore, the V79 cells showed liquid holding recoveries as measured by UV survival and induced mutation frequencies at oua^r and DT^r mutation loci, but aph^r-4-2 cells did not.

Spontaneous mutation rates were also determined in aph^r-4-2, aph^r-4-R2, aph^r-4-RP4 and -RP5 and V79 cells. The spontaneous mutation rates at 3 genetic markers determined by fluctuation analysis were 1-14 x 10^{-8} per cell per division in the V79 and aph^r-4-R2 cells, whereas those in the UV-sensitive aph^r-4 variants were

5 to 30 times higher. Similar results were obtained in DT^r and $6TG^r$ loci using a modified Newcomb's multiple replating technique.

It appears that the mutator activities of UV-sensitive aph^r-4 variants are not locus specific and cannot be explained by TdR auxotrophic phenotype nor by dCTP pools. A defective enzymatic function in these mutator mutants that rendered DNA replication or repair "error-prone" is suggested.

To

my mother, S. Y. Chao

and

my dear friend, Dr. C. M. Kao

ACKNOWLEDGMENTS

For their invaluable guidance, encouragement, support and friendship throughout the course of this study and my graduate training, I wish to express my appreciation to Dr. Chia-cheng Chang and Dr. James E. Trosko. I have benefited greatly from the perception and persistence shown by each of them. I thank Dr. Chang for providing me superior technical and intellectual training. I thank Dr. Trosko for his generous theoretical discussions and scientific freedom to pursue my ideas.

I also thank the other members of my graduate committee, Dr. Veronica M. Maher and Dr. Loren Snyder for the interest and time that they contributed to this project and my education. I am deeply grateful for their advice and friendship.

Special thanks to my colleagues and friends: Drs. Gen Tsushimoto, Peggy Wade, Ron Wilson, Thomas Glover, Larry Yotti, Roger Schultz, Stephen Warren and Andy Medcalf, as well as Ms. Judy Funston, Carolyn Hunt, Carol Lassila, Betty Dawson, Beth O'Malley, Gina Lu, Joyce Carter, Clara Rubinstein, Mr. Gary Ferenchick, and to Ms. Judy Copeman for her excellent typing assistance; last, but not least, to Ms. R. Severance who reviewed this manuscript and to Ms. Lily Chen for her assistance in computer programing.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The integrity of the genome requires a faithful replication and maintenance of deoxyribonucleic acids (DNA) in an organism. Mutations occur when the base sequences of DNA are altered or deleted. Mutations in somatic cells of the mammals have now been implicated in disease states, such as the carcinogenic (22,54,269,270), atherosclerotic (13) and aging processes (31,53,79,120,156,243,260). Recently, an increasing amount of attention has been focused on the toxicologic and mutagenic properties of chemicals present in our environment. Two observations have generated these interests. The first observation was the discovery that mutagenic compounds exist in our environment and most mutagens are carcinogenic in the mammals, and the second one was the awareness that environmental factors (epigenetic factors) are involved in the expression of genetic information during developmental processes (270,275). Thus, an understanding of mutagenesis and gene expression becomes essential for understanding the origin of chronic diseases.

Advances in mutation research during the past two decades have revealed two major mechanisms of mutagenesis: DNA misrepair during repair synthesis and directly induced base mispairing during DNA replication (64-67). Mutants sensitive to mutagens or deficient in DNA repair are especially useful for the analysis of the mechanism of mutagenesis. Studies in bacterial mutants have provided information about the complexity of mutagenic pathways including evidence for the existence of both constitutive and inducible "error-free" and "error-prone" DNA repair enzymes (111,296), but evidence for these pathways remains to be ascertained in the mammalian systems.

Because of the lack of useful mutants in mammalian cells, most of the studies are limited to mutant cells derived from some human syndromes found or suspected to be defective in DNA repair (5,49,86,117,160,202,216,217,232,286,287). Several techniques have been reported recently whereby mutagen-sensitive or repair-defective mutants of mammalian cells in culture have been induced, isolated and partially characterized (34,233-235,264,265). Similar to prokaryotic systems, three types of repair mechanisms have been reported to exist in mammalian cells: i.e., excision, photoreactivation and post-replication (or bypass replication) repair pathways (112). Studies with xeroderma pigmentosum and other normal or repair deficient mammalian cells have generally concluded that excision repair is an error-free process (167,186,234). Unlike bacterial cells, convincing evidence for the existence of an inducible error-prone repair mechanism in mammalian cells has not been demonstrated (37). Furthermore, the existence of a discrete post-replication repair system in mammalian cells has been questioned (50,51). However, in spite of the controversy on the mechanism of DNA repair pathways, some of those lesions seem to be substrates for mutation fixation.

It has been postulated that excision repair can be rendered error-prone if the deoxyribonucleoside triphosphates, i.e., DNA precursors, are not balanced (123). The effect of exogenous pyrimidines on induced mutagenesis could be attributed to enhanced base-mispairing (176) and/or inhibition of poly(ADP-ribose) polymerase which has been shown to be implicated in DNA repair process (70,71,237). The perturbation of nucleotide pools may also mediate spontaneous mutagenesis. Mutations induced by BrdU or MNNG have been shown to be modifiable by the presence of exogenous TdR or cytidine (61,207,208). Evidence suggests that bromodeoxyuridine (BrdU) mutagenesis is determined by the concentration of BrdU to which the cells are exposed instead of by the amount of bromouracil substitutions (140,257).

Genetic control of spontaneous mutator activity has been reviewed in prokaryotes (56,64-67,111,303) and eukaryotes (115,282,283). The mutator activity of these genetic loci may be mediated through a diversified function such as altered DNA precursor pools (175,288), defective DNA polymerase (56,67,124,240,246,247), a defective activity in post-replication methylation of DNA (90,91,171), or uracil-DNA glycosylase (69), so that errors of base mispairing are not recognized and effectively removed. Theoretically, misreplication might occur under three conditions: a) the presence of a defective DNA polymerase; b) the presence of base analogues or base modification; and c) the imbalance of deoxyribonucleotide pools. The involvement of altered DNA polymerase and DNA precursor pools in the mutagenic process in mammalian cells was not demonstrated until recent years.

In Chinese hamster cells, a high exogenous concentration of thymidine (TdR) is both toxic and mutagenic (23,257). On the other hand, Brennard and Fox (25) reported that an excess of TdR is toxic but not mutagenic to Chinese hamster cells. Evidence provided by Meuth et al., (175) and Weinberg et al., (288) suggests that an elevated dCTP level in the mammalian rodent cells may be associated with a spontaneous mutator activity. Although an elevated spontaneous mutation rate has been reported in cells derived from patients with Bloom's syndrome (97,282,283), the mechanism for this activity is not known.

Aphidicolin, a tetracylic diterpenoid antibiotic obtained from a species of fungi, is a specific inhibitor of polymerase α. The inhibition on purified α-polymerase is competitive with dCTP (106,125,193,194). Studies in human cells indicate that aphidicolin inhibits repair replication (16,47,110) or removal of pyrimidine dimers in ultraviolet light irradiated cells (248), although conflicting evidence has also been reported (89,205,236). The drug appears to be useful for the selection of mutants that have altered DNA polymerase α (190,259) or nucleotide

pools (7,8,10,40,226). Both types of mutations are conceivably able to influence the fidelity and accuracy of DNA replication and repair.

Recently, aphidicolin-resistant mutants have been isolated from several organisms (7,8,10,40,190,226,259). The mutants were found to have an elevated level of dATP (7) or altered DNA polymerase α activity (190,259). But none of them has been shown to be mutagen sensitive. In our laboratory, an UV sensitive aphidicolin-resistant mutant has been isolated (40). The mutant also possesses several other interesting phenotypes such as thymidine auxotrophy, cytidine sensitivity and site specific BrdU-dependent chromosomal abberrations.

The present study was undertaken to further characterize this mutant, especially to answer the following: a) is the induced mutability in this mutant different from that of V79 cells? b) does this mutant possess mutator activity? c) what are the phenotypes of its revertants? and d) what might be the basis for the phenotypic changes of this mutant?

LITERATURE REVIEW

Biological Consequences of DNA Damage and Mutation

The observable biological consequences of DNA damage depend not only on the types of lesions encountered by the cell but also on the location of the lesions in relation to each other or to the replication fork. The lesions in eukaryotes can be produced in non-transcribable regions such as introns that affect transcription of DNA to RNA, RNA processing, or nothing at all (silent mutation). The lesions may produce disruption to replication if they occur near replicons and cause the eventual generation of a change in the complementary DNA sequence. They can be located in the DNA strand near another lesion, such as pyrimidine dimers in the opposite strand. If these two lesions are repaired simultaneously, the gaps generated may overlap each other, producing a lethal or mutagenic double-strand break. Clearly, simply identifying and quantifying lesions will not be sufficient to predict the biological consequences with certainty.

Biologically, this alteration in DNA may result in cell death or gene mutation. The mutated gene may or may not show any biological effect. Some unexpressed mutations can be converted by some chemicals, such as tumor promoters, and become expressed (146,153,184,270,275). The reverse process can also be achieved by certain chemicals, such as antipromoters (141,270). The accumulated damage to DNA caused by mutagens is thought by some investigators to be a major contribution to most of the chronic diseases found in technological societies, such as cancer (144,211,269-271), teratogenic events (178,230), atherosclerosis and heart disease (13), neurological disorders (3) and aging (31,79,120,211,260).

Analysis of Gene Mutation

1. Quantitative Mutation Assays in Mammalian Somatic Cells In Vitro

The quantitative study of experimental mutagenesis in mammalian cells in vitro was first reported independently in 1968 by three laboratories, all using Chinese hamster cells (42,138,238). These and subsequent developments in experimental mutagenesis, cell hybridization and intercellular gene transfer using in vitro cell culture provide not only a simple method to study genetic control of mutagenesis and gene expression in human and mammalian cells, but also a new bioassay for environmental mutagens and carcinogens. The development of mutation assay systems requires that genetic markers for mutant selection be available. The selective systems in somatic cell genetics developed in the past have been thoroughly reviewed by Chu and Powell (46) and others (4).

Most of the genetic markers used in mutation assays are drug resistant markers, such as resistance to ouabain (Na^+/K^+-ATPase), 6-thioguanine or 8-azaguanine (HGPRT), diphtheria toxin (EF-2), α-amanitin (RNA polymerase), 2,6-diaminopurine or 8-azaadenine (APRT), BrdU (TK), toyocamycin or tubercidin (adenosine kinase), aminopterin or methotrexate (dihydrofolate reductase), trichoderm (60S ribosome subunits), emetine (40S ribosome subunits), thialysine (asn-tRNA synthetase), 5-fluorotryptophan (tryptophan transport system), ^{3}H-amino acid (affinity of α α-tRNA synthetase for amino acid), 2-deoxygalactose (galactokinase activity) and cycloleucine (methionine adenosyl transferase) [see Ref. 155 for review]. In Chinese hamster cells the most often used systems are $6TG^r$, oua^r and DT^r mutation loci (Tables 1 & 2).

Mutagenesis at these loci is different in terms of the nature of mutation(s) (dominant or recessive), location of the gene(s) (autosomal or X-linked), mutant expression time, the cell-density effect, concentration and duration of the selective

Table 1. Characteristics of Mutation Assays at Oua^r, DT^r and $6TG^r$ Loci

Locus	Chromosome Location	Dominance Recessiveness	Selective Agent	Optimal Expression Time (days)	Mutagen Specificity	Mutation Expression Affected by (Ref.)
Na^+/K^+-ATPase	Autosomal	Codominant	Ouabain	2-10	Induced by, EMS, MNNG, point mutagens; not induced by ionizing radiation and frameshift mutagens.	K^+ ion in the culture medium. Tumour promotors, cyclic Amp and temperature (11,37,38)
Elongation Factor-2	Autosomal	Codominant	Diphtheria toxin (DT) P.a. exotoxin	4-10	Induced by UV, X-ray, MNNG, EMS, ICR-170 etc.	Adenine-nucleosides affect DT binding on cell membrane. Cellular NAD level may affect inactivation of EF-2. (39,68,92,97, 99,104,105,127, 177,234)
HGPRT	X-Linked	Recessive	6-thioguanine 8-azaguanine HAT^s	>6	Induced by radiations and various chemical mutagens.	Cell-density. (35,38,39,44, 276,284,302, 304,305)

Adapted from Lewin (155)

Table 2. In Vitro Selection of Various Mammalian Somatic Cells and Forward Spontaneous Mutation Rates

Genetic Locus[1]	Selective Agents[2]	D/r[3]	Lethal Effect of the agent[4]	Mutagen Specificity[5]	Cell Line[6]	Spontaneous Mutation Rate[7,8] (Ref)
HGPRT	6TG, 8AZG	r.	Incorporated into DNA in place of G.	See Table 1.	Hum. Fibro. (2n)	$0.4\text{-}70\times10^{-5}$ (283)
					Hum. D98	4×10^{-4} (261)
					Mouse L	3×10^{-6} (183,185)
					CHL. (V79)	
					(2n±1)	1.5×10^{-8} (43)
					(2n)	2.5×10^{-5} (113)
					(4n)	4.7×10^{-5} (113)
					(8n)	1.3×10^{-5} (113)
					CHO (pro^-)	$4.7\text{-}5.6\times10^{-8}$ (175,288)
ATPase	Oua	cd.	Inhibits Na^+/K^+ ATPase.	See Table 1.	Mouse L	$5\text{-}7\times10^{-8}$ (11)
					CHO	$2\text{-}5\times10^{-8}$ (11,175)
					Hum. Fibro. (2n)	4×10^{-8} (29)
EF-2	DT, PAT	r/cd.	Inhibits protein synthesis.	See Table 1.	Hum. Fibro. (2n)	$5\text{-}6\times10^{-7}$ (97,99)
					CHL (V79)	$3\text{-}8\times10^{-8}$ (this thesis)
40S	EMT	r.	Inhibits chain elongation during translation.	EMS.	CHO	$1\text{-}5\times10^{-8}$ (98,100, 101,102)
AK	TOY, TUB	r.	Unknown, the agents are analogue of AR.	EMS.	CHO	4×10^{-5} (103)
TK	BrdU	r.	Incorporated into DNA in place of T.	EMS, MNNG.	CHL (V79)	4×10^{-7}[(f),225]
APRT	DAP, 8AZA	r.	Incorporated into DNA in place of A.	EMS, MNNG, BrdU/Black Light-UV.	CHO	2×10^{-7}[(f), 41]
					Hum. Epi (253J)	2×10^{-7}[(f), Liu, unpubl.]

Footnotes to Table 2.

1. HGPRT: hypoxanthine guanine phosphotransferase, ATPase: sodium-potasium-ATPase, EF-2: elongation factor-2, 40S: 40S ribosome subunit, AK: adenosine kinase, TK: thymidine kinase, APRT: adenine phosphotransferase.

2. 6TG: 6-thioguanine; 8AZG: 8-azaguanine, Oua: ouabain, DT: diphtheria toxin; PAT: P. aeruginosa toxin; EMT: emetine; TOY: toyocamycin; TUB: tubercidin; BrdU: bromodeoxyuridine; DAP: 2,6-diaminopurine; 8AZA: 8-azaadenine.

3. r: recessiveness, cd: codominance

4. G: guanine, AR: adenosine, T: thymidine, A: adenine.

5. EMS: ethyl methanesulfonate, MNNG: N-methyl-N'-nitro-N-nitrosoguanidine, UV: ultraviolet light.

6. Hum. Fibro.: human fibroblasts, CHL: Chinese hamster lung cells, CHO: hamster ovary cells, Hum. Epi: human epithelial cells, 2n: diploid, 2n ± 1 aneuploid.

7. Spontaneous mutation rate is given as per cell per generation. When no data are available, induced frequency (f) is given.

8. Adapted from Lewin (155).

agents, and medium composition of competitive analogues. A brief summary of the comparison of oua^r, DT^r, and 6TG^r mutation assay systems is presented in Table 1.

2. Biochemical Analysis of Gene Mutation

In addition to the biological assay systems mentioned above, a viral probe with an amber mutation can be used to study, quantitatively, the reversion of this mutation to wild type by infection of viruses to host cells. An excessive reactivation of viruses, as indicated by viral survival or cell death, is a result of changes in the amber mutation code. Deoxyribonucleic acid sequencing of this amber code, biochemically, would reveal the nature of mutation, such as transition, transversion, frameshift, deletion or duplication of base-pairs in the amber triplet code. With the advances in the restriction endonuclease enzymology, one can use viral probes without the amber mutation, rather, the profile-changes in restriction endonuclease sensitive-sites of the surviving virus after viral infection of host cells are studied. Furthermore, with DNA sequencing techniques, mutations in the introns of human hemoglobin genes of thalassemic patients have been studied (12,145) in order to understand whether these mutations cause differential expression during transcription or mRNA splicing. Biochemical studies of DNA sequences can be compared with biological studies so that a correlation can be drawn to reveal the mechanisms of thalassemia disorders in the humans.

Mutations can arise from two processes: spontaneous and induced mutagenesis. Spontaneous mutations usually occur during DNA replication when alterations in base-pairing properties are not recognized and effectively corrected. Induced mutations arise from treatments of mutagens and are relatively better understood compared to spontaneous mutations. These two types of mutations in mammalian cells will be discussed separately.

cha
res
mu
olo
tho
re
inc
als
see
pos
tio
bac
Sci

I.

ger
ion
ab
DN
Str
coo
be
les
stu
a [

Induced Mutagenesis in Mammalian Cells

Mutagenesis must be regarded as a biological process in which a chemical change in DNA is the primary and initial step. Induced mutations are produced as a result of the treatment of organisms with mutagens and usually are recovered at much higher frequencies. The idea that mutations result from errors in repair is an old one, because a class of mutations (i.e., chromosomal aberrations) has been thought to be the result of abnormal arrangement of broken chromosomes during reunion (75,76,121,147,228,229). A more recent hypothesis, which suggests that induced gene mutations arise as a result of error in repair due to base-pair alterations, has been developed by Witkin (295). Recent observations of bacteria seem to indicate that some mutations also result from repair replication error or post-replication repair error. In this section I will concentrate on recent observations on mutations due to repair in mammalian cells. For repair and mutagenesis in bacteria, the literature has been reviewed by Hanawalt et al., (111), Kimball (142), Schendel (231),and Witkins (296).

1. Mechanism of Actions of Various Mutagens.

Ultraviolet light (UV), at a wavelength of about 254 nm generated by a germicidal lamp, primarily produces cyclobutane rings in adjacent pyrimidines, while ionizing radiation generates strand breaks. Unlike physical mutagens mentioned above, each chemical mutagen produces its own specific array of lesions. However, DNA damage can be grouped into two categories: noncoding and coding lesions. Strand breaks, intercalations and cross-links do not seem to be recognized as a coding base during DNA replication. Alkylating agents, such as aflatoxin B_1, benzo(a)pyrene [B(a)P], 2-acetylaminofluorene (AAF), and UV, can cause bulky lesions in DNA. Among them, pyrimidine dimers produced by UV are the most studied lesion. Proflavin, acridine orange and ethidium bromide can intercalate into a DNA helix. Lastly, mitomycin C, nitrous acid and bifunctional nitrogen mustard

are known to react, chemically, with both strands of a helix causing a strand-to-strand cross-link. Psoralen requires photochemical reaction to cause cross-links. Ionizing radiation may cause cross-links if single-strand nicks on the opposite DNA strand are one base apart.

Coding lesions generally are produced by base analogues, while miscoding is related to alkylated base damage. These lesions occur by: a) incorporations of base analogues; b) thermal or chemical deamination of adenine or cytosine residues already in DNA that produces a hypoxanthine or uracil base; and c) alkylation of a base to alter base pairing properties during DNA replication.

Classification and mechanisms of chemical mutagens according to their reaction with DNA have been reviewed by J.J. Roberts (220). Chemical mutagens can react with DNA directly or indirectly. Direct mutagens are biological alkylating agents, such as β-propiolactone, sulfur and nitrogen mustards, alkyl sulfonate, alkyl nitrosamide, alkyl nitrosamidine; and arylalkylating agents, such as 7-bromomethyl benz(a)-anthracene. Indirect mutagens are synthetic chemicals, such as polycyclic hydrocarbons, aromatic amines, azo dyes, nitrosamines, 4-nitroquinoline-1-oxide, urethane, ethionine; and natural products such as cycasin, the pyrrolizidine alkaloids aflatoxin, safrole and various antibiotics.

Chemical mutagens undergo many chemical and/or enzymatic reactions in cells and react as electrophilic reagents with nucleophilic sites in DNA, RNA, and proteins in cells. Direct mutagens are themselves electrophilic. Indirect mutagens undergo metabolic activation to become electrophilic derivatives. The activation is also dependent on a cytochrome P-450-dependent membrane-based monoxygenase system which may be subjected to genetic control. Nucleophilic sites (which if involved in the formation of normal base-pairings are underlined) are in the $\underline{O}^6$, N3, N7, and C8 positions of guanine, the N1, N3, and N7 positions of adenine, the N1, N3 and $\underline{O}^2$ position of cytosine, the N3 and $\underline{O}^4$ positions of thymine, and the S atoms of

methionine and cysteine, the C3 position of tryosine, and the N1 and N3 positions of histidine. Chemical mutagens can react with the phosphate groups in polynucleotides to form phosphotriesters.

The reactions of chemical mutagens that have been shown to occur following direct reaction of DNA in vitro are: single base substitutions, depurinations, single- and double-strand breaks, esterification of phosphate groups, inter- and intrastrand cross-links and cross-links of DNA and proteins. Nitrous acid removes the amino group from adenine and cytosine which become hypoxanthine and uracil respectively. Deamination of adenine to hypoxanthine or of cytosine to uracil causes transition, while deamination of guanine to xanthine causes inactivation of DNA. Hydroxylamine and other hydroxyl (OH) donors also cause transition by addition of OH to the amino group of cytosine, so that cytosine undergoes a tautomeric shift to pair with adenine.

Alkylating agents, such as nitrogen mustard and ethyl ethanesulfonate, may produce mutations by a) addition of methyl or ethyl group to guanine which behaves like adenine analogues and pairs with thymine; b) depurination; and c) inter- or intrastrand cross-links. Special emphasis is given to N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and dimethyl sulfate (DMS), which have been used in the present studies. Both MNNG and DMS are direct acting alkylating compounds. It is believed MNNG has a low Swain-Scott substrate constant (s) of 0.42, and the ratio of O^6MeG to N^7MeG in DNA by MNNG is 0.1 (154) as compared to the s constant of 0.86 and an O^6MeG/N^7MeG ratio of 0.04 by DMS (170, 188): MNNG is a more potent mutagen than DMS is (170). Mutagenicity of O^6MeG in DNA has been thought to be due to its miscoding properties while the presence of N^7MeG does not lead to mispairings (170).

Compounds that require metabolic activation bind to specific sites of DNA. For example, activated AAF binds to C8 guanine (220). The modified bases are

shifte

(83,14

induc

deoxy

meta

Beryl

polym

crypt

HeLa

rate

ectiv

DNA

selec

nucle

2. R

fault

by D

and p

altere

repai

light

tion a

repai

There

shifted out of the double helix, while the fixed carcinogen is inserted in its place (83,148) and may cause inhibition of DNA methylation (209). Ionizing radiation induces changes in DNA mainly through free radical reactions that produce modified deoxyribose residues, modified bases and strand breaks.

Another class of mutagen is that of metal carcinogens (266), e.g., beryllium, a metal carcinogenic to animals and mutagenic to animal cells in culture (215). Beryllium inhibits the editing of 3'-5' exonuclease activity of M. luteus DNA polymerase, and decreases the fidelity of avian myelobastosis virus reverse transcriptase; beryllium (2 mM) can cause misincorporation of dAMP by purified human HeLa α, β, and γ DNA polymerases. Beryllium can increase the misincorporation rate of three human polymerases in UV-irradiated poly(dC) template. Since no editing exonuclease activity can be detected with purified mammalian α, β and γ DNA polymerases, the effect of beryllium may be due to a diminished nucleotide selectivity of polymerase, to the binding of beryllium to the template or to nucleoside triphosphates.

2. Repair Pathways.

Damaged DNA either prevents cellular proliferation (cell death) or it results in faulty protein production or regulation (mutations). Damaged DNA can be overcome by DNA repair (photoreactivation, base excision repair, nucleotide excision repair and post-replication repair). Repairable damages in DNA are missing, incorrect or altered bases, interstrand cross-links and strand breaks. The efficiency of DNA repair depends on several factor(s), such as repair enzymes, energy source (visible light in photoreactivation pathway), DNA precursors and post-replication methylation as suggested from studies in prokaryotes. Failure in one causes misrepair or no repair at all; thus cell death or mutation occurs.

The maintenance of the genetic integrity is the result of repair processes. There are two forms of repair, depending upon the requirement of light (light repair

and dam

exist in

functio

usually

repair

repair.

(5),.54,

patch (

nucleo

the da

templa

is "err

preven

the let

lexcis

if suff

Thus,

"quad

3. M

C

with h

uals.

activit

mutati

I

replica

damage

and dark repair). It has been demonstrated that a photorepair pathway seems to exist in some mammals (Dr. M. H. Wade, personal communication). However, the function of photorepair is not well understood in mammalian cells, which are not usually exposed to light. Maybe it is an analogue to the appendix in humans. Dark repair involves base excision repair, nucleotide excision repair and post-replication repair. The pathway of each repair is discussed in detail by several investigators (51,84,111, 112,114,142,220,231,296). Excision repair is characterized by short patch (X-rays) or long patch (UV and AAF) synthesis, depending upon the number of nucleotides excised. In either case, after the DNA lesions are excised (as long as the damage is confined to one strand), the other undamaged strand is used as a template for repair replication. Repair of DNA generally has two consequences; one is "error-free" and the other is "error-prone". "Error-free" repair mechanisms prevent lethality and mutation fixation, whereas "error-prone" repair removes only the lethal components of DNA damage. Photorepair in bacteria and dark repair (excision repair) in mammalian cells are believed to be "error-free" (51,111,169,220) if sufficient time is allowed for cells to perform repair before DNA replication. Thus, mutation frequency and/or lethality of irradiated cells can be reduced by "liquid holding" (48,107-109,137,160,167,168,186,241,286,287,299).

3. Mutations Due to An Unrepaired DNA Damage.

Our understanding of excision repair (nucleotide excision) comes from studies with human diploid fibroblasts of normal and xeroderma pigmentosum (XP) individuals. The XP cells of A, C, and D groups have a reduced level of the incision activity in the excision repair pathway. These types of cells have higher induced mutation frequencies.

If excision repair is error free and occurs mainly before fixation by DNA replication, then when DNA synthesis (replication) is inhibited, there will be more damaged DNA removed and repaired. Therefore, lethality and mutation frequency

will decrease. Indeed, liquid holding of UV-irradiated normal diploid fibroblasts reduces lethality and mutation frequency, whereas it did not change either biological end points in UV-irradiated XP cells (group A) (167-169). A similar conclusion (that excision repair is error-free in Chinese hamster V79 cells) was reached by Nakano et al., (186).

Furthermore, the role of thymine dimers in determining the biological effects of UV irradiation is less clear in mammalian cells than it is in bacteria. Excision of dimers has been reported for many cell lines, but ability to excise dimers does not seem to be associated with lower UV sensitivity, except in the case of certain repair deficient XP human cells and mutagen sensitive V79 mutants (167-169, 233-235).

If induced mutations are the result of unrepaired damaged DNA in cells, one can then measure and correlate mutation frequency and DNA adducts. There will be a direct relationship between increasing mutation frequency and mutagenic effectiveness. Although not all DNA adducts are responsible for mutation, one can compare the mutation frequency and the nature of adducts. For example, from studies by Newbold, et al., (188) carcinogenicity (as measured by mutagenicity) of aliphatic alkylating agents, such as the alkylnitrosamides and the alkylmethanesulphonates, is correlated with their ability to alkylate the nucleophilic oxygen atoms in DNA, particularly the O^6-atom of guanine. The O^6-alkylation of guanine is likely to interfere with DNA base-pair hydrogen bonding and is possibly the major DNA modification responsible for the induction of GC to AT transition.

Post-replication repair is possibly an "error-prone" mechanism. This repair process seems to account for by-passing the unrepaired damage present in the template of replication forks by means of recombination, strand displacement, direct misreplication, and gap formation with new initiation points. It has been proposed that the first two are "error-free" processes, though these two processes have not been conclusively demonstrated in mammalian cells (50,51,239). It has

been suggested that the gaps opposite to the damaged DNA (uninstructive template) are "filled-in" by de novo DNA synthesis (63,301). The gap-filling process in XP variant cells is slower. This process is caffeine sensitive in V79 (37,221,272), mouse, XP (201) and HeLa cells, but is not caffeine sensitive in normal human fibroblasts (220). Whether or not this is the same repair pathway in V79 and normal human fibroblasts is not known. However, others (51,93,263) have suggested that caffeine initiates more replication forks, to explain the phenomenon that caffeine inhibits formations of large-size DNA fragments (chain elongation). On the other hand, Waldren & Patterson, (280) suggest that caffeine acts to enhance UV killing by interference with the supply of purine nucleotides needed for repair. Thus caffeine increases the mutation frequency.

4. Mutations Due to a Misrepaired DNA Damage.

It is known that DNA synthesis involves enzymatic activities of "proof-reading". In prokaryotes, this function resides in the protein for polymerization. However, in eukaryotes, it is at a separate protein. There is experimental evidence that indicates exogenous pyrimidine (thymidine) additions can alter BrdU- or MNNG-induced mutation (176,207,208). It is not known which enzymatic activities, polymerization or proofreading, are altered by unbalanced pyrimidine pool, because these two activities are coupled. Evidence also suggests that dCTP analogue, ara-dCTP is inhibitory to polymerase (57). Superabundance of exogenous thymidine alone is also both toxic and highly mutagenic to Chinese hamster cells (23), but additions of deoxycytidine reversed the toxicity and mutagenicity of thymidine (61, 176,207,208). The effect of exogenous pyrimidine has been attributed to perturbation of pyrimidine metabolism (140) or to the fidelity of base pairing during normal replication repair of DNA (123). Durkacz et al., (70) reported that thymidine also reduces repair capacity of mouse cells.

Deoxyribonucleic acid synthesis is a fine-tuned process which can be blocked by additions of deoxyribonucleosides such as TdR, AdR, GdR, or BrdU (140,172, 219) to the culture medium. This inhibition of DNA synthesis is believed to be mediated via an inhibition of ribonucleotide reductase. This inhbition can be reversed by additions of 2'-deoxycytidine (172,207,208,219). It appears that overnight (16 hrs) treatments of GdR, AdR or TdR (1 mM) to Chinese hamster V79 cells result in higher mutation frequencies on $6TG^r$ locus (257). On the other hand, hydroxyurea inhibits ribonucleotide reductase and araCTP inhibits polymerase but neither chemical is mutagenic (23).

Incorporations of BrdU into DNA also sensitize the DNA to visible light (black light). Mutation induction by combination of BrdU/black light treatments has been reported (45), and the same techniques have been used to select DNA repair deficient mutants (235), nutritional auxotrophic mutants (45) and $APRT^-$ mutants (Liu, unpublished results). Large quantities of cancer chemotherapeutic drugs are pyrimidine analogues; these drugs are used in addition to radiation therapy. The pyrimidine analogue will certainly change the deoxyribonucleotide-pool balance, and thus produce certain physiological and genetic effects. Thus far, there are few experimental data from studies in mammalian cell mutagenesis to support the hypothesis that the fidelity of DNA repair is also dependent on the availability of a physiologically balanced deoxyribonucleotide pool. Apparently, a mutant with an altered deoxyribonucleotide pool is needed for this study.

Lastly, an inducible repair process has been demonstrated in prokaryotes and it is "error-prone". Whether or not an inducible "error-prone" repair process exists in mammalian cells is not settled.

Other conditions that may effect misrepair are: a) the nutritional state of the cell (70,134) during excision repair process; and b) oxygen tension for repair of damage by the radicals (6). The physiological state of the cell can change

nicotinamide adenine dinucleotide (NAD) levels by addition of thymidine (70,71,134, 237) and they have been implicated in the repair of strand breaks.

5. ADP-ribosylation and DNA Repair.

Poly(ADP-ribose) polymerase is responsible for ADP-ribosylation of nuclear proteins. This is defined as a postsynthetic modification of protein by the covalent attachment of the ADP-ribose moiety of NAD^+. In the cytoplasm, ADP-ribosylation of elongation factor-2 is responsible for the inhibition of protein synthesis by both diphtheria and Pseudomonas aeroginosa toxins (52,122,128,199). The activation of membrane adenylate cyclase by cholera toxin is thought to occur by ADP ribosylation of the adenylate cyclase-associated GTP-binding protein (88). The inhibition of protein synthesis by the toxin is thought to be NAD dependent (127). ADP-ribosylation of nuclear protein in eukaryotic nuclei is thought to correlate with semi-conservative DNA synthesis, cell proliferation, DNA transcription, DNA repair, cell cycle, cellular differentiation and development (197,213), though the exact function is unknown. The possible involvement of ADP-ribosylation in DNA repair is based on six observations: a) alkylating agents and ionizing radiation cause depletion of intracellular NAD^+ (70,94,244,293); b) fragmented DNA stimulates the activities of poly(ADP-ribose) polymerase (14,15,17,19,191,237,242); c) mouse 3T3 cells grown in NAD^+ depleted medium are unable to perform alkylating agent-(MNNG) induced unscheduled DNA synthesis (UDS) (134); d) inhibitors of poly(ADP-ribose) polymerase, such as benzamide and nicotinamide, enhance UDS of UV-irradiated repair proficient cells (2,18,73,165,179,187,196,212); e) inhibitors of post-replication repair, such as caffeine and theophylline, also are inhibitors of the polymerase (213); and f) benzamide causes enhanced cytotoxic effects of MNNG and DMS to the wild type V79 cells (Liu, unpublished results), while caffeine causes enhanced cytotoxicity and mutagenicity in UV-irradiated V79 cells (36,37). To date,

no studies in correlating mutation induction and the effect of benzamide have been reported.

6. Mutation Fixation.

To understand the relationship between mutation to repair and to replication is to determine the time of fixation or stabilization of mutation relative to these cellular events. Photoreversibility of pyrimidine dimers in bacterial system has been used to determine or infer the time of fixation of UV-induced mutations. Studies in E. coli indicated mutations are fixed prior to or at replication (214). Another method for drawing an inference about fixation is to apply the mutagen at various times relative to DNA replication. This method was used with synchronized cells to infer that mutations induced by several different agents (UV, X-rays, NAcAAF) were fixed either solely or at least most efficiently at replication. The yield of mutations is highest when the cells were treated in late G_1, significantly less when the treatment was given earlier in G_1, and is the least when in G_2. A lower cytotoxicity and mutation yield will result when there is a longer interval between mutagen-treatment and DNA replication (107,137,159). Using this inference, a number of different agents and conditions, such as metabolic inhibitor or non-nutrient conditions, can influence the mutation yield produced by a given mutagen. Delayed DNA replication by the conditioned medium has been reported to enhance repair capacity of a normal excision repair (error-free) pathway by reducing mutation frequencies and increasing survivals (48,160,186). However, this treatment produces no difference in repair deficient cells after UV, NAcAAF or activated B(a)P (160,168,241,287,299) treatments.

Spontaneous Mutagenesis

Spontaneous mutations are rare events and occur at a constant rate for a given locus. Examination of this problem was triggered by the mechanism suggested by

Watson and Crick's model of the DNA helix in the 1950's (285) and by early research in microorganisms. Both of them have implicated that spontaneous mutations arise during DNA replication.

1. Base-mispairing Hypothesis.

The mechanism of point mutations by base replacement, as suggested by the Watson and Crick model, is that, during DNA replication, a base may assume an occasionally transitory, rare tautomeric configuration, and pair with another base with which it is not normally paired.

A tautomeric shift occurs when an amino group ($-NH_2$) in C6 of adenine (or in C4 of cytosine), that provides a hydrogen atom for bonding with the complementary keto (C=O) group of thymine, is changed to imino (-NH) form so that adenine bonds in a complementary way with cytosine. A tautomeric shift also occurs in keto forms of thymine (or guanine) to enol (COH) form. Possible transitions due to tautomeric shift are A-C imino, A imino-C, G-T enol and G enol-T. These types of copy errors require DNA replication and are affected by temperature (158).

The tautomeric shift generally accounts for transition mutation. However, the mechanism with which transversion mispairings arise is not clear, because purine to purine mispairs generally cause a distortion in the helix unless they are short-lived intermediates. Topal and Fresco (268) proposed a scheme in which a rotation in the entering base from the more probable anti configuration to syn configuration occurs while the template base is in the enol or imino configuration. For example, a base-pair of G syn and A imino with two hydrogen bonds and normal Wastron-Crick interstrand dimensions (268,285) can be constructed. Such an intermediate does not cause strand distortion and upon replication, causes transversion. Possible transversion due to Topal and Fresco mispairings are A imino-A syn; A imino-G syn; G enol, imino-G syn and G enol, imino-A syn. Evidence supporting this hypothesis includes the mutation studies with 5-bromouracil (5BU) and 2-aminopurine (2AP).

Both agents cause transitions but not transversions (123,222). This is so because a) Topal and Fresco's model does not involve rotation of pyrimidine or its analogue, and 5BU also exhibits a greater tendency to assume enol form than its natural counterpart thymine and b) 2-aminopurine lacks a substituent on C6 so that purine-purine mispairing potential is reduced. The mispairing by the entering 2AP syn to template A or G, forms only one hydrogen bond which is also interfered with by interaction between the C8 hydrogen of 2AP and the C2 amino group of the template G. Once 2AP is incorporated into the template, 2AP amino cannot pair with either A syn or G syn, while 2AP imino can pair only one hydrogen bond with either purine syn.

These two hypotheses suggest that transitions should occur most frequently, while transversions should occur less frequently, because the former requires an one-step process, with keto-enol or amino-imino shift, and the latter requires a two-step process, the first being the tautomeric shift followed by a rotation. The equilibrium constant for a tautomeric shift is about 10^{-4} to 10^{-5}; but for rotation, which is dependent on the purine, it is about 10^{-1} (G syn) to 10^{-2} (A syn) (268). DNA synthesis is generally believed to be a two-step catalytic process involving a base incorporation reaction followed by a checking reaction (56,111,135,268). If base mispairings are due to mistakes in both catalytic activities, the transition mutations, according to Watson-Crick tautomerization, should be 10^{-8} to 10^{-10} per base pair because tautomeric equilibrium constants are the same at both catalytic reactions. According to Topal and Fresco's mispairing model, the fidelity for transversions should be 10^{-9} to 5×10^{-12} per base-pair (268), because tautomeric re-equilibrium of template residues is expected in both catalytic steps, while the re-equilibrium of syn configuration of the terminal residue on the daughter strand is not expected in the checking step due to a much higher activation energy for

rotation about the glycosyl bond of an incorporated residue stacked on its neighbor than in the entering monomer.

It follows that in systems lacking the exonuclease function on checking step of DNA synthesis, the rate for copy-error due to transition or transversion would be expected from the first step alone. Mutation studies in a mutator mutant, mutD5, of E. coli suggest that the mutant may lack the checking process because the mutation frequencies for transition, transversion or frameshift are about $x10^{-5}$ - 10^{-6}, compared to 10^{-9} - 10^{-10} of the wild type. Nevertheless, Topal and Fresco's model did not explain the mechanism from which frameshifts arise.

2. Base Slippage-realignment Hypothesis.

Studies by Degnen and Cox (62) indicate that mutD5 is active only during DNA replication. This mutD5 strain is sensitive to 5-amino-acridine, a frameshift mutagen known to act at the replication fork. Furthermore, mutD5 mutants have an increased frequency of transition, transversion and frameshift mutations, and frequencies due to transitions are higher than those due to transversions. Although Topal and Fresco's model (268) suggests mutD5 mutants are defective in checking step of DNA synthesis, the frameshift mutations in this mutD5 strain cannot be explained either by the model proposed by Watson and Crick or by Topal and Fresco. In order to explain the enhanced frameshift mutations, Fowler et al, (81) proposed a mechanism that involves slippage and realignment of base in the template and/or daughter strand at the replication fork. This mechanism is similar to that proposed by Streisinger et al., (258) to explain the origin of frameshift through mispairing error during repair of single-strand breaks in the DNA molecule. Slippage of template base at the replication fork causes a deletion, while slippage in the terminal base of the daughter strand causes an addition, and frameshifts therefore may be dependent on a replication enzyme complex.

3. Replicative Enzyme-dependent Hypothesis.

Studies in bacteriophage T4 mutants have identified a number of genes whose functions in DNA replication and repair have been implicated in the genetic determination of both spontaneous and induced mutageneses. Mutant genes having mutator or antimutator effect are (65-67): genes px (generalized repair and recombination), genes v (pyrimidine dimer exonuclease), genes hm (unknown function), genes 30 (DNA ligase), genes 32 (Albert's protein which functions in DNA replication, recombination and repair), genes 42 and td (pyrimidine metabolism, 42 codes for dCMP hydroxymethylase, td codes for thymidine synthetase to synthesize hydroxymethyl dCTP and dTTP respectively), and gene 43 (DNA polymerases). Extensive review on mutator mutants of T4 phage has been given by Drake (65-67) and Rosamond (223). For mutator mutants in prokaryotes, Cox (56) and Hanawalt et al., (111) have also presented a thorough review.

While there are reports indicating replication-independent mutations may occur in prokaryotes and lower eukaryotes (6), there is no such evidence suggesting the existence of a replication-independent mutation in mammalian somatic cells. Although recent reports indicate that transposable elements in corn and fungi (6,164) do control expressions of certain phenotypes that may be viewed as the replication-independent mutation through recombination, the existence of transposable elements in mammalian cells is unknown. Mutation-like events due to extranuclear DNA, e.g., virus, can be detected as genetic variation and regarded as replication-independent mutation. For example, integration and loop-out of viral DNA in cellular DNA can possibly introduce duplications or deletions by non-precise excision of viral DNA. Recent demonstrations on three distinct genes in human DNA related to the transforming genes of mammalian sarcoma retroviruses may be of importance for elucidation of this mechanism on cell growth, differentiation, and neoplastic transformation (150,297,300).

Evidence supporting enzyme(s)-dependent mutation mechanism is from studies in T4 bacteriophage, prokaryotes and lower eukaryotes (20,56,67,115). The replication-dependent spontaneous mutation produces most frameshifts (20,67) and accounts for the majority of spontaneous mutation in these organisms. Works from mutator gene analysis of T4 phage (65-67), bacteria (56,111), yeast (115) and in vitro fidelity anlaysis of DNA replication enzyme(s), including that from mammalian cells (254), clearly suggest that DNA polymerase, DNA precursors, and post-replication methylation enzymes may play an active role in the selection of bases or correction of base mispairings during replication. The following review will concentrate on mutation mechanism assumed to be single base-pair alterations.

4. Post-replication Methylation

In prokaryotes, there has been demonstrated that post-replication methylation of base residues (N^6 of adenine, C5 of cytosine) prevents cleavage of DNA by nucleases. The methylation provides a mechanism with which foreign DNA or mismatched base pairs are recognized and effectively excised. The methylation also provides a protective function so that foreign DNA is eliminated from the host DNA. Mutants such as dam$^-$(dam-3 or dam-4) or dcm$^-$(90,91,171) with defective/deficient methylation function, have been reported to contain single-stranded breaks which are amplified in dam-3 polA12 and dam-3 lig-7 double mutants (171). Combinations of dam-3 with polA, recA, recB and recC are lethal. Mutant dam-3 has increased UV-sensitivity, spontaneous mutation and spontaneous induction of prophage. It appears that the function of a dam-specific enzyme also involves "mismatch" repair such that dam$^-$ strains are deficient in a post-replicative error-avoidance pathway which allows a specific elimination of mismatched lesions. A new approach has been used (91) to isolate mutants with a second mutation defective in adenine-methylation-instructed mismatch correction in a dam mutant.

Because dam mutants are 2-aminopurine sensitive due to the excision of unmethylated, mismatched base pairs, a dam strain with a second mutation lacking mismatch correction could be expected to restore resistance to 2-aminopurine in a dam mutant with the mutator properties.

The methylation of eukaryotic DNA has been reviewed by Ehrlich and Wang (74). The methylation of eukaryotic DNA may control transcription, and function in maintenance of chromosome structure, repair of DNA, hotspot mutagenesis, and oncogenic transformation. However, no mammalian mutant with a defective "dam" or "dcm" specific enzyme has been reported. Recent demonstration on reactivation of an inactive X chromosome by 5-azacytidine provides a new research direction (180).

5. Fidelity of Replication

A. Polymerase Base-selectivities.

The fidelity of replication concerns the accuracy with which incorrect base-pairs are excluded during or immediately after deoxyribonucleoside triphosphate (dNTP) incorporation. Mismatchings due to tautomeric forms of the bases have been estimated from the tautomeric equilibrium constants of the bases, and are higher than the observed in vivo mutation rate in T4-phage (2×10^{-10}) per base pair (21,67). Hydrogen bonding alone cannot explain the observed fidelity of replication. It is, therefore, proposed that DNA polymerase may participate in the selection of a correct base during the base incorporation step and a 3'-5' exonuclease activity may be involved in removal of the misincorporated base.

Evidence supporting these base selectivities of polymerase was gained by isolating T4 phage mutants defective in DNA polymerase that also have a temperature sensitive mutator or antimutator phenotype (67,82,118,250-253). In vitro studies on the DNA polymerase from this mutant suggest that the polymerase is

responsible for the elevated mutation rate, and that the purified polymerase incorporates incorrect bases into a template four times higher than does a wild-type polymerase. Base selectivity of the polymerase in a mutator T4 mutant shows mutator activities and temperature sensitivity for DNA replication, i.e., the misincorporation of nucleotides increases with increasing temperature by a factor of six as compared to the wild type polymerase.

Studies on higher eukaryotes provide further support for the base selection activity of polymerase during DNA replication. Weymouth and Loeb (292) demonstrated that the misincorporation frequency of the in vitro system by polymerae is 1.25×10^{-4}. This frequency may reflect mispairings at the first catalytic step of replication because the polymerase does not seem to have error-correction properties, such as the associated 3'-5' exonuclease in prokaryotes. Springgate and Loeb (254) further reported that a polymerase from acute lymphoblastic leukemic cells produces a tenfold higher polymerization error than the polymerase from normal lymphocytes does. This inaccurate incorporation of bases is indicative of possible alteration in the polymerase base selectivity.

Studies on T4 polymerase mutants indicate there are at least two types of mutations on polymerase that can alter base selectivity. If base selectivity of polymerase is involved in replication-dependent spontaneous mutations, the polymerase must contain binding sites for both the template base and the entering monomer dNTP. The first hypothetical copy-error mechanism as proposed by Drake (65) relates to the monomer base acceptor. A mutation of this type generally can recognize the template base but incorrectly accepts a different base residue, e.g., accepts guanine instead of adenine to couple with a thymine, and causes a transition mutation. Mutants of this type have a high transition rate but a low transversion rate, and would be sensitive to certain chemical mutagens because the probability of accepting a chemically altered base would be increased. A second hypothetical

copy-error mechanism by a polymerase mutant relates to communications between two binding sites. A structurally altered polymerase of this type might produce transitions as well as transversions. This type of polymerase mutant might not be sensitive to chemical mutagens. Studies on T4 polymerase mutants generally support these two hypotheses (21,65). However, in mammalian somatic cells no such polymerase mutants have been reported, though a temperature sensitive mutant of mouse FM3A cells may be defective in one of the two subunits of polymerase (274).

Other enzymes that have been implicated in producing mutator phenotype are the associated 3'-5' exonuclease activities in polymerase of bacteriophage, prokaryotes and polymerase II of yeast, and ligase of T4 phage (66). MutD5 mutants possibly are defective in exonuclease activities (111,135,268). Purified eukaryotic replicative polymerase α does not have this associated 3'-5' exonuclease, and mutation of this activity on a separate protein has not been reported in the eukaryotes.

B. Base Analogues and Deoxyribnucleotide Pools

Alterations in DNA precursors have been demonstrated to change spontaneous mutation rates or induced mutation frequencies in prokaryotes and eukaryotes (61,67,175,176,198,207,208,257). In prokaryotes and T4 phage, thymidine (TdR) deprivation causes an enhanced mutation rate either by mass action or by inhibition of pyrimidine metabolic pathways (26,66,140). Thymidineless mutagenesis is synergistic with 2-aminopurine (2AP) mutagenesis in bacteriophage T4 (123,222). It has been suggested from accumulated evidence that perturbation of normal deoxyribonucleotide triphosphate pools (95,119,151) and mispairs due to base analogues in DNA, lead to mutagenic effects of 5'-bromodeoxyuridine (BrdU) and 2AP. Hopkins and Goodman (123) further suggested that the configuration of the neighboring nucleotides surrounding the base analogue mispair is responsible for the antipolarity of BrdU and 2AP mutageneses. In mammalian cells, evidence has been shown that

BrdU

been

hypo

deox

ribon

(174)

anal

pool

resi

(doe

tropi

retu

AdR

and

acti

not

arab

(23)

it is

poly

not

to

of

BrdU mutagenesis is mediated by the concentration of BrdU to which the cells have been exposed instead of the amount of bromouracil substitution (140). This hypothesis is consistent with the hypothesis that triphosphates of BrdU, TdR, 2'-deoxyadenosine (AdR) or 2'-deoxyguanosine (GdR) inhibit cytosine diphosphate (CDP) ribonucleotide reductase and decrease deoxycytidine triphosphate (dCTP) pools (174). It is this imbalance of pyrimidine pools which mediates the base (or base analogue) mutagenesis.

Recent evidence suggests that in the rodent cells, endogenously elevated dCTP pools cause an increase in spontaneous mutation rate in ouabain- and 6-thioguanine-resistant loci (175,288). This elevated mutation rate seems to be locus specific (does not occur in emetine resistant locus or reversion of proline auxotrophs to prototrophs). Additions of TdR to the culture medium so that the dCTP/dTTP ratio is returned to normal, reverse the mutagenic effect. Furthermore, excess exogenous AdR, GdR or TdR in the culture medium of V79 Chinese hamster cells is both toxic and mutagenic (23,257). It appears that an imbalance of dNTP pools causes mass action so that mispairings are elevated, as proposed by Drake (66) and others.

On the other hand, Brennard and Fox (25) reported exogenous TdR is toxic but not mutagenic. Hydroxyurea, inhibitor of CDP ribonucleotide reductase, or cytosine arabinoside (araC), inhibitor of the reductase or DNA polymerase, is not mutagenic (23). Although 3'-5' exonuclease activities do not reside on the replicative enzyme, it is generally believed that this editing enzyme exists in a replication complex with polymerase in the mammalian systems. The reason that exonuclease activities did not correct the mispairing due to increased dCTP by mass action is unknown.

C. Uracil-DNA Glycosylase

Duncan and Weiss (69) reported that spontaneous mutation to nalidixic acid or to rifampicin resistance is increased 5-fold in uracil-DNA glycosylase (*ung*) mutants of *E. coli*. In addition, G-C to A-T transition in *tryA* reversion analysis is increased

by 15-fold and this type of transition mutation seems to be specific. Moreover, uracil-containing phage are viable in ung$^-$ bacteria, thus suggesting that the uracil is not removed from matured DNA, contrary to what is generally believed (95,112). It is suggested that uracil-DNA glycosylase appears to edit (mutagenic) deaminated cytosine residues, but does not prevent uracil misincorporation into DNA as AU base pair. The function of the ung gene seems to preclude the use of uracil as a normal DNA constituent.

6. Mutator Activities Associated with Unknown Mechanisms

Reports from studies in E. coli K-12 mutator mutants, as summarized by Cox (56) and Hanawalt et al., (111), indicate that elevated spontaneous mutations occur in mutations in genes such as uvrD, uvrE, dam, dcm, dnaQ, mutD, mutL, mutR, mutS, mutT and mutU, in which uvrD, uvrE and mutU may be at the same gene. Mutants of the mutD gene cause generalized hypermutability (transitions, transversions and frameshifts) which is stimulated by TdR (2 μM) in the TdR$^+$ strain and suppressed by nalA, or sumD genes. Mutants of mutT genes are specific mutator mutants (transversions only) while transitions and transversions occur in mutS, mutR, mutL and mutU (uvrE). Furthermore, polA mutants (polymermase I mutation) with mut-S, R, -L, or -D background increase frameshift mutation rate, and mutations in polymerase III (polC) also have an elevated frameshift mutation rate.

In lower eukaryotes such as yeast, there are four interrelated repair pathways (24,116); and mutator activities in yeast are dependent on channelling DNA lesions to repair pathways which may be "error-prone" when there is a deficiency in "error-free" repair pathways (115). A mutable gene in maize, reported by McClintock (164), has now been demonstrated to be a transposable element which regulates expressions of genes. Such a transposable element has not been demonstrated in mammalian cells.

Regarding the mammalian somatic cell system, three reports to date indicate that an elevated spontaneous mutation rate has been observed in TdR^- Chinese hamster ovary cells (175), in ribonucleotide reductase mutants of mouse lymphosarcoma cells (288) and in diploid fibroblasts from patients with Bloom's syndrome (BS) (97,282). The hypermutabilities of the former two mutants are associated with an endogenously elevated dCTP level while that of the latter is unknown. The mutator activities of the rodent mutants are locus specific ($6TG^r$ and oua^r loci), while that of BS fibroblasts are found in $6TG^r$ (282) and DT^r (97) loci. Emerit and Cerutti (78) and Warren (283) reported that the concentrates of culture medium from BS fibroblasts contain a clastogenic factor which causes $6TG^r$ mutation and chromosomal aberrations but not oua^r mutation in the wild type V79 cells. Speculation has been that BS fibroblasts lack a detoxification factor and that this clastogen accumulates in the culture medium.

In humans, cells having spontaneous mutations as defined by chromosomal instabilities, are found in individuals with Fanconi's anemia (FA), Bloom's syndrome, and Ataxia-Telangiectasia (AT). Fibroblasts from these patients are sensitive to mutagens such as X-ray (FA, AT), and/or UV (BS). Other human fibroblasts with in vitro mutagen-sensitivity are found in patients with xeroderma pigmentosum (UV and UV-like mutagens); hereditary retinoblastoma (X-ray); and Cockayne's [UV, EMS, (279)] and Gardner's [X-rays, mitomycin C, UV and no liquid holding recoveries (160)] syndromes. All of these patients have an increased incidence of spontaneous and/or induced cancer [see reviews by Arlett and Lehmann (5), Paterson and Smith (202)].

Recent studies, using restriction endonuclease mapping on human hemoglobin genes from thalassemic or sickle cell anemic patients (12,145), suggest that α thalassemias are due to a deletion of α gene loci, possibly caused by an unequal crossing-over between tandemly repeated α genes; and that a distinctive polymorphism due to base substitution in a HpaI cleavage site of the β^S gene occurs in 60 to 80

percent of sickle cell anemic patients with this gene. As to β thalassemia, it is more complicated. In β^+thalassemias (low production of normal β globin from a decreased amount of β mRNA), the defect is due to either defective regulation of transcription of β globin genes or abnormal processing of mRNA. In β^o thalassemias (with no normal β globin or mRNA), the defect is probably at the level of defective transcription, abnormal mRNA processing, deletions or base substitution in the β structural gene. For example, some patients with β^o thalassemias have either untranslatable β globin mRNA, or specific nonsense mutation in the β mRNA. In δβ thalassemia and hereditary persistence of fetal hemoglobin (HPFH), synthesis of δ and β globin does not occur, while only fetal hemoglobin is synthesized. Using recombinant DNA cloning techniques, the deletion of δ and β globin genes from δβ thalassemic and HPFH patients have been mapped and shown to regulate the relative expression of γ globin genes. It appears that mutations on the introns of human genomes also affect the expressions of structural genes and give rise to hereditary disorders in the humans.

Isolation and Characterization of Mutagen-Sensitive Mutants in Mammalian Cells

A number of methods have been used to induce, and select for mutagen-sensitive mutants in mammalian cells [see review by R. Schultz (233)]. The methods are: a) nonselective procedures with massive screening or replica plating techniques (152,227,255,267) to avoid the harsh processes necessary for mutant enrichment; b) selection techniques using differential incorporations of BrdU and subsequent exposure to visible light so that excision repair proficient cells become lethal (133,143,218). The second method provides some success in the isolation of repair deficient mutants. But a combination of BrdU incorporation and black light-exposure is also mutagenic as reported by Chu et al., (45). A more recent

development using irradiated viruses to select for UV-sensitive and repair deficient mutants which cannot undergo host cell reactivation of the virus and thus survives through selection. This viral reactivation may be a better technique, except that certain viruses have a host cell specificity and the kind of viruses that can be used is also limited. For example, Chinese hamster cells do not appear to incorporate the viruses commonly used for such studies (233).

An alternative method of enrichment and selection of mutagen sensitive and excision repair deficient mutants has been described by Schultz et al., (233,235). In this method, wild type V79 cells were induced to mutate by BrdU/black light and UV (20 J/m^2). The two mutagenic inductions are necessary if one assumes that gene(s) responsible for the repair function is (are) autosomally recessive (264). Selections of repair deficient mutants were carried out by the use of differential incorporation of ^{3}H-TdR immediately following exposures to low dose UV (10 J/m^2) in a replication-inhibited population. After incubation with ^{3}H-TdR, the selection was performed at 4°C to accumulate ^{3}H damage. Following selection, the surviving cells were incubated at 34°C in a medium containing hypoxanthine, aminopterin and thymidine so that thymidine kinase deficient cells were eliminated. Using this method, Schultz et al., (233-235) have isolated several DNA repair deficient mutants which have been shown to be mutagen sensitive and have mutagen-induced hypermutability. However, none of the DNA repair mutants is reported to be deficient in polymerase or spontaneously hypermutable.

Aphidicolin-resistant Mutants

1. Aphidicolin: Mode of Action.

The tetracyclic diterpenoid aphidicolin ($C_{20}H_{34}O_4$, m.p. 227-233°C) is an antibiotic and antiviral metabolite from Cephalosporium aphidicola Petch (28,59). It is a potent inhibitor of nuclear and mitochondrial DNA synthesis and it strongly

inhibits the growth of herpes simplex, vaccinia, SV40 and adenoviruses (30,106,149, 162, 166,204). It inhibits DNA but not RNA or protein synthesis (110,131,294). Aphidicolin is active against iododeoxyuridine-resistant herpes virus and does not induce mutation in herpes virus or prokaryotes directly (30) or indirectly through metabolic activation by rat liver microsomes (203). When aphidicolin (0.5 μM) was added to the culture medium during mutant expression or selection of $6TG^r$ cells, aphidicolin did not enhance recoveries of $6TG^r$ mutants (Liu, unpublished results). It suggests that aphidicolin does not have mutagenic activities.

Aphidicolin has been shown to be a specific inhibitor of polymerase α (131,193-195,294) but not of polymerase β or γ (129-131,294) at levels which inhibit mitosis (130,131,205). It does inhibit polymerase II of yeast [unlike most polymerases in eukaryotes, it has 3'-5' exonuclease activities (210)] but it has no effect on polymerases from E. coli or T4 phage (206). The fact that vaccinia virus DNA polymerase, which is a single polypeptide chain, is sensitive to aphidicolin suggests a direct interaction between polymerase and aphidicolin. On the other hand, that the sensitivity of yeast DNA polymerases I and II and of sea urchin DNA polymerase α to aphidicolin decreases upon purification (131,210) also indicates an interaction between aphidicolin and an accessary subunit. Recent demonstration of an aphidicolin-sensitive protein stimulatory factor for DNA polymerase α from rat giant trophoblast cells or calf thymus (136,298) supports the indirect interaction hypothesis. The effect on polymerase α of this stimulatory factor is abolished by aphidicolin but is unaffected by 2',3'-dideoxythymidine triphosphate (inhibitor of β-, γ polymerase).

Kinetic analyses of the mechanism of inhibition on purified polymerase α by aphidicolin, using activated DNA as template/primer, show that the inhibition is competitive with respect to dCTP (106,193,194,206), noncompetitive with respect to other dNTPs and uncompetitive with DNA. When synthetic homopolymers are used,

the degree of sensitivity of DNA polymerase α to aphidicolin is modified (193). DNA synthesis in isolated nuclei of sea urchins, permeabilized mouse FM3A cells or intact cells is inhibited competitively with all four dNTP, but noncompetitively with the individual dNTP (9,194) or with three of the four dNTP (193). In adenovirus-infected KB cells, the inhibition by aphidicolin to the endogenous DNA polymerase activity is competitive with dTTP (106).

The competitive interaction suggests that the inhibition of DNA replication by aphidicolin is brought about by inhibiting the binding of one or more of the dNTP to DNA polymerase. This is so because aphidicolin probably binds only to DNA polymerase molecules which have not yet bound dNTP molecules, and forms a catalytically inactive aphidicolin-polymerase complex.

2. Use of Aphidicolin to Identify Eukaryotic Replication and/or Repair Enzyme(s).

Five lines of evidence suggest that α or α-like DNA polymerase is required for DNA replication. The first is that α-polymerase activity increases during the S phase when DNA undergoes replication, or in actively dividing cells (27,58,126,131, 161, 205, 289-291). Secondly, aphidicolin inhibits DNA replication and cell growth in vivo, and α-like, not β-, or γ-like polymerase in vitro (27,47,204,205). Thirdly, the fact that the concentration of aphidicolin required to inhibit DNA replication in vivo is almost identical to the concentration required to inhibit DNA polymerase α in vitro, further suggests that the enzyme is the target of aphidicolin (205). Moreover, aphidicolin does not have the inhibitory effects similar to inhibitors of β-,γ-polymerase, such as dideoxy-GTP or -TTP [ddTTP (57,136,166,298)]. This suggests that β- and γ-polymerases are probably not replicative enzymes. Lastly, animal cells resistant to aphidicolin either have an increased level of DNA polymerase α (190), or contain an aphidicolin-resistant DNA polymerase-α (259).

Indirect evidence from studies on sister chromatid exchange (SCE) in a Chinese hamster V79 cell line suggests that aphidicolin acts on the replication fork

(139). Ishii and Bender (132) reported that DNA polymerization inhibitors, such as aphidicolin, hydroxyurea, and cytosine arabinoside (araC), increase spontaneous SCE frequency and have a synergistic effect on UV-induced SCE frequency, while ddTTP, neocarzinostatin, and novabiocin have no effect, and cycloheximide decrease both SCE frequencies.

The most interesting findings come from the studies on the effects of aphidicolin during unscheduled DNA synthesis (UDS) in mutagen-treated (UV, MNNG, MNU) mammalian cells. Independent investigations from four laboratories (16,47,110,248) showed that aphidicolin inhibits UDS in human diploid fibroblasts (47,248), normal lymphocytes (16), and HeLa cells (110). While these studies were performed in replication-inhibited cells [replication is inhibited by hydroxyurea and/or araC (110,248), or by cell contact in confluent culture (47)], the results from mitotic cells show that aphidicolin does not inhibit UDS in UV-irradiated HeLa cells (89,205), bleomycin treated permeable SR-C3H/He or mouse ascites sarcoma cells nor in isolated rat liver nuclei (236). Rather, the later studies indicate that aphidicolin can be substituted for hydroxyurea to inhibit scheduled DNA synthesis and that it allows measurement of repair synthesis (205).

Attempts have been made in many laboratories to isolate aphidicolin-resistant mutant cells. Most mutants were induced by MNNG (7,8,10,190), EMS (226,259), or by combined treatments with BrdU/black light and UV (40). Among these mutants, aphidicolin resistance is reported to be associated with a) an altered polymerase α (259); b) an inducible amplification of polymerase α (190); and c) an alteration in dNTP pools, such as i) by an elevation in dATP (7); ii) thermolabile thymidylate synthetase (8); iii) by alteration (10) or iv) by amplification (226) of ribonucleotide reductase; and v) by an elevation of dCTP or all four dNTP (40) in the rodent cells. An elevated UV-induced cytotoxicity and mutability are reported in an aphidicolin resistant mutant from the V79 Chinese hamster cell line (40). The mutant (aph^r-4,

or aph^r-4-2) isolated by Chang et al., is reported to be: a) slow growth; b) auxotrophic to TdR, deoxycytidine or deoxyuridine; c) sensitive to UV light; d) hypermutable by UV irradiation, e) sensitive to AdR, GdR and TdR (100 μM); f) of having high BrdU-dependent chromosomal aberrations; g) a high SCE frequency; and h) a high "reversion" rate. One of the "revertants", aph^r-4-R2, is not sensitive to UV and has a fast growth rate. Also, it is resistant to aphidicolin and has the same dCTP pool as the original mutant. Thus, the UV sensitivity in the aph^r-4-2 probably is not associated with the endogenous dCTP levels.

Concluding Remarks

With the exception of large deletions, insertions, and inversions, both spontaneous and induced mutations arise from errors in DNA synthesis during repair or replication. On the basis of the mechanism of mutagen action, there are two groups of mutagens: a) base modifiers (BrdU, 2AP), and b) DNA attackers: these agents, directly (MNNG) or indirectly (AAF) through metabolic activation, modify DNA or attack DNA, cause bulky DNA lesions and produce bulky distortions in DNA. They cause mispairings and inhibit DNA synthesis. The lesions, if not repaired or if misrepaired, cause lethality and mutation. The fidelity of DNA replication could be achieved by at least two enzymatic mechanisms: a) nucleotide selectivity of DNA polymerases, i.e., the slower the polymerization rate, the longer the checking time between nucleotide pairs, and the higher the fidelity; b) "proof-reading" or "editing" by the 3'-5' exonuclease activity. In mammalian cells, this latter activity is separated from the enzyme harboring polymerization activity.

Unlike spontaneous mutagenesis, where DNA polymerases are mistaken by the tautomeric and isomeric forms of nucleotides, induced mutagenesis is provoked by mutagen-induced modifications of DNA template strands. Excision repair of potentially lethal and/or mutagenic DNA lesions can occur only in double-strand

DNA, and then only when the segment of the complementary strand is intact for excision repair to be "error-free". In the case of lesions in the single-strand DNA and in the case of nonrepaired DNA lesions encountered by the DNA replication machinery, there is no way for DNA polymerase to produce an "error-free" repair.

Error-prone repair pathway is inferred from studies in bacterial systems and is thought to involve post-replication repair and/or polymerase fidelity. In mammalian cells, due to lack of suitable mutants, the speculative existence of an error-prone repair pathway has not yet been verified.

Enzymatic functions to ensure the accuracy of normal DNA replication are base selectivity, editing, uracil-DNA glycosylase, post-replication methylation, and DNA precursor pools. Although mutants defective in polymerase α have been isolated, mutator activities are reported to be associated with endogenously elevated dCTP.

Aphidicolin is a specific inhibitor of polymerase α and aphidicolin resistant mutants have been isolated. One of the mutants (aph^r-4-2) is sensitive to UV and is hypermutable at oua^r locus. The enhanced UV-sensitivity in this mutant is not associated with the endogenous dNTP.

MATERIALS AND METHODS

Cell Strain

A partially transformed aneuploid lung fibroblast line (V79) derived from a male Chinese hamster, Cricetulus griseus (2n=22), (80) was used as the parental cells for isolation of potentially mutant cell lines. This cell line has an indefinite life span and can be grown in cultures attached to the bottom surface of plastic or glass culture flasks or plates. These V79 cells divide every 14-16 hours and can form colonies from single cells with a cloning efficiency of 70% or more. The parental cell line will be regarded as the wild type cell line and used as the control group to study the nutritional requirements and the mutabilities of the mutants in this dissertation.

Culture Medium

Unless otherwise indicated, all cells in the experiments to be described below were grown in modified Eagle's Minimum Essential Medium (MEM) (72) with Earle's salts supplemented with a 50% increase of all vitamins, all essential amino acids except glutamine, a 100% increase of all non-essential amino acids and 1 mM sodium pyruvate. The concentration of sodium bicarbonate in the medium was adjusted to 1.0 gm/L. This medium was referred to as growth medium. The selection medium for ouabain-resistant mutants or 6-thioguanine resistant mutants was the same as the growth medium except that it was supplemented with 1 mM ouabain or 10 μg/ml 6-thioguanine (Sigma Chemical Co.). The medium used in the experiment studying

liquid holding recoveries was prepared from Eagle's MEM (GIBCO, Grand Island, New York, Cat. No. F-11) containing 1.5 gm/L sodium bicarbonate.

The medium was sterilized by a positive pressure filtration (Nuclepore Corporation, Pleasanton, California) and stored in a dark, cold room at $4^{o}C$. Prior to use, each bottle of 500 ml medium was supplemented with fetal calf serum (FCS, 5% v/v; GIBCO, Grand Island, New York; Flow Laboratories, Inc., Rockville, Maryland; Pel Freeze, Rogers, Arkansas; or Sterile Systems, Inc., Logan, Utah) which had been stored at $-20^{o}C$, thawed and heat inactivated, if not heat inactivated by the manufacturers, at $56^{o}C$ for 30 minutes prior to use. In addition, penicillin G (100 units per ml) and streptomycin (100 µg per ml) (Eli Lilly and Co., Indianapolis, Indiana) were added to the medium. For testing the nutritional requirements of the aph^{r}-4 variants, dialyzed FCS prepared with an Amicon hollow fiber dialyzer (HIP10, Amicon Corporation) was used.

Conditioned medium was prepared (186) by growing confluent wild type cells [5-6 x 10^{5} per cm^{2}; 20 ml of medium per flask (75 cm^{2})] in this Eagle's MEM supplemented with 10% FCS. Twenty-four hours later, the conditioned medium was pooled, sterilized by negative filtration (0.22 µM, Falcon Plastics, Oxard, California) immediately and stored at $-20^{o}C$ until use.

Culture Vessels and Incubation Conditions

Stock cell cultures were grown in plastic flasks (Corning Glass Works, Corning, New York). The cells were grown in fresh medium, either by refeeding or subculturing to ensure a log phase growth, the day before an experiment. Cells were dissociated with 0.01% crystalline trypsin (Sigma Chemistry Co.) in phosphate buffered saline (PBS) without calcium and magnesium ions but with 0.1% (wt/v) methylcellulose. Unless otherwise indicated, cells were grown in plastic culture dishes (9 cm, Corning Glass Works) and incubated in water-jacketed incubators

which provide a stable temperature of 37°C with humidified air and 5% CO_2. The wild type cells have a generation time of 14-16 hours at log phase growth (233).

Cell Growth Rates

For each cell line, approximately 0.5-1 x 10^5 cells were plated in a sufficient number of culture dishes at time zero; two plates from each cell line were trypsinized at various times thereafter and counted. The values were averaged.

Cell and Colony Counts

After trypsinization, cells were suspended in medium and stored on ice and counted with a hemacytometer. Serial dilutions were carried out to obtain cells at different concentrations. Less than a four-hour-exposure to cold did not significantly affect cloning efficiencies. For all experiments, cells were scored after 7 (the V79 cells) or 12 (slow growing mutants) days of growth. Colonies were rinsed with saline, fixed with 95% ethanol and stained with Giemsa (2.5% Giemsa in 3% methanol, Gurrs Improved R68, Sarle, Santa Monica, California). Only colonies with 30 or more cells were visually scored using a colony counter (American Optical Company).

Chemicals

Unless otherwise indicated, all chemicals were dissolved in PBS. The following chemicals were obtained from Sigma Chemical Company: 12-O-tetradecanoyl phorbol-13-acetate (TPA), hypoxanthine, thymidine (TdR), 5-bromodeoxyuridine (BrdU), caffeine, hydroxyurea, cytidine (CR), benzamide, cytosine-1-β-D-arabinofuranoside (araC), 2'-deoxyadenosine (AdR), 2'-deoxyguanosine (GdR) and their 5'-triphosphates (dTTP, dCTP, dATP and dGTP). Aminopterin was from Nutritional

Biochemical Corporation and 6-thioguanine (2-amino-6-mercaptopurine, 6TG) was from ICN Pharmaceutical Inc. (Cleveland, Ohio). Purified diphtheria toxin (Lot No. D343, 2000 floculating unit per mililiter, 1 lf = 2.5 μg of protein or 40-60 minimum lethal doses) was from Connaunght Laboratories Limited (Willowdale, Ontario, Canada, M2N5T8). Diphtheria toxin (DT) was divided into aliquots of 0.15 ml per glass vial and stored in liquid nitrogen until use. All other chemicals were stored according to recommendations by the manufacturers. Aphidicolin ($C_{20}H_{34}O_4$, M.W.:338) was a gift from the Development Therapeutics Program, National Cancer Institute and was dissolved in 100% dimethylsulfoxide (DMSO, Mallinckrodt Inc., Paris, Kentucky). Solutions of 6TG, AdR, or GdR were prepared by dissolving the chemicals in NaOH (1N) and then in double distilled water (final pH=11.35). Benzamide and TPA were dissolved in 100% ethanol. N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG) and dimethyl sulfate (DMS) were purchased from Aldrich Chemical Company, Inc., Milwaukee, Wisconsin. And N-acetoxy-2-acetylaminofluorene (NAcAAF) was a gift from Dr. James Miller, University of Wisconsin. All chemical mutagens (MNNG, DMS and NAcAAF) were dissolved in 100% DMSO and added onto cell culture immediately. All chemical solutions were filter sterilized (except when 100% ethanol or DMSO was used as a solvent) with a disposable, negative pressure filter (0.22 μM, Falcon Plastic) or a positive pressure filter unit (Swinnex-GS, Millipore Corp., Bedford, Massachusetts) and stored in -20°C until use. The final concentration of 6TG used in mutant selection was 10 μg/ml (6 μM).

For deoxyribonucleoside triphosphate measurements, primer template double strand poly(dI-dC)-poly(dI-dC) and poly(dA-dT)-poly(dA-dT) were from P-L Biochemicals, Inc. (Milwaukee, Wisconsin) and E. coli DNA-polymerase I (in 50% glycerol) was from Boehringer Mannheim. Tritiated-dATP (28 ci/mmol), -dGTP (11.7 ci/mmol) and -dCTP (19 ci/mmol) were from the Radiochemical Center (Amersham,

England) and ^{3}H-dTTP (82.4 ci/mmol) was from New England Nuclear (Boston, Massachusetts). All templates, polymerase, and ^{3}H-dNTP were stored at -20°C.

Cytotoxicity and Cloning Efficiency Determinations

Sensitivities of the cells to various forms of radiation and chemicals were determined by plating 400 or more cells per plate to allow an estimated 100-400 surviving colonies. Survival rates were determined in triplicate plates for each treatment. Four hours after cell plating, chemicals in microliter (µl) quantities were added to the medium on the attached cells. For ultraviolet light (UV) irradiation, with the medium removed, the attached cells were irradiated with a germicidal lamp (General Electric, 25T8-25 w) which was positioned to deliver 1.4 Joule per meter squared per second (J/m^{2}/sec). The UV-irradiated cells were grown in 5% dialyzed FCS for 24 hours so that replication and repair of DNA were dependent entirely on endogenous nucleotides.

The effects of caffeine (0.25 - 0.5 mM) or benzamide (2 mM) during DNA repair period were tested. Benzamide was added at the time of cell plating. Four hours later, chemical mutagen (MMNG, NAcAAF or DMS) in µl volume was added or cells were irradiated with UV. Caffeine was added immediately after UV. Caffeine was changed every 4 days until cell colonies were fixed and scored. Benzamide was present for 4 days. Cells were grown in medium supplemented with 5% FCS when chemical mutagens were used.

For X-ray irradiation, cells were diluted to various concentrations so that one ml contained the number of cells to be plated onto one plate. Cells were then suspended in ice-cold medium during X-ray irradiation (184 R/min, 250 Kvolts, 20 mA with 3 mm Al filtration) and were plated and grown in medium with 5% FCS after irradiation. The doses of chemicals are expressed in micromoles (µM), or µg/ml whereas UV and X-ray doses are in Joule per square meter (J/m^{2}) and rads (r)

respectively. To test the effect of benzamide (2 mM) on the survival of X-ray irradiated cells, the chemical was added to the medium after irradiation and was removed 4 days later. Unless otherwise indicated, chemicals and medium were changed every four days. Cloning efficiencies of controls were determined concurrently by plating 1,200 cells in three plates without chemicals or radiations. Colony-forming abilities of control and treated cells were calculated by dividing the total number of colonies recovered by the total number of cells plated, for each treatment in each cell line. All experiments were repeated at least once.

Mutant Isolation

Portions of this work have been reported elsewhere (40,226). Mutants resistant to aphidicolin were induced in V79 cells (30×10^6) by the combined treatment of bromodeoxyuridine (1×10^{-4} M) and black light (40,45,235). After sufficient expression time (one week) the mutagenized cells were pooled, replated, and irradiated with UV (16 J/m^2). Seven days later, these cells (30×10^6) were selected with aphidicolin (1 μM) for 23 days at a cell-density of 1×10^6 per plate (40). This concentration of aphidicolin (1.0 μM) was used because the wild type V79 cells are very sensitive to this drug, e.g., they have a relative plating efficiency (by single-cell platings) of 4×10^{-4} at 0.5 μM of aphidicolin (40,226). A lengthy treatment (longer than 10 days) of V79 cells in mass-culture with 0.4 μM of aphidicolin resulted in no clonable cells (personal observation). The combined mutation induction was performed because it is assumed that gene(s) responsible for aphidicolin resistance (aph^r) is (are) autosomal recessive.

Four aph^r colonies at a frequency of 4.5×10^{-7} survived the selection. The surviving colonies were isolated by removing the medium and attaching a glass cylinder with autoclaved adhesive (Cellseal, Fisher Scientific) around the colony; the encircled cells were dissociated from the plate surface with trypsin. One of the

aph^r mutants, aph^r-4, is a slow-growing thymidine auxotroph. It continuously gave rise to fast growing "revertants" when cells were grown in medium supplemented with 5% FCS. Some slow-growing thymidine prototrophic colonies were also recovered when cells were grown in medium supplemented with 5% dialyzed FCS. These revertants were isolated and characterized. The aph^r-4 mutant has been recloned. One of the recloned population, aph^r-4-2, has been thoroughly characterized in this study together with a fast-growing thymidine auxotrophic revertant aph^r-4-R2, and the slow-growing thymidine prototrophic revertants, aph^r-4-RP4 and RP5.

To reduce the frequency of fast-growing revertants, in the aph^r-4-2 population, aph^r-4-2 cells were grown in 1.0 μM aphidicolin for one week. A large population of these cells was frozen and stored in a liquid nitrogen tank. No cell culture was used for an extensive period of time: a new culture from storage stocks was initiated for each experiment.

Deoxyribonucleoside Triphosphate Measurements

The endogenous deoxyribonucleoside triphosphates (dNTP) were measured according to L. Skoog and his colleagues (157,245). To obtain partially synchronized (276,281) cell extracts, cells from confluent cultures were plated at a density of 1.5×10^6 per plate (9 cm, 2.3×10^4 per cm^2) in growth medium with 5% FCS one day before extraction. Collection of cells was made with a rubber policeman and dNTP was extracted by 60% methanol at -20^oC overnight. The extracts were separated from cell debris with centrifugation (29×10^3 xG, Survall) and were lyophilized with a lyophilizer (Model 10-MR-TR, the Virtis Co., Gardiner, New York). The dried samples were further treated with perchloric acid (0.5 N) to reduce interference from enzymes (e.g. nucleases, nucleoside diphosphokinases and deoxyribonucleoside monophosphokinases which phosphorylate dAMP, dGMP and

dCMP), and were then treated with potassium hydroxide (1.5 N) to neutralize perchloric acid. The extracts were further lyophilized and dissolved in double distilled water (1-2 ml), then stored in aliquots at -20°C until dNTP determinations. The cell debris in the precipitation was used for determinations of DNA contents.

The dNTP pools were measured, using the defined copolymers poly(dI-dC)-poly(dI-dC) and poly(dA-dT)-poly(dA-dT) as primer templates and E. coli DNA polymerase I. This method works on the ability of DNA-polymerase in the presence of primer template to incorporate dNTP into an acid-insoluble product. When dCTP was measured, poly(dI-dC)-poly(dI-dC) and excess ^{3}H-dGTP were used. That is, when dCTP in the cell extracts was a limiting factor in the reaction, the extent of polymerization was measured by the incorporation of ^{3}H-dGTP into the primer template as an acid-insoluble product. The incorporation of radioactivity was directly proportional to the amount of the limiting deoxyribonucleoside triphosphate. When dGTP was measured, excess ^{3}H-dCTP was used. For measurement of dATP or dTTP, primer template poly(dA-dT)-poly(dA-dT) and excess ^{3}H-dTTP or ^{3}H-dATP, respectively, were employed. The dNTP pools are expressed as picomole (pmol) per μg DNA for each nucleotide. For detailed dNTP measurment, see Appendix A.

Unscheduled DNA Synthesis

The quantitative assay for the excision repair capacities, that is, the unscheduled DNA synthesis assay, was originally developed by Trosko and Yager (273), and it measures incision, excision and polymerization of DNA following damages by radiations or chemical mutagens. Cells were plated at a density of 3-6 x 10^{6} per plate (6 cm, Corning Glass Works) in growth medium with 5% FCS and incubated overnight. The next day, cells were maintained in tyrosine deficient medium (GIBCO) and 5% dialyzed FCS for 24 hours; then they were maintained in a medium

lacking arginine and isoleuccine (GIBCO), but also supplemented with 5% dialyzed FCS (GIBCO), for another 48 hours. One hour prior to UV irradiation, hydroxyurea (5 mM, Sigma) was added to the medium to further arrest scheduled DNA synthesis. Before UV irradiation, the medium was collected and reserved. Cells were then exposed to ^{3}H-TdR (5 µci/ml, 45 ci/mmol) in the reserved conditioned medium following UV irradiation. Cells were harvested (in PBS with a rubber policeman) after 1.5 or 3.0 hours of ^{3}H-TdR incorporation, centrifuged and stored in the freezer for further analysis. The control cells received the same treatment except that they had no UV irradiation. All treatments were measured in duplicate or triplicate plates. Radioactivities were measured in DNA extracted by trichloracetic acid. The specific activities (dpm/µg DNA) of DNA repair capacities are expressed as the amount of radioactivities per unit of DNA.

The radioactivities were measured in a counting fluid containing toluene and dioxene by a liquid scintillation spectrophotometer (LS 9000, Beckman).

Mutation Induction

1. Mutagenesis with Ultraviolet Light Radiation.

Cells were plated for attachment in medium supplemented with 5% FCS. Four hours later, culture medium was removed and cells were irradiated with various doses of UV from a germicidal lamp (General Electric, 25T8-25w) which delivers 1.4 J/m^{2}/sec. Enough cells were plated so that each treatment resulted in survivors of at least 1 x 10^{6} cells. Irradiated cells were then incubated in fresh medium supplemented with 5% dialyzed FCS for 24 hours so that replication and repair of DNA were dependent entirely on endogenous nucleotides. After the DNA repair period, cells were grown in 5% FCS and refed with fresh medium every day to ensure optimal expression of mutants. In some experiments, thymidine (TdR) at 2-4 µM or aphidicolin at 0.5 µM was added to the growth medium 3 days after UV

irradiation to study the effect of TdR or aphidicolin on mutation expression. Survival rates of cells receiving the same UV doses were also determined concurrently (see Cytotoxicity and Cloning Efficiency Determinations).

In experiments to test the effects of error-free excision repair to the mutation induction by liquid holdings with conditioned medium, both the control and UV irradiated cells were incubated in the conditioned medium for various times during the DNA repair period, and then in the regular growth medium thereafter. The survival of UV irradiated cells was corrected by the plating efficiency of control cells which were grown in the same conditioned medium for the same duration but without the UV irradiation.

When mutagenized cells reached 90% confluence, the cells were subcultured (one to 8 dilutions for the wild type and aph^r-4-R2 cells; one to 4 dilutions for aph^r-4-2 cells) into flasks (75 cm^2, Corning Glass Works) and similarly subcultured every two days thereafter.

2. Mutagenesis with X-ray Radiation.

Cells were suspended in an ice-cold medium during X-ray irradiation (184 R/min), and then were grown in flasks (75 cm^2, Corning Glass Works) with medium and 5% FCS. Cultures were never allowed to exceed 90% confluence without subculture and the growth medium was changed every day. Survival rates were determined concurrently.

Mutation Assys

Quantitative forward mutations were measured by 3 assay systems: mutations from sensitive to resistance to 6-thioguanine, ouabain and diphtheria toxin. The genetic loci involved are genes for hypoxanthine guanine phosphoribosyl transferase

(HGPRT), sodium-potassium adenosine triphosphotase (Na^+/K^+ ATPase), and elongation factor-2 of protein synthesis respectively.

Replating techniques were used throughout the experiments. After sufficient expression time, cells were trypsinized and replated at appropriate densities. Enough plates were used so that induced mutants were selected from a population of at least 10^6 cells. For ouabain resistant (oua^r) cell selection, 4×10^5 cells were plated into each plate containing 1 mM ouabain (Sigma) with or without 4 μM TdR. For selection of $6TG^r$ mutants, cells were plated either at a density of 1×10^5 per plate (low cell-density selection) without TPA, or at a density of $1.0\text{-}1.5 \times 10^6$ per plate (high cell-density selection, for X-ray induced mutation) with TPA (0.01 μg/ml, 4 days); and $6TG^r$ mutants were selected by 6TG (6 μM) with or without 2 μM TdR. At these cell densities and drug concentrations, mutant-recoveries were more than 90% (37,38,92,153,284,302). Selective medium was renewed every 3-4 days. For diphtheria toxin resistant (DT^r) mutant selection, 1×10^5 cells were plated into each plate, and these cells were allowed to grow for 2 days so that each attached cell developed into 4-16 cell-stage. The medium was changed and DT (0.1, 0.2 or 0.6 lf/ml) was added to each plate. The medium contained no nucleosides except those from 5% FCS. Four days later, DT was removed and colonies were allowed to grow for another 6-7 days. Under this selection procedure, the recovery of DT^r colonies was identical to the recovery of those with DT present for the entire period of development (unpublished results).

All resistant colonies were fixed with ethanol, stained with Giemsa and scored after 7,8 or 12 days of growth. Two or three replatings were performed in each experiment, and each experiment was also repeated at least twice. Some resistant colonies were isolated with a glass cylinder and trypsin, and retested for their resistance to individual drug or toxin.

Spontaneous Mutation Rate Determinations

1. Fluctuation Analysis.

Spontaneous mutation rates in ouabain (oua^r), diphtheria toxin (DT^r) and 6-thioguanine ($6TG^r$) resistant loci were determined by using fluctuation analysis (163,175) or modified Newcomb's replating technique (189,277,282). Clones derived from single cells of each cell line were isolated from culture dishes and were grown in 24-well plates (16 mm diameter per well, Costar, Massachusetts) or in plates (9 cm, Corning Glass Works, Corning, N.Y.) when more than 1×10^6 were desired. Generally, 72 to 150 clones (replicate or sib cultures) were isolated per experiment and no more than 10 clones were from the same culture dish. When cells reached 90% confuence, these cultures were individually trypsinized and replated equally in three tissue culture plates. Mutants resistant to ouabain were selected by ouabain (1 mM) containing medium with 4 μM TdR that was changed every three days. For determinations of mutation rate at the $6TG^r$ locus, cells were plated in triplicate plates ($1\text{-}1.5 \times 10^6$ cells per plate) containing fresh medium and 2 μM TdR. Four hours later, TPA (Sigma Chemical Co., 10 ng/ml) and 6TG were added. The TPA was removed by medium change 4 days later. This concentration of TPA and duration of exposure are not toxic to V79 cells, but are able to eliminate metabolic cooperation which decreases the recovery of $6TG^r$ mutants (153,284,302). The 6-thioguanine or ouabain and TdR were present in the medium for the entire period of colony development (7 to 12 days).

The DT^r mutants were selected by a modified replating technique reported previously (234). Cells from each sib culture were plated at a density of less than 2×10^5 cells per plate; two days later, the medium was changed to a fresh one, and DT (0.6 lf/ml) was added. The toxin was removed four days later. The commonly used replating technique (39,92) was utilized in one experiment in which DT (0.2 lf/ml) was added four hours after replating and was present for three days. All DT^r

clones thus selected were allowed an additional 6 days for colony development. No purine or pyrimidine except that in 5% FCS, was present in the medium. The cell number and plating efficiency were estimated simultaneously from a sample of randomly selected clones. The mutation rate was calculated according to Luria and Delbruck (163) using the Po estimation.

2. Multiple Replating Technique.

Because expression of $6TG^r$ or DT^r mutants has a relatively long phenotypic lag, it is possible that the mutation rates in these loci may be underestimated. A modified Newcomb's technique (189,233,277,282) was utilized to determine the spontaneous mutation rates at these loci. This technique consists of multiple mutation frequency determinations and concurrent cell-division monitorings at different times, and obtains the mutation rate from the increase in the mutation frequency of a cell population over a known number of cell divisions.

A cell population of $2\text{-}5 \times 10^7$ cells derived from two single cells of each cell line was grown in medium supplemented with 5% FCS. For mutation frequency determinations, a total of $2.4\text{-}3 \times 10^6$ cells from each cell line was replated for mutant selection (cell density: 1×10^6 cells per plate for $6TG^r$ mutants; 4×10^5 per plate for DT^r mutants). 6-Thioguanine and TPA, or diphtheria toxin (0.6 lf/ml) were added four hours after cell plating. In addition to the cells that were plated for an estimation of mutation frequency, a total of $10\text{-}20 \times 10^6$ cells (aph^r-4-2, 20×10^6; V79, 10×10^6) was distributed to two culture flasks (150 cm^2, Corning Glass Works). Cells in the flasks were subcultured when necessary and the total number of cells at each subculturing was determined as described above. The cell counts allow determinations of cell divisions occurring between two time points when mutation frequencies are estimated. The spontaneous mutation rate (a) was calculated from the equation: $a=\ln 2[2(MF_x-MF_{x-1})/\ln(N_x/N_{x-1})]$ where MF and N are mutation frequency and cell number respectively. The calculated mutation rate using this

equation allows a paired t-test comparison (249) between cell lines. All determinations were repeated.

Mutation Frequency and Mutation Rate Calculations

1. Mutation Frequencies.

The frequency was determined by dividing the number of mutants selected from mutation assays by the total cells plated in each treatment, corrected by the plating efficiency. The frequency is expressed as numbers of mutants per 10^5 or 10^6 survivors (i.e., clonable cells).

2. Mutation Rates.

A. Fluctuation Analysis.

The average number of cell divisions (d) was calculated from the formulae: $(N_f-N_o)/\ln 2$, where N_f and N_o are final and initial cell numbers per replicate culture. The mutation rate was calculated on the basis of the mean number of mutants per replicate culture. There are three ways to obtain this mean: a) the Po estimation [mean = $\ln(1/Po)$] (163), where Po is the fraction of cultures with no mutants; b) the likely mean, CaN_f/C, which is the average mutants per replicate culture assumed to arise from certain generations back when no mutation has yet occurred, and which can be obtained indirectly from the observed mean; and c) the arithmetic mean, r, which is the observed mutants per replicate culture. The means calculated from these methods are presented in RESULTS. Because $r = aN_f\ln(CaN_f)$ (163) and $Cr = CaN_f\ln(CaN_f)$ (32) where a is mutation rate and C is the number of replicate cultures, the mutation rate, a, is $Cr/CN_f\ln(CaN_f)$ or CaN_f/CN_f. The value CaN_f can be obtained from the tables provided by Capizzi and Jameson (32) if r and C are known.

The mutation rate was calculated from the equation: mean/cell divisions, and is expressed as mutants per cell per division. Only the mutation rate calculated by the Po estimation has been presented.

B. Multiple Replating Technique.

The mutation rate is calculated from the equation derived from the original equation (the difference in mutation frequencies divided by the difference in cell numbers) by Newcomb (189,233,277,282). Because the number of mutants (m) observed in one particular replating time point (x) arise in one or more prior generation(s), mutation frequency (MF_x) can be expressed as $m_x/½N_x$ and the difference in frequency would be $m_x/½N_x - m_{x-1}/½N_{x-1}$, or $2(MF_x - MF_{x-1})$. The change in cell generation is $\ln(N_x/N_{x-1})/\ln 2$ (282). The mutation rate can be obtained from $[2(MF_x - MF_{x-1})/\ln(N_x/N_{x-1})]\ln 2$ (282). The mutation rate can also be calculated from the slope of the mutation frequency curve as a function of cell divisions (256). The mutation rate calculated from this equation is expressed as per cell per generation.

RESULTS

Isolation of UV-sensitive Aphidicolin-resistant Mutant and Its Revertants

Cells (3×10^7), which were previously mutagenized with BrdU-black light and UV, were plated for selection with 1.0 μM aphidicolin. Four colonies survived the selection. One of these four aph^r mutants is found to be sensitive to UV (40). This mutant (aph^r-4) has been recloned. One of the recloned lines, aph^r-4-2, was extensively studied in this dissertation. This UV sensitive mutant is slow-growing compared to V79 cells. Upon single cell plating, larger size colonies were observed. These were found to be TdR auxotrophic and UV-normal revertants. One of these revertants with a normal growth rate (designated aph^r-4-R2) was chosen for study. In addition, when the mutant cells were plated in medium with 5% dialyzed FCS, few slow-growing survivors were found. These appear to be thymidine prototrophic revertants. Two revertants of this category designated aph^r-4-RP4 and aph^r-4-RP5 were also studied. The colony-forming abilities of these mutants and V79 cells in the presence of various concentrations of aphidicolin are shown in Figure 1. The results indicate that aph^r-4-2 and its revertants were resistant to aphidicolin compared to V79 cells. The V79 cells cannot grow in medium with more than 0.4 μM aphidicolin, whereas the revertants have more than 80% plating efficiency in the same medium. The figure also indicates that the colony-forming abilities of aph^r-4-R2 cells decreased at 0.8 μM and those of aph^r-4-2 and aph^r-4-RP cells decreased at 1.0 μM aphidicolin. The results found here are consistent with the results reported by others in CHO (226) or V79 cells (40).

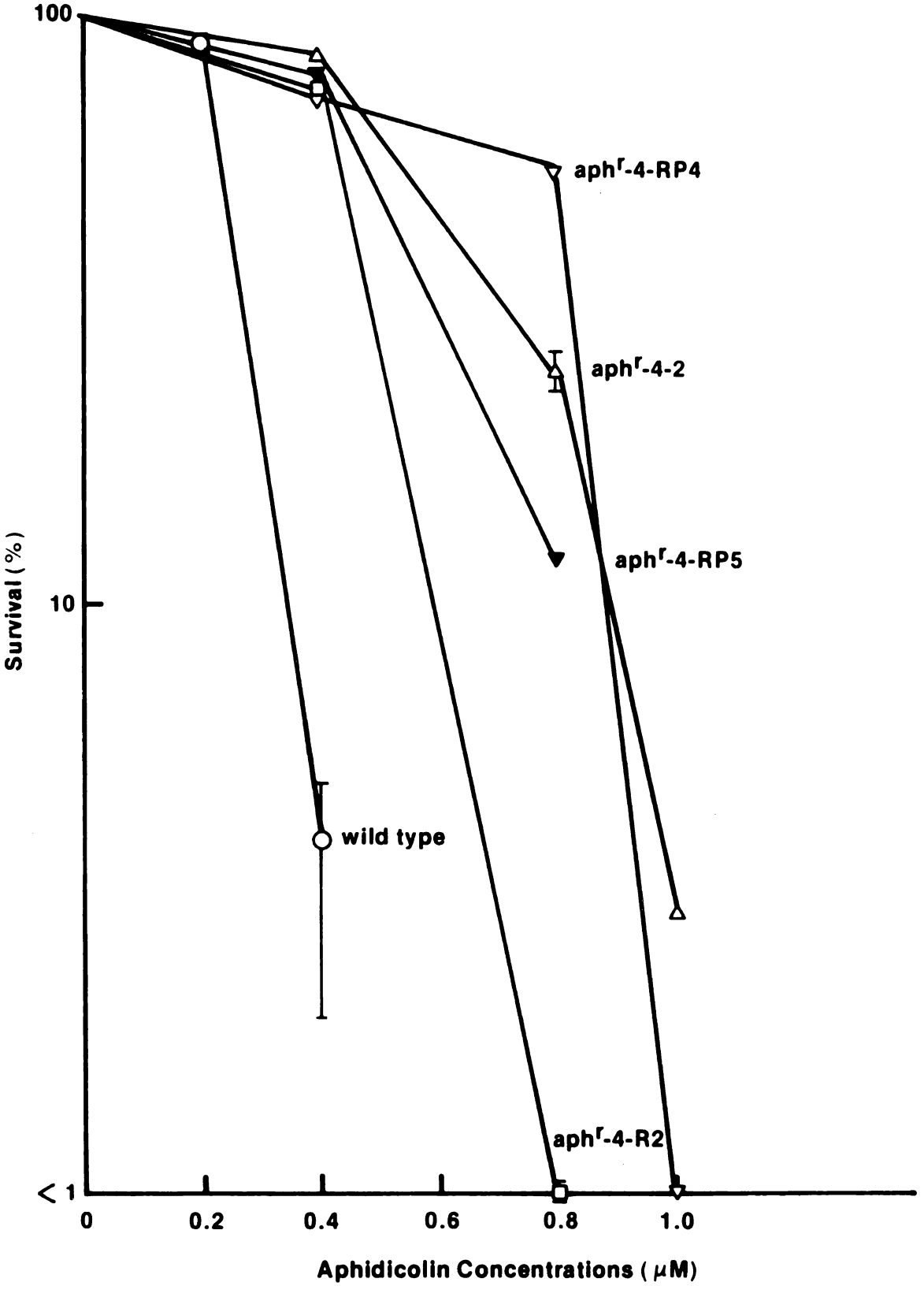


Figure 1. Colony-forming abilities of aph^r-mutants and wild type V79 cells in the presence of various concentrations of aphidicolin. Aphidicolin was present for the entire period of colony development.

Growth Rates

Cells were plated at a density of 400 per plate (about 6 cells per cm^2). Four hours after plating, 6 single cells per plate were chosen for observation of cell division through the microscope every 24 hours. The following results were found. The V79 cells divided every 14 hours, aph^r-4-2 divided every 24 hours and aph^r-4-R2 divided every 18 hours. The aph^r-4-RP cells divided every 24 hours in the presence of 5% dialyzed FCS. In another experiment, the V79 and aph^r-4-2 cells were plated in a density of 0.75 and 1.4 x 10^3 per cm^2, respectively (0.5 and 1 x 10^5 cells per 9 cm plate) in medium with 5% FCS. Cells in duplicate plates were trypsinized and counted every 24 hours after plating. Figure 2 shows that aph^r-4-2 cells double every 24 hours and the V79 cells every 14 hours. Cell counts at 72 hours showed non-exponential growth which may be due to nutrient depletion.

Deoxyribonucleoside Triphosphate Pool Measurements

The inhibition of aphidicolin on purified α-polymerase has been shown to be competitive with deoxycytidine triphosphate (dCTP). To test the possibility that the resistance of aph^r-mutants to aphidicolin might be due to elevated endogenous dCTP pools, the deoxyribonucleoside triphosphates (dNTP) were measured in aph^r-4-2, aph^r-4-R2 and the V79 cells. When the dNTP were extracted with methanol (60%) and assayed for dNTP levels, it was found that dCTP pools in aph^r-4 and aph^r-4-R2 are 3 to 8 times higher than those in V79 cells (40). However, when the methanol extracts were treated with 0.5 N perchloric acid which has been reported to eliminate enzymatic activities, such as nuclease, nucleoside diphosphokinase and deoxyribonucleoside monophosphokinase involved in the conversion of dAMP, dGMP, and dCMP to its dNTP (192), the results (Table 3) were different from those reported previously. There appears to be no significant difference in dCTP in the V79, aph^r-4-2, and aph^r-4-R2 cells. In these experiments the exogenous dNTP was

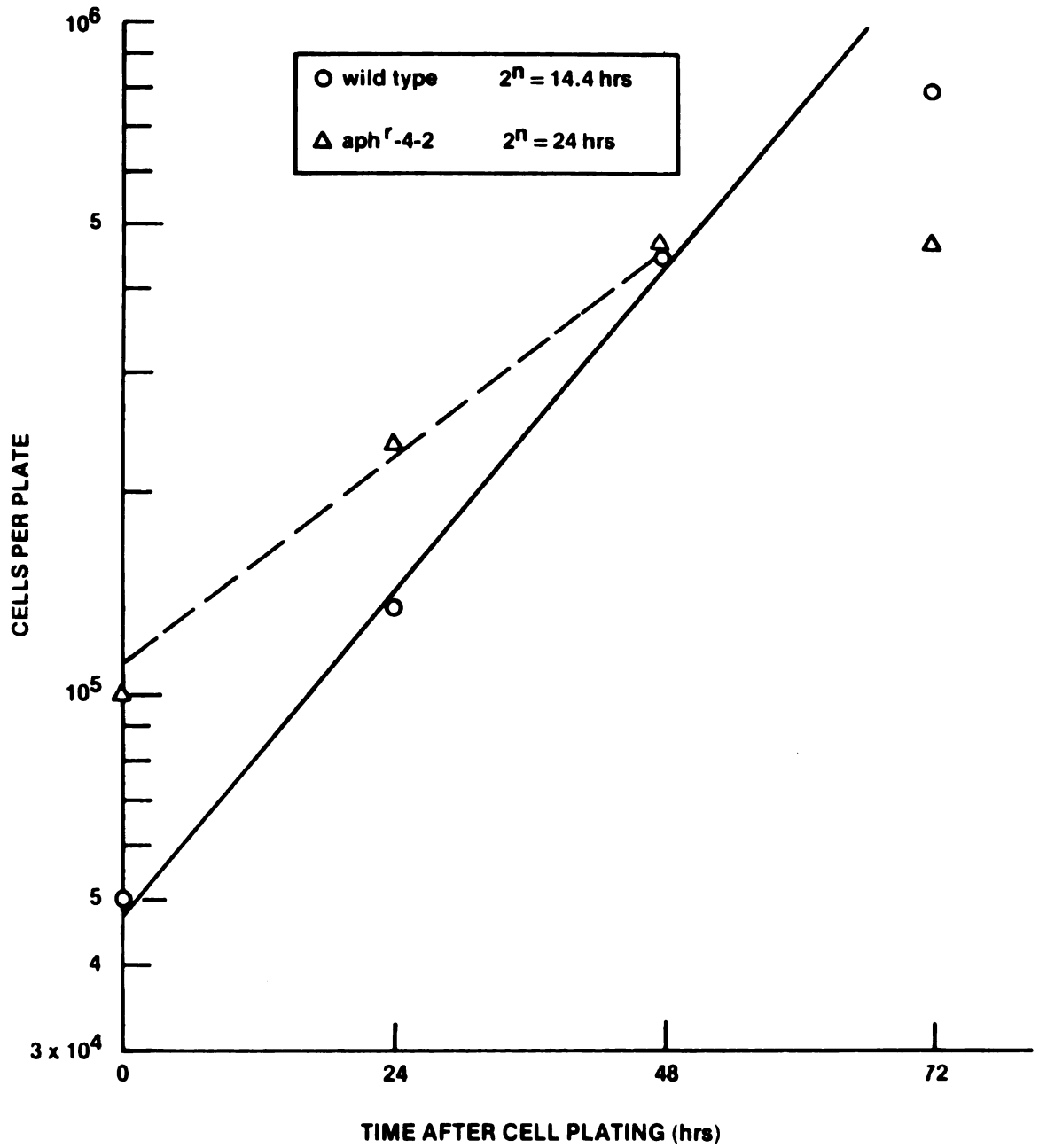


Figure 2. Growth rates for aph^r-4-2 and the wild type V79 cells in the growth medium supplemented with 5% FCS. 2^n = doubling time.

Table 3. Deoxyribonucleoside Triphosphate Pools in Aph^r-mutants and V79 Cells[a.b.]

Cell lines	dCTP	dTTP	dATP	dGTP
(Experiment #1)				
V79	15.6	2.8	2.24	0.02
aph^r-4-R2	7.9	2.9	0.94	0.04
(Experiment #2)				
V79	4.9	2.4	-	-
aph^r-4-2	8.6	1.25	-	-
aph^r-4-R2	4.0	10.0	-	-
V79 with Markers[c.]	17	7.5	-	-

a. Cells in confluency were plated at a density of 1.0-1.5 x 10^6 per plate to obtain log phase growth. One day (24 h) after replating in 5% FCS, cells were harvested; and dNTP were extracted and assayed according to methods described in the text.

b. pmole/μg DNA. -: not assayed.

c. Deoxyribonucleotides (pmole) were added to V79 cells during cell harvesting: dCTP, 10; dTTP, 5.

also added to the wild type V79 cells during harvesting. The results indicate that exogenous dCTP and dTTP were not degraded by treatment with 0.5 N perchloric acid.

Nutritional Requirements

In a series of studies, Chang et al., (40) have reported that aph^r-4 requires deoxyuridine, deoxycytidine or thymidine for growth in medium with dialyzed FCS. It appears that the mutant is a thymidine auxotroph. Results presented in Tables 4 and 5 confirm that aph^r-4-2 and aph^r-4-R2 cannot grow in medium with 5% dialyzed FCS. In one experiment, aph^r-4-2 had 38% colony-forming ability compared to 0.3% for aph^r-4-R2. This may be due to the presence of prototrophic revertants in aph^r-4-2 cell population. When the medium was supplemented with 2 or 4 μM TdR, these two mutants grew as well as the wild type V79 cells. Addition of 5 μM of adenine deoxyribonucleoside (AdR) or guanine deoxyribonucleoside (GdR) did not support the growth of aph^r-4-2 or aph^r-4-R2 cell in 5% dFCS.

Additions of TdR, AdR or GdR at a concentration of 100 μM resulted in drastic cell killing for aph^r-4-2 and aph^r-4-R2. The cytotoxic effects of TdR, AdR or GdR at 100 μM were significantly less in V79 than in aph^r cells. Additions of TdR (2 μM) to the medium, supplemented with AdR (5 or 100 μM) or GdR (5 μM), reversed the cytotoxic effects of AdR or GdR. Thymidine at 2 μM, however, did not reverse the cytotoxicity of 100 μM GdR. It is clear that aph^r-4-2 and aph^r-4-R2 are auxotrophic to TdR but are also sensitive to 100 μM TdR or GdR, and to 100 μM AdR if TdR (2 μM) is not added.

Since fast-growing revertants of aph^r-4 are all TdR auxotrophs and aph^r-4 may harbor more than two mutations, it is possible that a second type of revertant exists. In order to search for this second type of prototrophic revertant, aph^r-4-2 cells (400 cells/plate) were grown in medium supplemented with 5% dialyzed fetal

Table 4. Relative Colony-forming Abilities of Aph^r-mutants and V79 Cells in Medium Supplemented with 5% Dialyzed FCS and Various Deoxyribonucleosides at 37°C

Cell lines	None										With 2 μM TdR			
		TdR			AdR		GdR		CR	CR(4mM)	AdR		GdR	
		2μM	4μM	100μM	5μM	100μM	5μM	100μM	4mM	TdR(4μM)	5μM	100μM	5μM	100μM
Experiment #1														
V79		100	-	-	-	-	-	-	-	-	103	74	97	35
aph^r-4-2		100	-	-	-	-	-	-	-	-	93	61	87	2
aph^r-4-R2		100	-	-	-	-	-	-	-	-	76	45	86	0
aph^r-4-RP4		100	-	-	-	-	-	-	-	-	117	79	110	2
aph^r-4-RP5		100	-	-	-	-	-	-	-	-	110	79	102	0
Experiment #2														
V79	84	100	87	90	71	53	73	22	0	100	102	93	96	90
aph^r-4-2	38	100	92	8	29	0	22	0.1	0	2	117	83	107	21
aph^r-4-R2	0.3	100	72	0	4	0	4	0	0	100	65	64	63	4
aph^r-4-RP4	86	100	139	0	72	2	83	0	0	3	135	35	118	84
aph^r-4-RP5	77	100	115	0	45	0.3	52	0	0	88	103	81	89	8
Experiment #3														
V79	100	100	-	100	94	-	81	-	-	-	100	88	93	70
aph^r-4-2	1.3	100	-	1.3	0.4	-	0.4	-	-	-	58	8	46	0
aph^r-4-R2	1	100	-	0	0	-	0	-	-	-	74	44	75	0
Experiment #4														
V79	100	100	100	100	-	0	-	0	0	74	-	68	-	-
aph^r-4-R2	0	98	100	0.3	-	0	-	0	0	100	-	79	-	-

The relative colony-forming abilities were determined by plating 1,200 cells of each cell line in medium supplemented with 5% dialyzed FCS and various deoxyribonucleosides for the entire period of colony development.

TdR: thymidine, AdR: adenine deoxyribonucleoside, GdR: guanine deoxyribonucleoside, CR: cytidine. Results presented here are percent survival; -, not tested.

Table 5. Relative Colony-forming Abilities of Aph^r-mutants and V79 Cells in Medium Supplemented with Various Deoxyribonucleosides at 37°C and 34°C

Cell lines	(°C)	Medium supplemented with: 5% dialyzed FCS				5% dialyzed FCS + 2 μM TdR		
		None	AdR 100 μM	GdR 100 μM	BrdU 100 μM	None	AdR 100 μM	GdR 100 μM
V79	37	92	0	0	0	100	68	54
	34	100	2	0	0	100	40	12
aph^r-4-2	37	21	0	0	0	100	54	3
	34	18	0	0	0	100	16	3
aph^r-4-R2	37	0.1	0	0	0	100	29	0
	34	0	0	0	0	100	23	0
aph^r-4-RP4	37	76	0	0	0	100	58	0
	34	53	0	0	0	100	23	0

		5% dialyzed FCS: thymidine (μM)			Cytidine 4mM		5% FCS: AraC (μM)	
		2	4	100	-	TdR (4μM)	0.2	0.5
V79	37	100	84	90	0	74	17	0
	34	100	74	70	0	100	-	0
aph^r-4-2	37	100	97	7	0	10	66	0
	34	100	69	0	0	3	-	0
aph^r-4-R2	37	100	100	0.4	0	100	8	0
	34	100	98	0	0	20	-	0
aph^r-4-RP4	37	100	100	13	0	2	48	0
	34	100	70	3	0	17	-	0

The colony-forming abilities were determined by plating 1200 cells of each cell line in medium with various deoxyribonucleosides at either 34° or 37°C for the entire period of colony development. BrdU = bromodeoxyuridine. AraC = cytosine-1-β-D-arabinofuranoside.

Results are percent survival; -, not tested.

calf serum. Ten surviving colonies were isolated two weeks later. All of them are sensitive to UV-irradiation as aph^r-4-2 cells are (data not shown). Two of the ten prototrophic revertants (aph^r-4-RP) were tested for their nutritional requirements. The results presented in Tables 4 and 5 indicate that aph^r-4-RP4, and -RP5 grew well in medium with 5% dialyzed FCS, and that like the parental mutant they were sensitive to 100 μM of TdR, AdR or GdR in the absence of 2 μM TdR. Thymidine at 2 μM reversed the cytotoxicity of AdR for both aph^r-4-RP revertants. Aph^r-4-RP5 is sensitive to GdR (100 μM) whether or not TdR is present. In one of three experiments, aph^r-4-RP4 was not sensitive to GdR (100 μM) when TdR was present. The reason for the discrepancy is not clear.

To test whether the mutation expression is temperature sensitive, the nutritional requirements of aph^r-4-2, and aph^r-4-R2 were observed at both 34^{o}C and 37^{o}C. Table 5 shows that at 34^{o}C, the nutritional requirements, and TdR-, AdR-, and GdR-sensitivities of aph^r mutant and revertants, were not at variance with the phenotype expressed at 37^{o}C.

When bromodeoxyuridine (BrdU) at 100 μM was added to growth medium supplemented with 5% dialyzed FCS, all the aph^r mutants and revertants were as sensitive as the V79 cells to BrdU at 34^{o}C or 37^{o}C (Table 5). It appears that aph^r-4-2, its revertants and the wild type cells are not deficient in thymidine kinase. Table 5 also shows that aph^r-4-2 and its revertants were as sensitive to 0.5 μM cytosine arabinoside (araC) as the wild type.

Because aph^r-4-2 and aph^r-4-R2 cells are TdR auxotrophs and sensitive to TdR and GdR, they may be defective in ribonucleoside diphosphate reductase. Robert de Saint Vincent *et al.*, (278) reported that in a Chinese hamster CCL39 mutant deficient in deoxycytidine monophosphate (dCMP) deaminase, a TdR auxotrophic phenotype can be created if uridine diphosphate reductase is inhibited by a high concentration of cytidine. Chang *et al.*, (40) reported that the wild type V79 cells

used in our laboratory become thymidine auxotrophic in the presence of a high concentration of cytidine. The survivals of aph^r-4-2, aph^r-4-R2, aph^r-4-RP4, -RP5 and the wild type V79 cells in 4 mM cytidine, with or without 4 μM TdR, were compared. The results, presented in Table 4, show that all cell lines could not grow in 4 mM cytidine. Additions of 4 μM TdR reversed the cytotoxicities of cytidine in the wild type, aph^r-4-R2 and aph^r-4-RP5, but not in those of aph^r-4-2 and aph^r-4-RP4. When cells were grown at 34^{o}C, TdR partially reversed the cytotoxicity of cytidine in aph^r-4-R2 (20% at 34^{o}C compared to 100% survival at 37^{o}C).

Mutagen Sensitivities

Since the aph^r mutant could be due to a mutation at the gene encoding DNA polymerase α and since α-polymerase could be involved in the repair process, I was interested in the mutagen sensitivity of the aph^r mutant. The results may provide some preliminary indication concerning the molecular basis of mutation. For this purpose the repair capabilities of aph^r-4-2 and aph^r-4-R2 were measured by determining the survival of these mutants after exposure to direct acting mutagens such as UV, X-rays, dimethyl sulfate (DMS), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and N-acetoxy-2-acetylaminofluorene (NAcAAF). Figures 3A and 3B show the results from 6 determinations using UV as a mutagen. As shown in Figures 3A and 3B aph^r-4-R2 was as sensitive to UV as the wild type V79 cells, whereas aph^r-4-2 and aph^r-4-RP4 cells were more sensitive to UV irradiation. In one experiment, V79 cells had a higher UV survival (Fig. 4) than they did in the 6 previous experiments (Figs. 3A,3B); the reason is not known. The survival of the aph^r-4 mutants and the wild type cells after X-ray irradiations or DMS-treatment is shown in Figures 4 and 5A respectively. The results clearly show that aph^r-4-2 and aph^r-4-R2 were not more sensitive to X-rays or DMS than were V79 cells. At doses higher than 500 rads, aph^r-4-R2 had a slightly lower survival than V79 cells. When MNNG

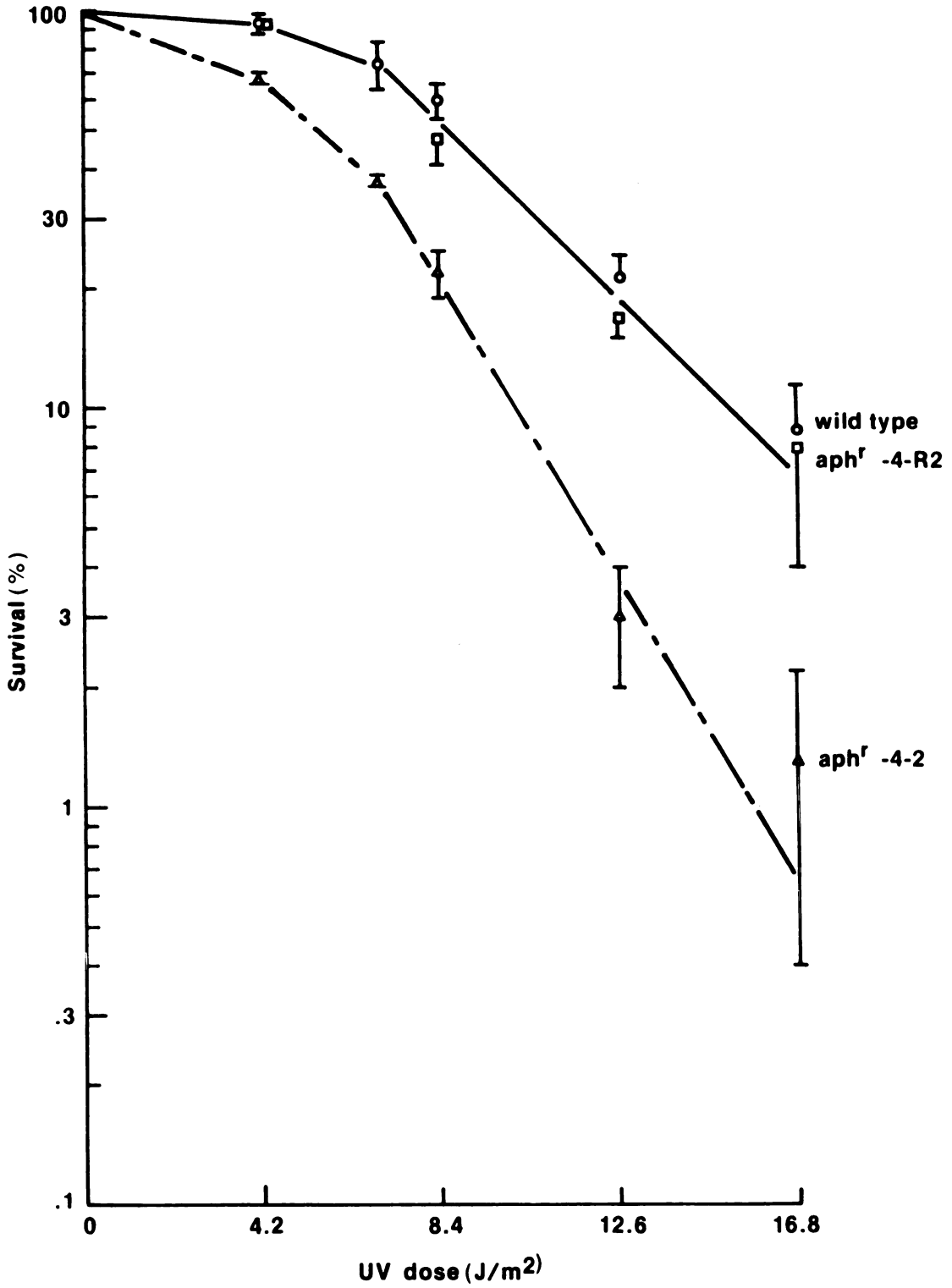


Figure 3A. Survival curves of UV-irradiated aph^r-4-2 (△), aph^r-4-R2 (□) and wild type Chinese hamster V79 cells (○). Cells were allowed to repair in the growth medium supplemented with 5% dFCS for one day and then were grown in the same medium supplemented with 5% FCS. Mean and standard errors of 4 experiments are shown.

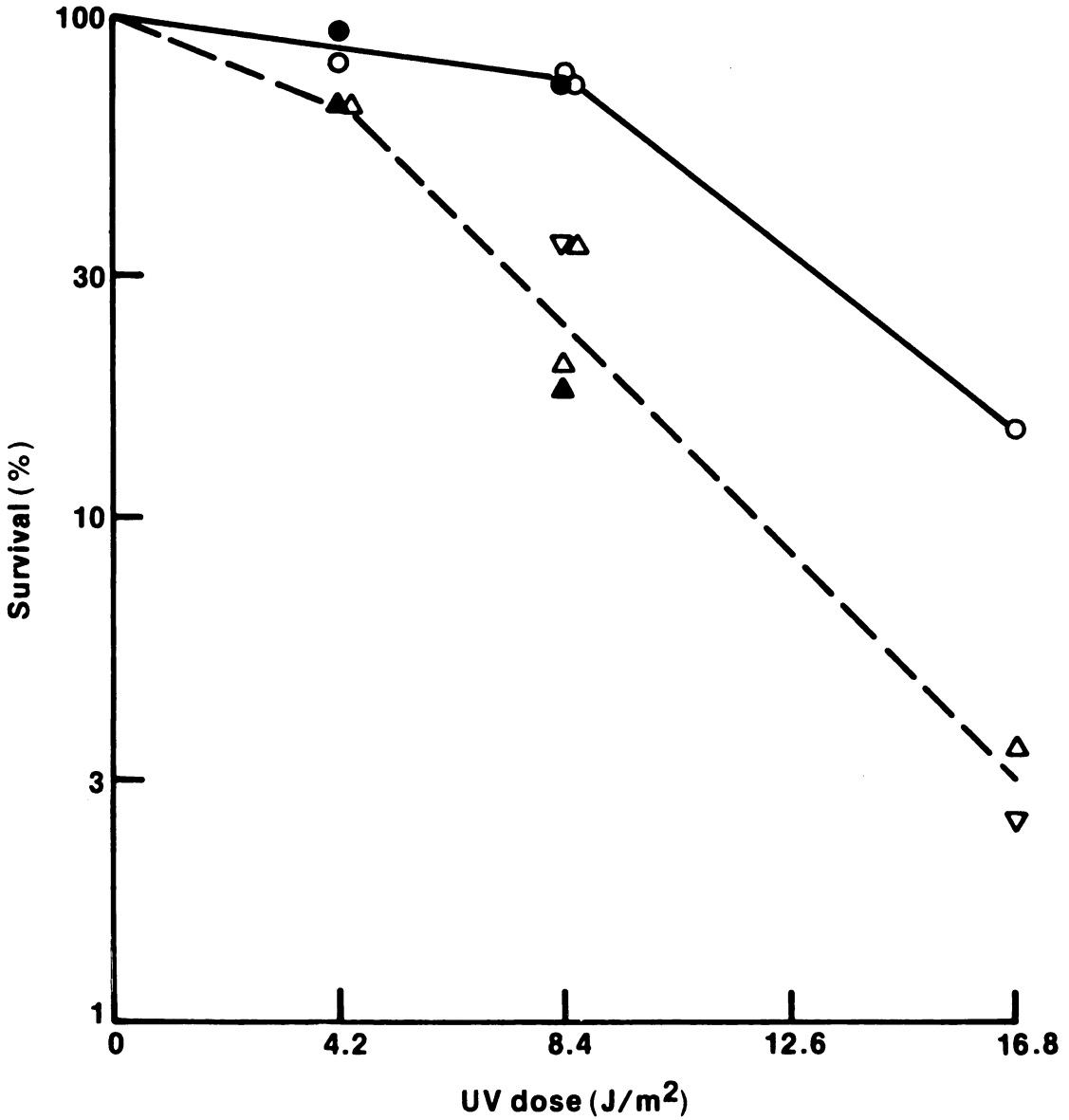


Figure 3B. Survival curves of UV-irradiated aph^r-4-2 (△,▲), aph^r-4-RP4 (▽) and wild type V79 cells (○,●) in medium with (closed symbols) or without (open symbols) TdR (4 μM).

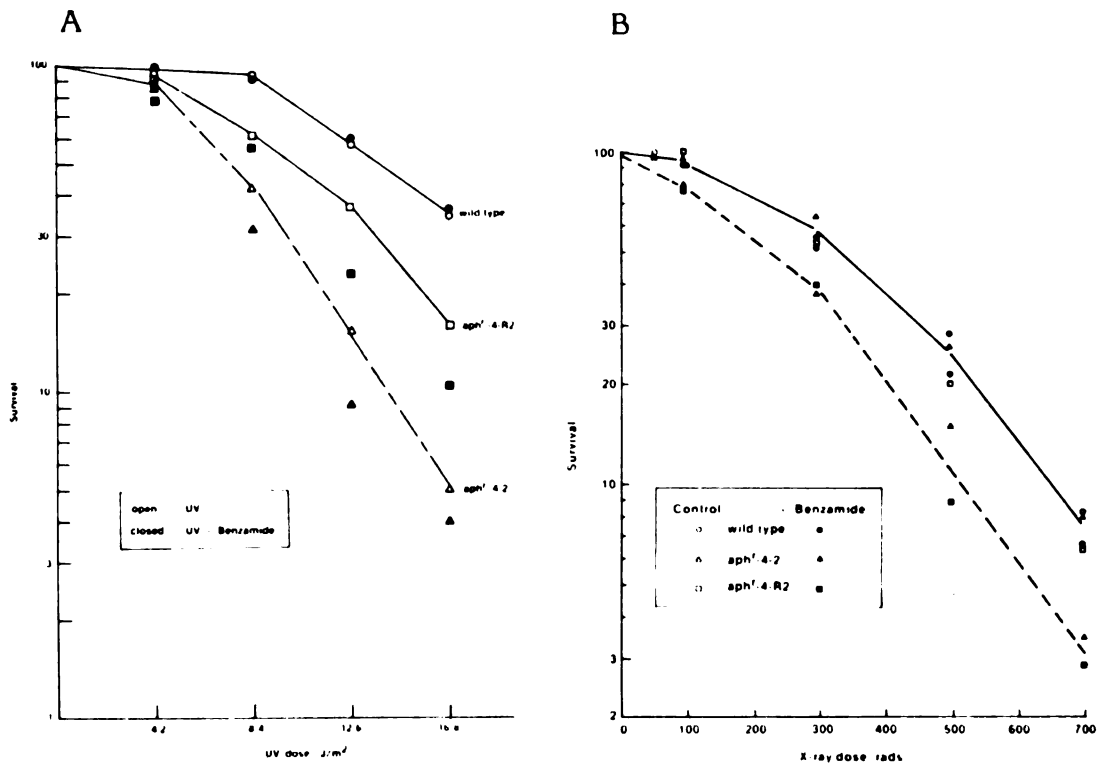


Figure 4. Survival curves of UV-(left), X-ray-(right) irradiated aph^r-mutants and wild type V79 cells. Benzamide (2 mM) was added during the repair period for 3 days in medium supplemented with 5% FCS.

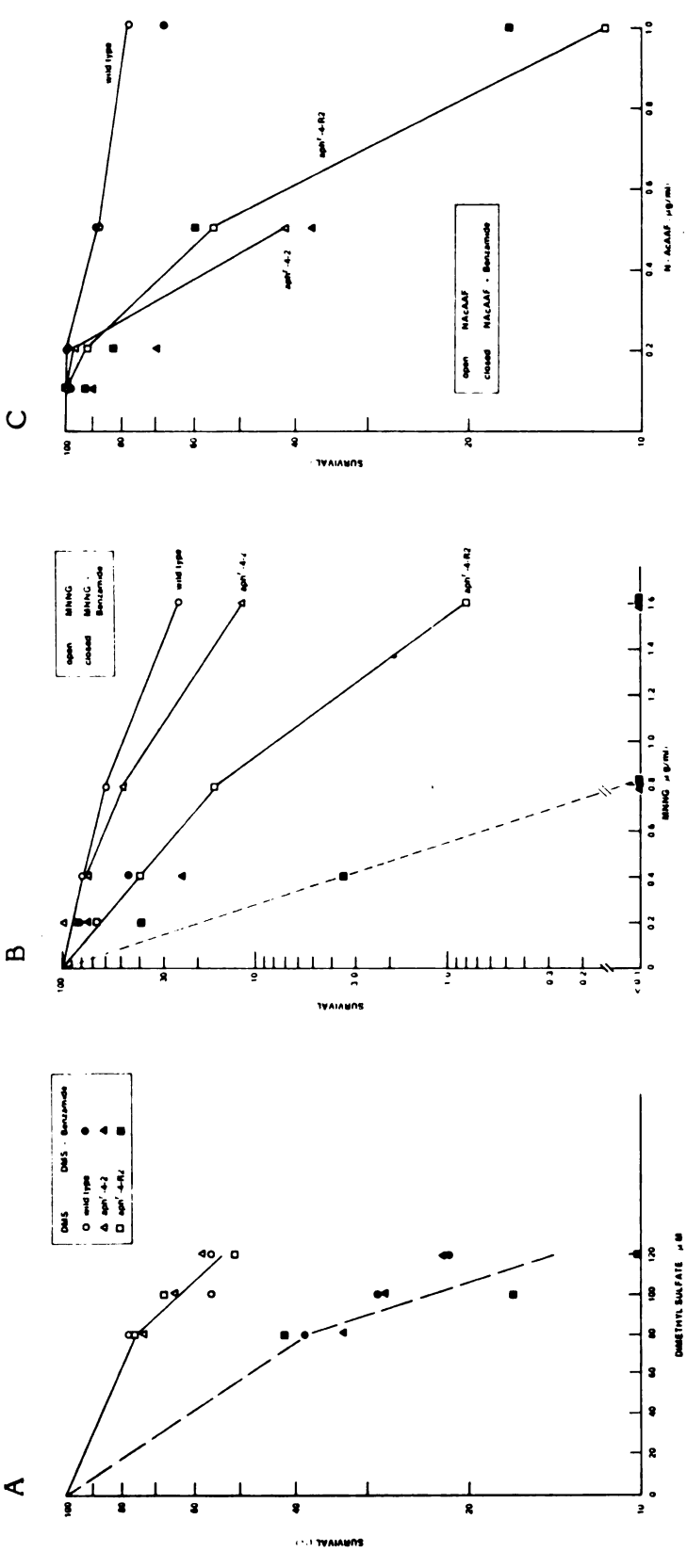


Figure 5. Survival curves of DMS-(A), MNNG-(B) and NAcAAF-(C) treated aph^r-mutants and wild type V79 cells. Benzamide (2 mM) was added during repair period for 3 days in the growth medium supplemented with 5% FCS.

was used as a mutagen, the results (Fig. 5B) showed that aph^r-4-R2 was significantly more sensitive to MNNG. In the second experiment (data not shown), the MNNG-sensitivity of aph^r-4-2 cells was similar to that of the aph^r-4-R2 cells. Furthermore, both aph^r-4-2 and aph^r-4-R2 were very sensitive to NAcAAF compared to the wild type cells (Fig. 5C). The survival curves of X-ray, MNNG- and NAcAAF-treated V79 cells were similar to those reported by Schultz et al., (233,235).

In summary, compared to the wild type cells, aph^r-4-2 is sensitive to UV, MNNG and NAcAAF, whereas aph^r-4-R2 is sensitive to MNNG and NAcAAF. Neither of the cells is more sensitive to X-rays or DMS than the wild type V79 cells.

Effects of Benzamide and Caffeine on Survival of DNA Damage

The previous section indicated that, as compared to the V79 cells, aph^r-4-2 was sensitive to UV, MNNG and NAcAAF whereas aph^r-4-R2 was sensitive to MNNG and NAcAAF. In an attempt to obtain evidence concerning the mechanism of mutagen sensitivity of the mutant, the effects of benzamide [a strong inhibitor of poly(ADP-ribose) polymerase] and caffeine (a weak inhibitor and also an inhibitor of "by-pass" repair pathway in V79 cells) on cytotoxicities of mutagens, were determined. Table 6 shows that benzamide at 2 mM was not toxic to the cell lines studied. Dimethyl sulfoxide at 0.25% or 0.5%, with or without benzamide, did not significantly affect cell plating efficiencies. Results presented in Figures 4 and 5 show that benzamide sensitized the lethal effects of MNNG and DMS, but not of UV, X-ray or NAcAAF in V79 cells. For aph^r-4-2 and aph^r-4-R2 cells, benzamide further enhanced the lethal effect of UV, X-ray, MNNG, or DMS but not that of NAcAAF. Table 7 shows that the enhanced cytotoxic effects of benzamide in aph^r-4-2 or aph^r-4-R2 cells were greater than in V79 cells, when cells were treated with

Table 6. Effects of 12-O-Tetradecanoyl Phorbol-13-acetate (TPA), P
on the Plating Efficiency (%) of A-1

Table 6. Effects of 12-O-Tetradecanoyl Phorbol-13-acetate (TPA), Benzamide, or Caffeine on the Plating Efficiency (%) of Aph^r-mutants and Wild Type V79 Cells[a]

A.	(DMSO %)	Control			with Benzamide (2 mM)		
Cell line		V79	aph^r-4-2	aph^r-4-R2	V79	aph^r-4-2	aph^r-4-R2
Expt. #1	0	87	81	31	77	69	29
#2	0	63	80	71	52	86	63
#3	0	73	55	50	-	-	-
	0.25	79	53	47	80	49	41
	0.50	70	58	50	82	72	43

B.	Control			Caffeine (0.25 mM)			0.5 mM	
	V79	aph^r-4-2	aph^r-4-R2	V79	aph^r-4-2	aph^r-4-2	V79	aph^r-4-2
Expt. #1	88	59	88	74	45	81	-	-
Expt. #2	58	50	-	-	-	-	47	31

C.	Control		with TPA (0.01 μg/ml)	
	V79	aph^r-4-2	V79	aph^r-4-2
Expt. #1	90	51	90	64
Expt. #2	62	76	60	81
	86	60	87	58
	75[b]	87[b]	65[b]	97[b]
	77[b]	79[b]	76[b]	83[b]

a. Cells were plated at a density of 400 cells per plate (9 cm) in triplicates with medium and 5% FCS. Benzamide was added at the time of cell replating, TPA and caffeine were added 4 hours after replating. Benzamide and TPA were present for 3 and 4 days respectively. Caffeine was present for the entire period of colony development. Results were presented as the average plating efficiency of triplicates.

b. Cells were irradiated with UV (8.4 J/m^2).

Table 7. Effects of Benzamide (2 mM) on the Relative Colony-forming Ability of Mutagen-treated Aph^r-mutants and V79 Cells[a]

Mutagen (dose)		Ratio[b]		
UV (J/m^2)		V79	aph^r-4-2	aph^r-4-R2
	0	1.00	1.00	0.96
	4.2	.95	0.98	0.83
	8.4	.98	0.79	0.89
	12.6	1.00	0.56	0.62
	16.8	1.00	0.77	0.59
X-ray (rads)				
	0	1.00	1.00	1.00
	100	0.99	0.82	0.77
	300	0.96	0.60	0.74
	500	0.76	0.56	0.45
	700	0.88	0.38	0.50
MNNG (µg/ml)				
	0	1.00	1.00	1.00
	0.2	1.00	.75	0.60
	0.4	.58	.32	0.10
	0.8	<.02	<.01	<.01
	1.6	<.01	<.01	<.01
NAcAAF (µg/ml)				
	0	1.00	1.00	1.00
	0.1	0.99	0.91	0.93
	0.2	1.00	0.72	0.90
	0.5	1.11	0.90	1.07
	1.0	0.87	-[c]	1.50
DMS (µM)				
	0	1.00	1.00	1.00
	80	.49	.59	.54
	100	.51	.44	.25
	120	.38	.38	.20

a. Benzamide (2 mM) was added to medium with 5% FCS in triplicate plates at the time of cell plating. Four hours later, cells (> 1200 cells/3 plates) were exposed to mutagens except X-rays. Cells were exposed to benzamide one hour after X-ray irradiation. Benzamide was present for 2-4 days after mutagen treatments.

b. Ratio = Survival (%) with benzamide/survival (%) without benzamide.

c. Not determined.

UV, X-ray or MNNG. Benzamide essentially gave similar enhancements of cell-killings to V79 and aph^r-4-2 cells when DMS was used. It did not enhance the lethal effect of NAcAAF in any cell line.

Table 8 shows the effect of caffeine (0.25 or 0.5 mM) on the survival of UV-irradiated V79 and aph^r cells. The concentrations of caffeine used were not toxic to V79 and aph^r-4-R2 cells, but they appear to be slightly toxic to the aph^r-4-2 cells (Table 6). Although caffeine at either 0.25 or 0.5 mM enhances the lethal effect of UV in both cell lines, it appears that the effect was slightly greater in aph^r-4-2 cells than in V79 cells. This is shown (Table 8) by the ratio of survival with caffeine to survival without caffeine at low UV doses (4.2-12.6 J/m^2), and the difference may not be statistically significant.

Cytotoxicities of Chemicals Used In Mutation Assays

In mutation assay studies, mutants were selected with different selecting agents in the presence or absence of exogenous thymidine or TPA. Experiments were carried out to determine whether there would be a differential effect of these chemicals on the plating efficiency of mutant and V79 cells.

Table 6 shows that TPA at a concentration of 0.01 µg/ml did not affect the plating efficiency in V79 and aph^r-4-2 cells, whether they were irradiated or not irradiated by UV (8.4 J/m^2). Table 9 shows that the three selection agents, i.e., ouabain (oua), 6-thioguanine (6TG) or diphtheria toxin (DT) are equally toxic to aph^r-4-2, aph^r-4-R2 and V79 cells. Additions of TdR to oua, 6TG and DT media did not alter the cell-killing effect of these drugs. Aph^r-4-2 and aph^r-4-RP4 cells apparently had a high frequency of spontaneous mutants resistant to oua, 6TG or DT.

Resistant cells selected by these drugs were also isolated and grown in 24-well plates in the absence of the drugs for several generations, before being retested

Table 8. Effects of Caffeine on UV-irradiated Chinese Hamster Cells[1]

Expt. No.	Cell lines	UV dose (J/m^2)	Survival (%) Control	+ Caffeine[2.3.]	Ratio[4]
1	V79	0	100	100	1.00
		4.2	58	45	.76
		8.4	41	6.4	.16
		12.6	13	0.6	.05
		16.8	3	0.2	.07
	aph^r-4-2	0	100	100	1.00
		4.2	55	32	.58
		8.4	22	5	.23
		12.6	7	1	.14
		16.8	2	0.1	.05
2	V79	0	100	100	1.00
		4.2	98	77	.79
		8.4	47	22	.47
		12.6	11	5	.45
		16.8	3	1	.33
	aph^r-4-2	0	100	100	1.00
		4.2	67	40	.60
		8.4	22	7	.32
		12.6	3	1	.33
		16.8	0.7	0.3	.43
	aph^r-4-R2	0	100	100	1.00
		4.2	95	63	.66
		8.4	51	27	.53
		12.6	19	8	.42
		16.8	7	2	.29

1. Cells were grown in 5% dFCS ± caffeine for 24 hours after UV-irradiation, then were grown in medium with 5% FCS and caffeine.
2. Expt. #1 caffeine = 0.5 mM, plating efficiency (%) without UV: V79 60 (control), 47 (with caffeine), aph^r-4-2: 50 (control), 31 (with caffeine).
3. Expt. #2 caffeine = 0.25 mM, plating efficiency (%) without UV: V79 88 (control) and 74 (with caffeine), aph^r-4-2: 59 (control) and 45 (with caffeine), aph^r-4-R2: 88 (control) and 81 (with caffeine).
4. Ratio = % survival with caffeine/% survival without caffeine.

Table 9. Cytotoxicities of 6-Thioguanine, Ouabain and Di
and V79 Cells in Media

Table 9. Cytotoxicities of 6-Thioguanine, Ouabain and Diphtheria Toxin to Aph^r-mutants and V79 Cells in Media Supplemented with 5% FCS with or without 2 µM Thymidine

Cell Line	Media Supplemented with										
	Thymidine	6-Thioguanine (µM)[a.b.]			Ouabain (mM)[a.c.]		Diphtheria Toxin (Lf/ml)[d.]				
	2 µM	3	6	12	0.5	1.0	0.05	0.1	0.2	0.5	1.0
V79	-	<2	3	5	4	<4	less than 0.03 in all				
	+	2	2	<2	4	<4	concentrations tested				
aph^r-4-2	-	8	8	14	17	13	2	3	3	4	2
	+	17	15	10	25	6	2	<2	<2	5	5
aph^r-4-R2	-	1	<1	<1	<3	<3	n.t				
	+	2	1	3	2	<2	n.t				
aph^r-4-RP4	-	7	6	4	31	16	n.t				
	+	4	4	1	22	22	n.t				

[a]The colony-forming abilities were determined by plating cells in triplate plates (9 cm) in media containing individual drugs for the entire period of colony development.

[b]3×10^5 cells were plated and the numbers were expressed as numbers of colonies per 10^5 viable cells.

[c]1.2×10^6 cells were plated and the numbers were expressed as colonies per 10^6 viable cells.

[d]3×10^5 cells were used. Diphtheria toxin was present for 4 days then was removed by medium change. Numbers were expressed as colonies per 10^4 viable cells. n.t = not tested. The cytotoxicity of DT did not change when DT was added 4 hours or 2 days after cell plating.

with the same selective agent. Table 10 shows that all mutants tested are stable and heritable for their mutant phenotype. Two $6TG^r$ mutants from aph^r-4-RP4 were resistant to HAT medium (46). To determine whether cell densities used for mutation assays in the present studies would affect the recoveries of oua^r, $6TG^r$ or DT^r mutants, reconstruction experiments were carried out, using spontaneous or UV induced mutant clones. Table 11 shows that when oua^r cells were cocultivated with oua^s cells and selected by ouabain medium, the recovery rate of oua^r was more than 80%, compared to the experiment in which only oua^r cells were plated. The recovery of $6TG^r$ or DT^r mutants from the cocultivating of mutants and $6TG^s$ or DT^s cells was 100%. The experiment using DT^r mutants, derived from aph^r-4-2, gave the same results.

Ultraviolet Light Induced Mutation

A preliminary mutation study at the ouabain-resistant locus has been reported by Chang et al., (40). These studies, using an in situ technique, have shown that aph^r-4-2 is hypermutable at the Na^+/K^+-ATPase locus. In the studies presented in this thesis, a replating technique was used to determine the mutation frequencies in three loci, i.e., ouabain-, diphtheria toxin- and 6-thioguanine- resistant loci.

1. Ouabain-resistant Mutations.

The mutation frequencies at the optimal expression time for different UV doses from 4 experiments are presented in Figure 6. Results within each individual experiment or from the combined data clearly showed that the UV-sensitive aphidicolin-resistant mutant, aph^r-4-2, is hypermutable at the Na^+/K^+-ATPase locus. The range of mutability of aph^r-4-R2 was found to be overlapping with that of V79 cells. Figures 7 and 8 show the mutation frequencies at different expression times from two representative experiments. In general, the mutation expression time at this locus is not significantly different between mutant cell and V79 lines.

Table 10. Stabilities of Spontaneous Oua^r, 6TG^r and DT^r Mutants

A. Oua^r Clones

Cell line	Medium Supplemented with: 5% FCS, None	5% FCS, 4 μM thymidine	Ouabain (1 mM), 5% FCS, None	Ouabain (1 mM), 5% FCS, 4 μM thymidine
V79	13	13	13	13
aph^r-4-2	39	39	39	39
	18	18	18	18
	17	17	17	17
aph^r-4-R2	6	6	6	6

B. 6TG^r Clones

	Medium Supplemented with: 5% FCS, None	6TG (6 μM)	HAT	5% dFCS
V79	9	9	0	7
aph^r-4-2	8	8	0	8
aph^r-4-R2	7	7	0	4
aph^r-4-RP4	9	9	2	8

C. DT^r Clones

	Medium Supplemented with: 5% FCS	5% FCS + DT (2 lf/ml)
aph^r-4-2	22	22
aph^r-4-RP4	9	9

Clones were isolated individually with a glass cylinder and replated onto muti-well plates in medium without selection agents. Three days later, individual selection agents were added to the well with fresh medium. The numbers presented here are the number of viable clones.

HAT: medium contains hypoxanthine, aminopterin and thymidine (46).

Table 11. Recoveries of Ouabain-, 6-Thioguanine- and Diphtheria Toxin-resistant Cells in Drug-sensitive Aph^r-mutants and V79 Cells

		Cells per plate[1]			Cells Recovered (%)
I. Ouabain Locus:[2]		oua^s (Cell Line)	oua^r	(Origins, Cell Lines)	oua^r
	a.	0	50	(Spontaneous, V79)	29
		3.14×10^5 (V79)	50	(Spontaneous, V79)	23 (80)
	b.	0	50	(UV-induced, V79)	58
		3.14×10^5 (V79)	50	(UV-induced, V79)	61 (100)
	c.	0	50	(Spontaneous,	27
		2.2×10^5 (aph^r-4-2)	50	aph^r-4-2)	32 (100)
	d.	0	50	(UV-induced,	45
		2.2×10^5 (aph^r-4-2)		aph^r-4-2)	48 (100)
	e.	0	50	(UV-induced,	36
		2.5×10^5 (aph^r-4-R2)		aph^r-4-R2)	34 (94)
II. 6-Thioguanine Locus:[2]		$6TG^s$ (Cell Line)	$6TG^r$	(Origins, Cell Lines)	$6TG^r$
	a.	0	50	(UV-induced,	31
		7.9×10^4 (V79)	50	aph^r-4-2)	31 (100)
	b.	0	50	(UV-induced,	31
		5.4×10^4 (aph^r-4-2)	50	aph^r-4-2)	34 (100)
III. Diphtheria Toxin Locus:[2]		DT^s (Cell Line)	DT^r	(Origin, Cell Lines)	DT^r
	a.	0	50	(Spontaneous,	15
		5×10^4 (aph^r-4-2)	50	aph^r-4-2)	53 (>100)
	b.	0	50	(UV-induced,	24
		1×10^5 (aph^r-4-2)	50	aph^r-4-2)	42 (>100)

1. Cells per plate were corrected by plating efficiency in all sensitive cell lines. Selective agents were added 4 hours after cell plating.
2. Number of sensitive cells plated: Oua^s, 4×10^5; $6TG^s$, 1×10^5; DT^s, 1×10^5 (per 9 cm plate).

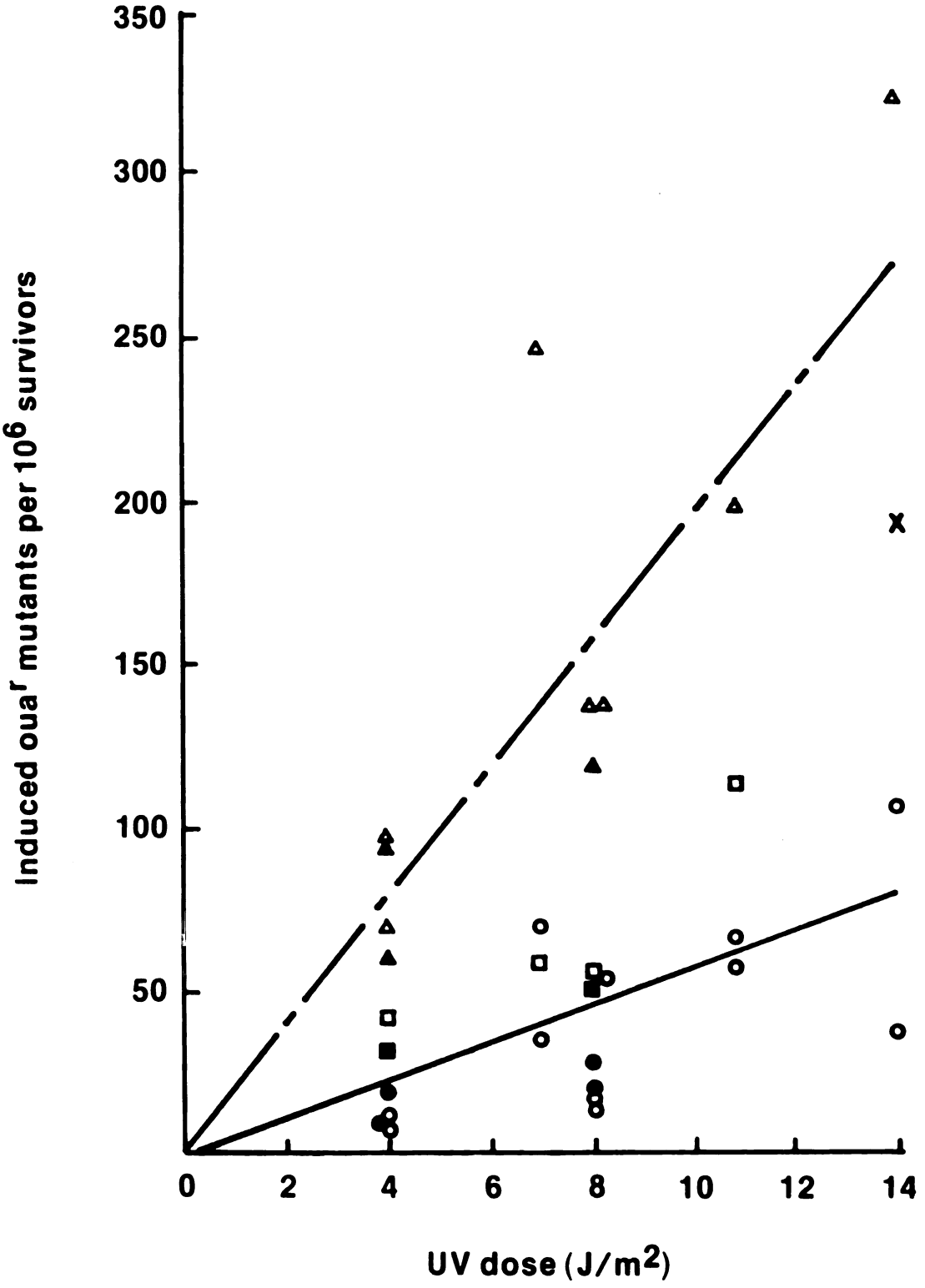


Figure 6. Dose response of UV-induced oua^r mutation frequencies in aph^r-4 (X), aph^r-4-2 (△,▲), aph^r-4-R2 (□,■) and V79 (○,●) cells. Mutagenized cells were grown in medium with (closed symbols) or without (open symbols) 4 mM TdR during expression and selection period. The background frequencies in all cell lines were less than 20 x 10^{-6} and were subtracted from the induced frequencies (4 experiments).

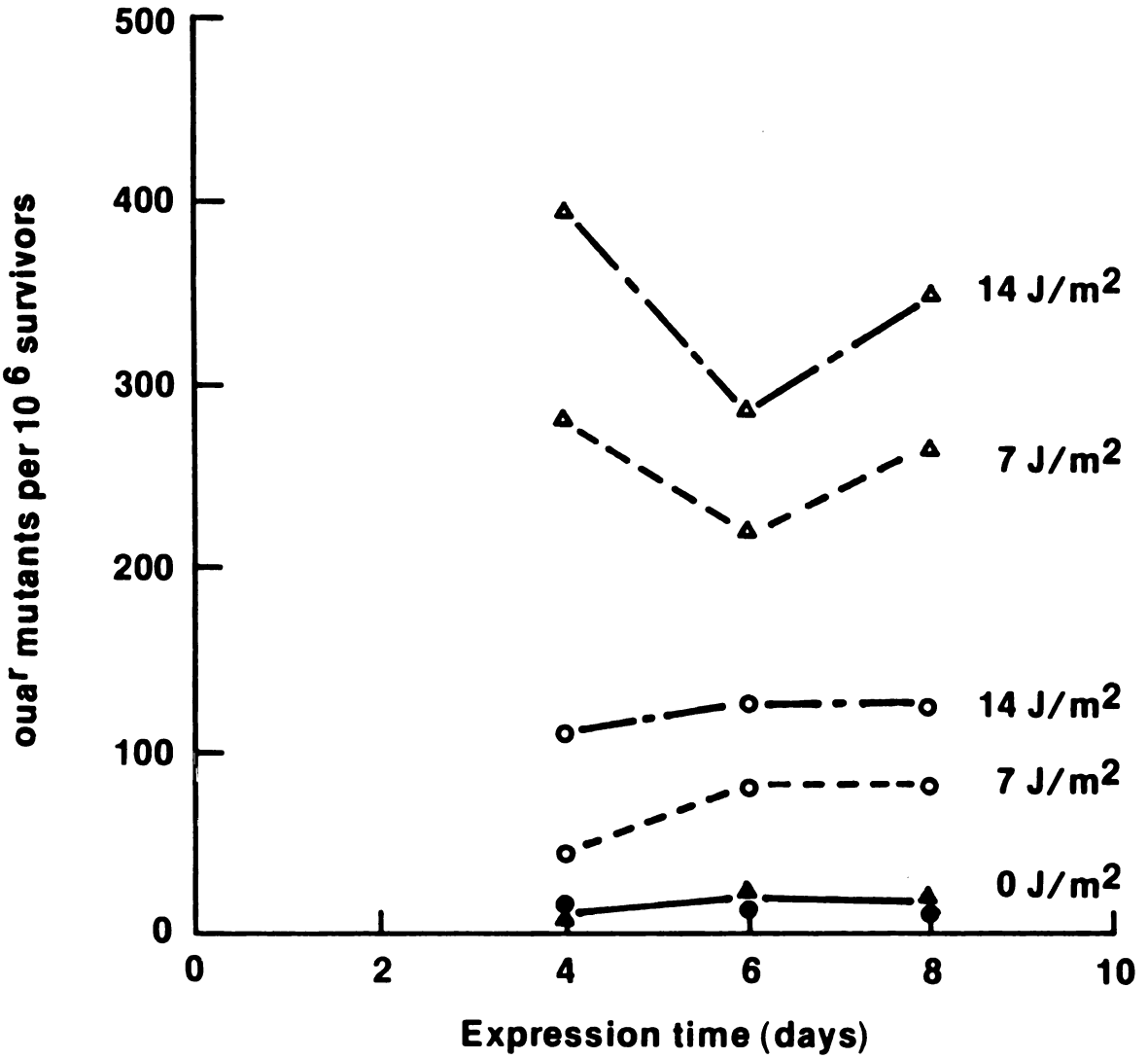


Figure 7. Expressions of UV-induced oua^r mutants in aph^r-4-2 (△,▲) and V79 (○,●) cells. Results from control (closed symbols) and UV-irradiated cells (open symbols, 7 J/m^2 and 14 J/m^2) are presented.

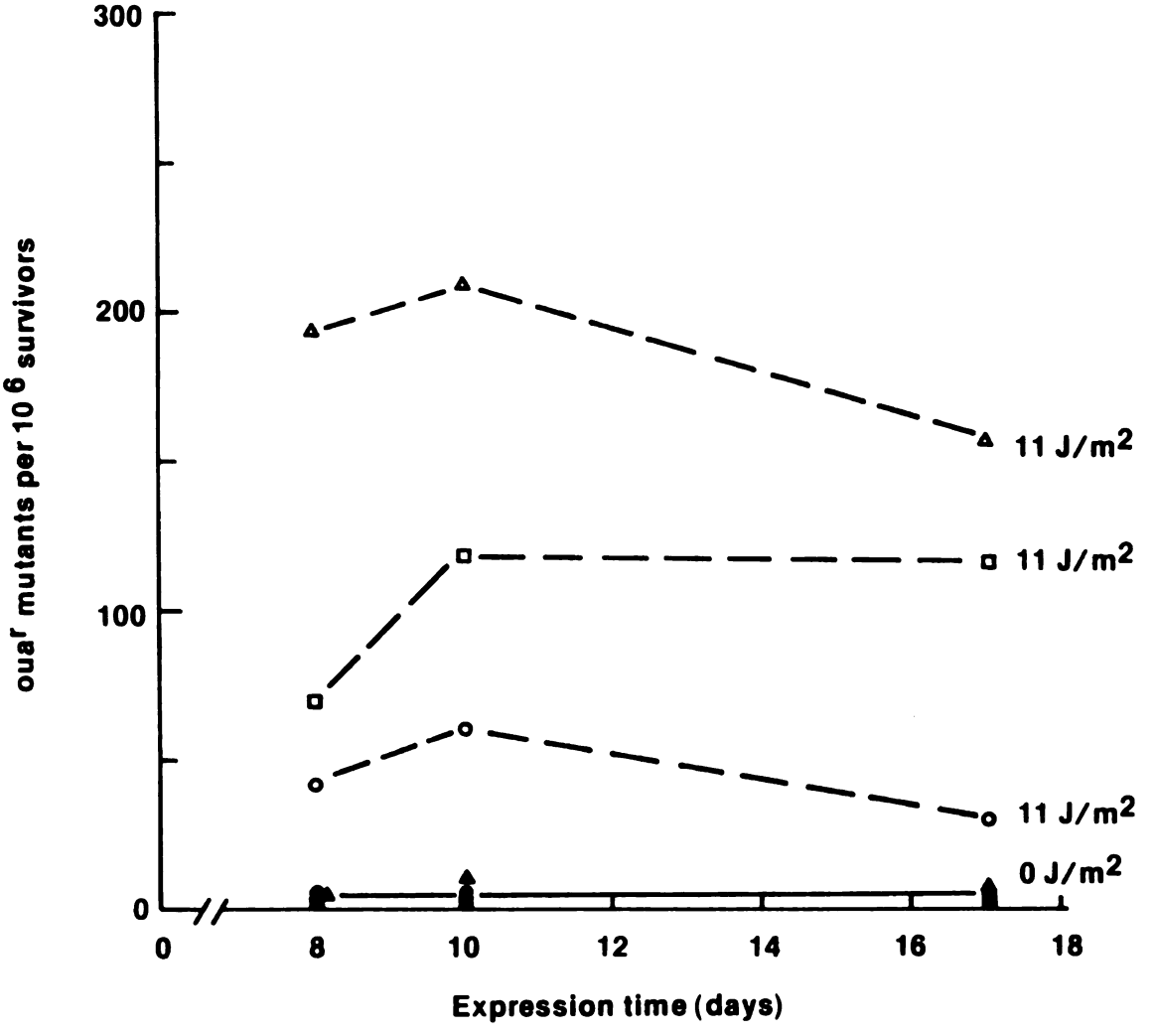


Figure 8. Expressions of UV-induced oua^r mutants in aph^r-4-2 (△,▲), aph^r-4-R2 (□,■) and V79 (○,●) cells. Results from control (closed symbol) and UV irradiated cells (open symbols, 11 J/m^2) are presented.

When mutation frequencies are presented as a function of cytotoxicity of UV damage (Fig. 9), aph^r-4-2 showed a higher mutation frequency than did V79 and aph^r-4-R2 at the same level of survival. The results are similar to those plotted according to UV doses, except that a biphasic curve was obtained for aph^r-4-2 (Fig. 9) instead of a linear curve (Fig. 6).

2. Diphtheria Toxin-resistant Mutations.

The UV-induced mutability of aph^r-4-2 and its revertant, aph^r-4-R2, was also studied in the assay system involving mutations from diphtheria toxin sensitivity to resistance. The results (N=4) presented in Fig. 10 indicate that aph^r-4-2 is also hypermutable at the diphtheria toxin-resistant locus. Similar to a previous report (234), the optimal mutation expression time in this system was about 5 to 7 days for both wild type V79 and the mutant cell lines (Fig. 11). When mutation frequencies were plotted according to survivals in logarithmic scales, the aph^r-4-2 was found to have a higher mutation frequency than both the wild type and its revertant at the same survival level (Fig. 12). Unlike the ouabain-resistant mutations, the mutation induction curve at the diphtheria toxin-resistant locus is linear instead of biphasic.

3. 6-Thioguanine-resistant Mutations.

The hypoxanthine guanine phosphoribosyl transferase (HGPRT) mutation assay system in the Chinese hamster cell is perhaps the most commonly used and the best described in vitro mammalian assay system. The prominent features of the system are the cell density effect due to metabolic cooperation, the long expression time (about 7 days) and the stable expression of mutation frequencies after the optimal expression time. This mutation assay system was also used (low cell-density selection) to study the mutability of aph^r mutants. The results showed (Figs. 13 and 14) that the wild type V79 cells indeed had stable mutation frequencies after the six-day expression time. Unexpectedly, the mutation frequencies of the mutant,

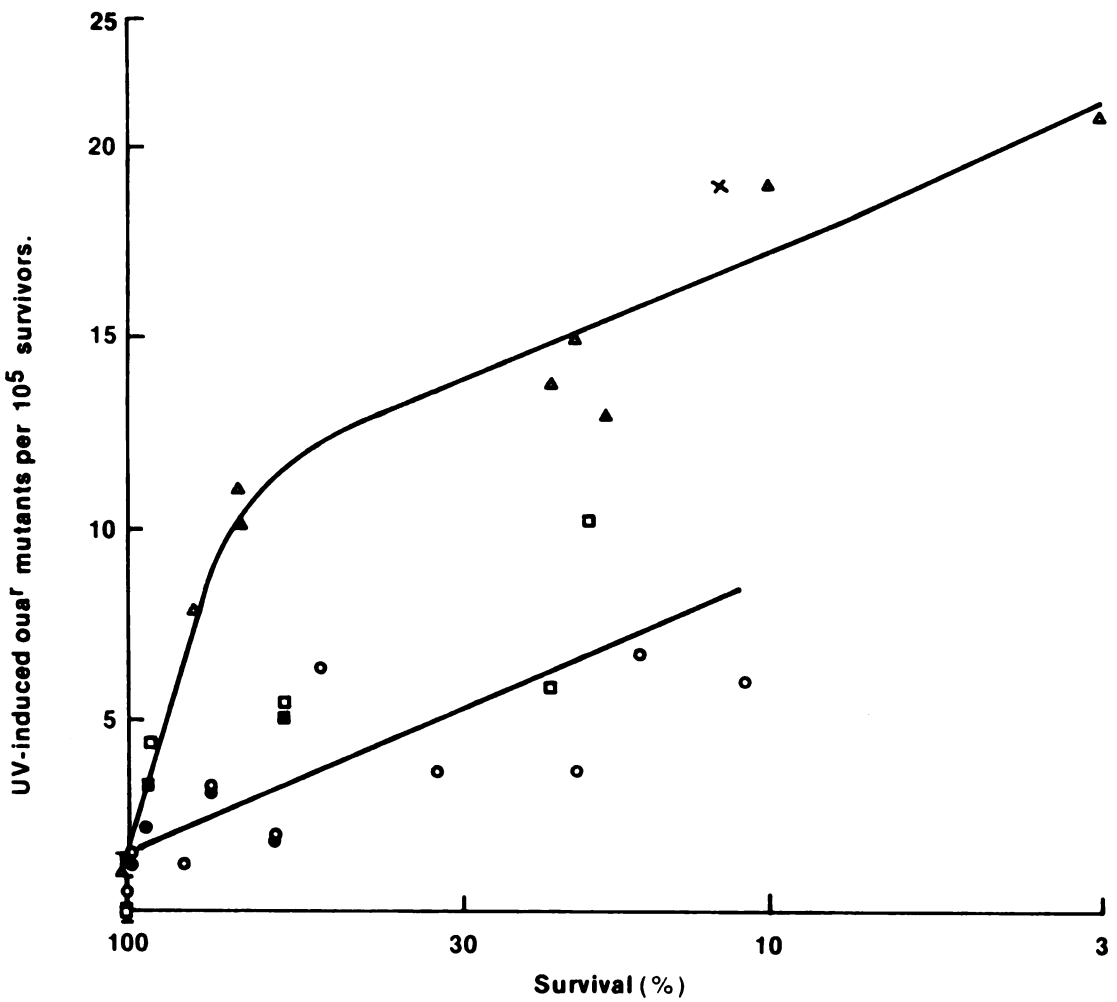


Figure 9. UV-induced oua^r mutant frequencies of aph^r-4 (X), aph^r-4-2 (△), aph^r-4-R2 (□) and V79 (○) cells as a function of cytotoxicities of UV damage. Mutagenized cells were grown in medium with (closed symbols) or without (open symbols) 4 μM TdR during expression and selection period.

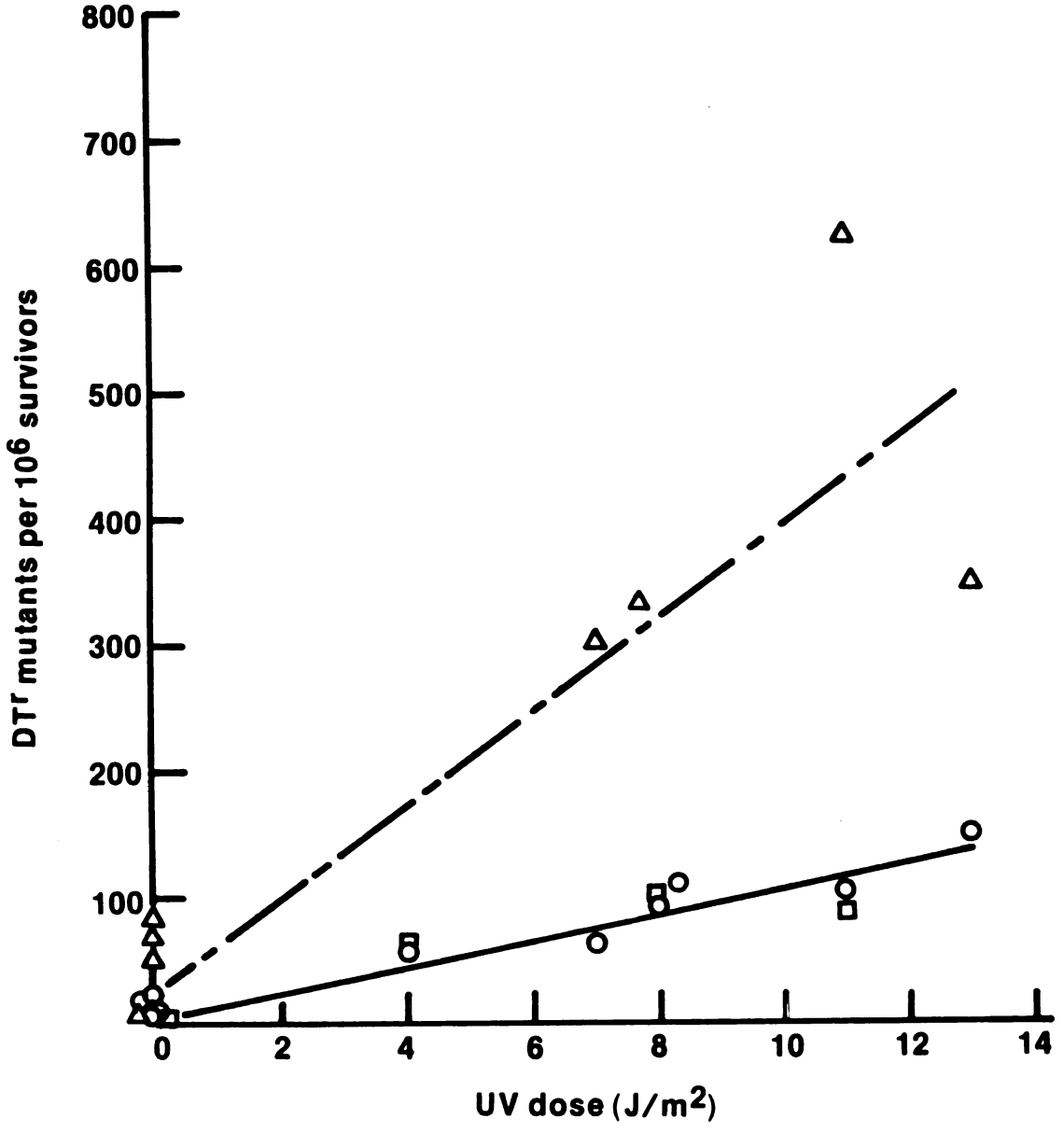


Figure 10. Dose response of UV-induced DT^r mutation frequencies in aph^r-4-2 (△), aph^r-4-R2 (□) and V79 (○) cells. The background frequencies were not subtracted from induced mutation frequencies.

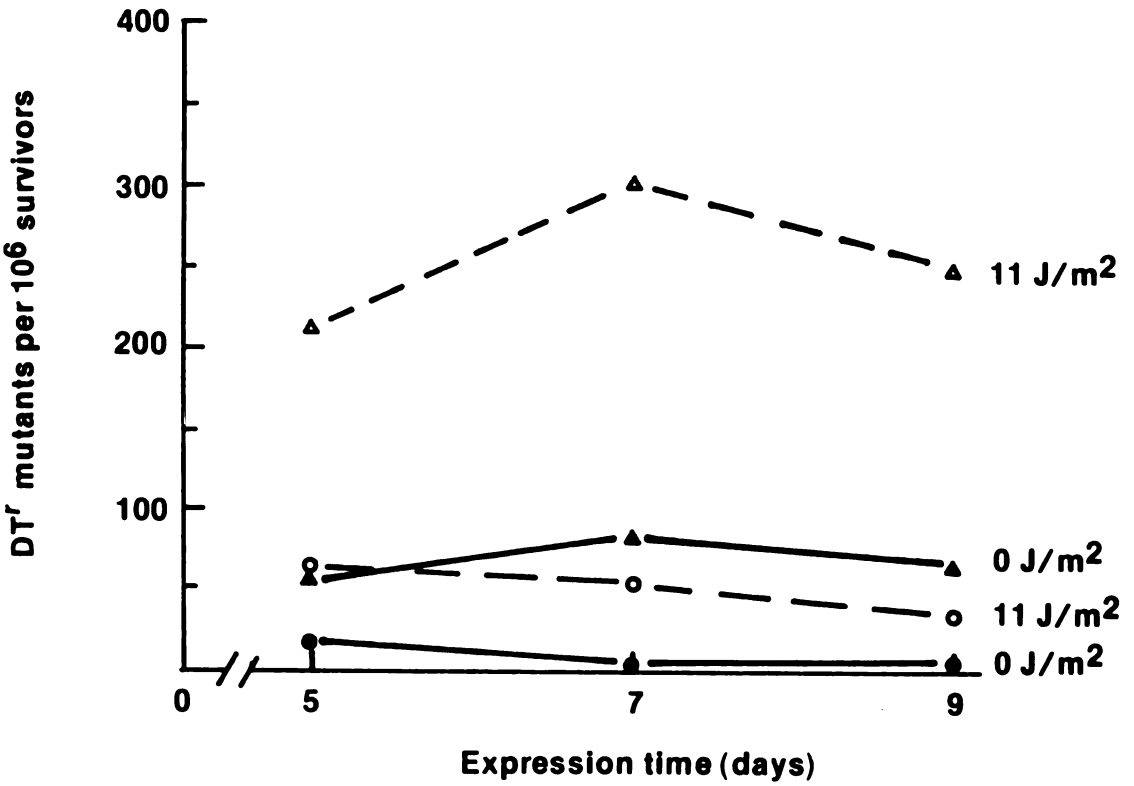


Figure 11. Expression of UV-induced DT^r mutants in aph^r-4-2 (△) and V79 (○) cells. Results from control (closed symbols) and UV-irradiated cells (open symbols, 11 J/m^2) are shown.

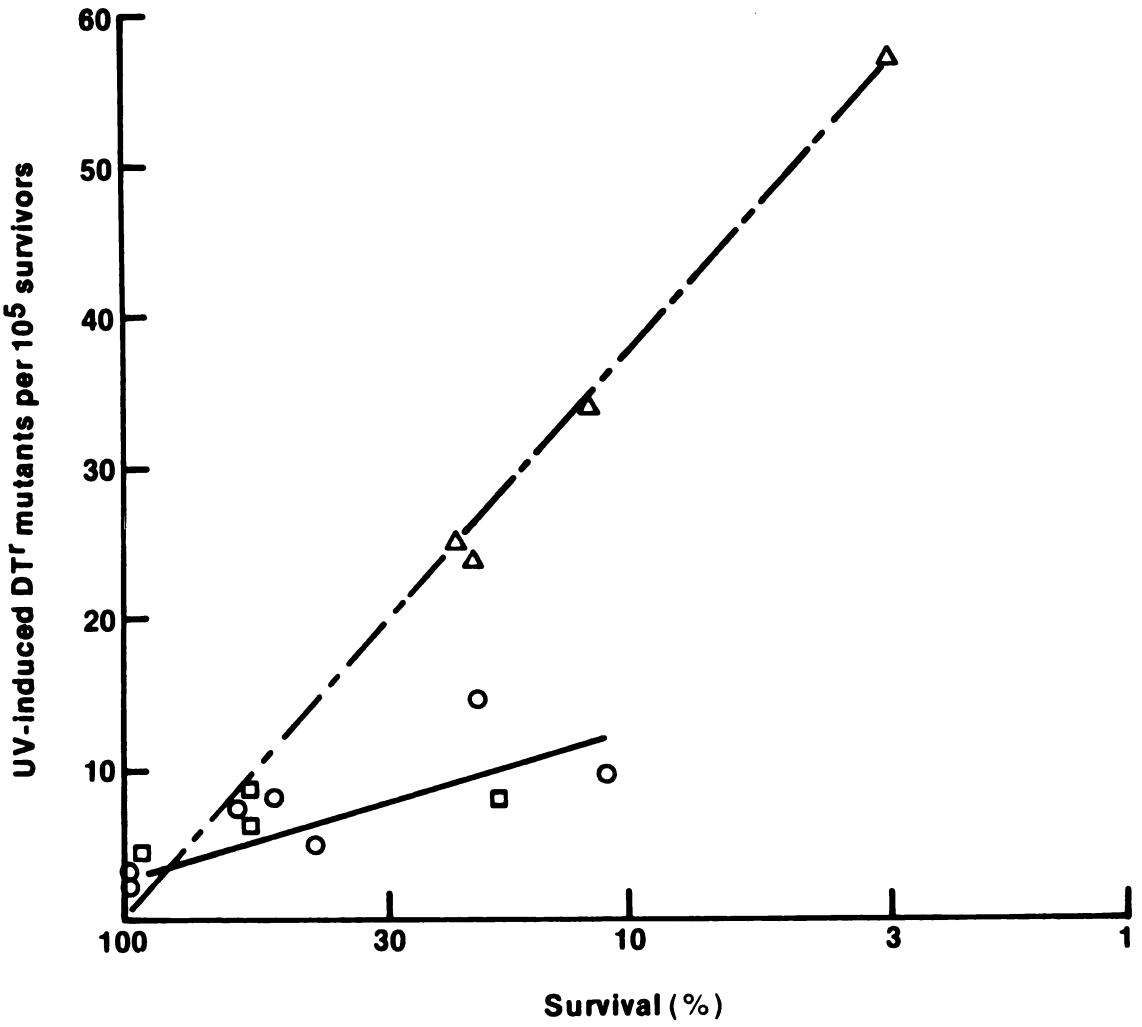


Figure 12. UV-induced DT^r mutant frequencies of aph^r-4-2 (△), aph^r-4-R2 (□) and V79 (○) cells as a function of cytotoxicities of UV damage. Results from 4 experiments are shown. Background frequencies have been subtracted.

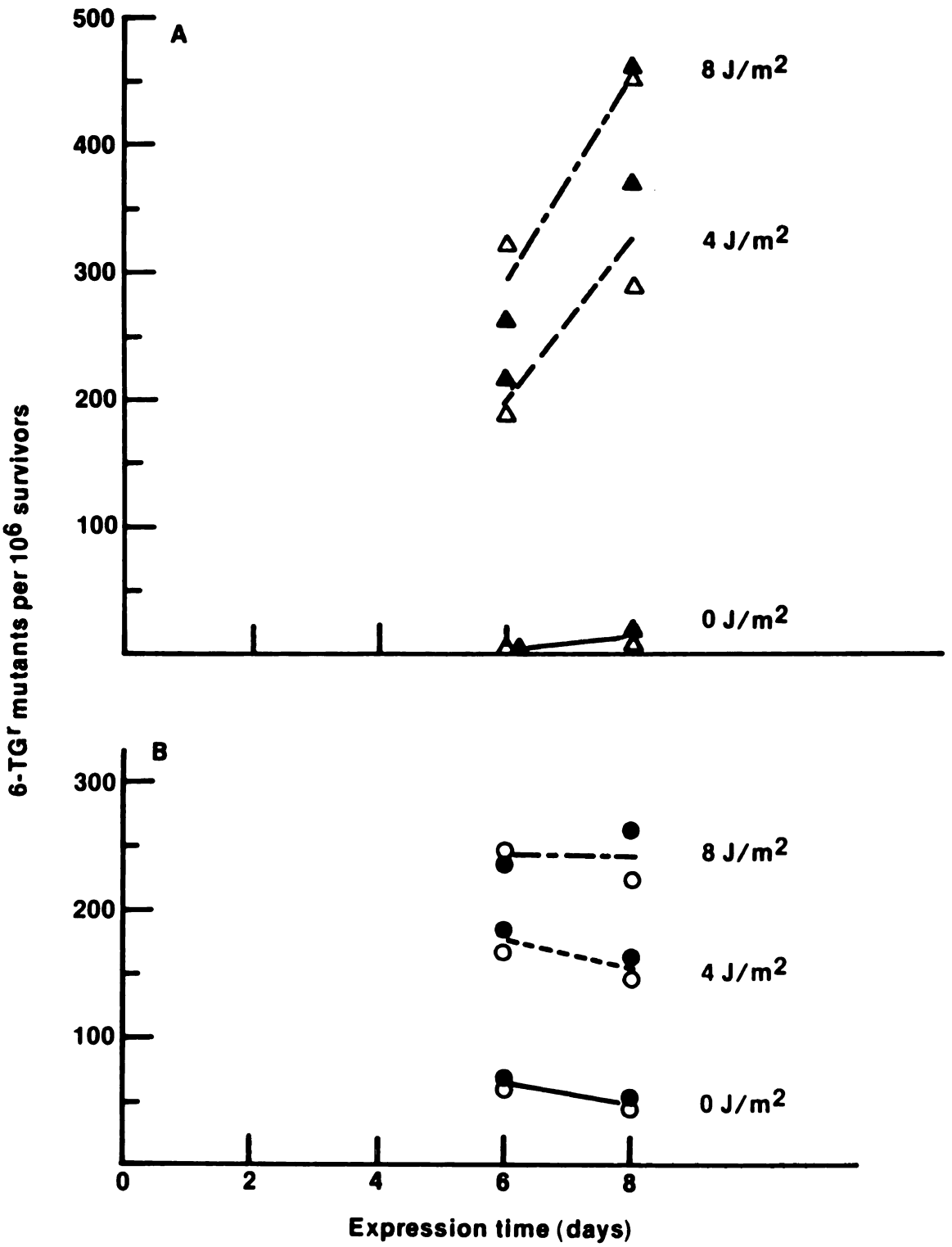


Figure 13. Expressions of UV-induced 6TG^r mutants in aph^r-4-2 (△, ▲) and V79 (○, ●) cells. Mutagenized cells were grown in medium with (closed symbols) or without (open symbols) 2 μM TdR during expression and selection period.

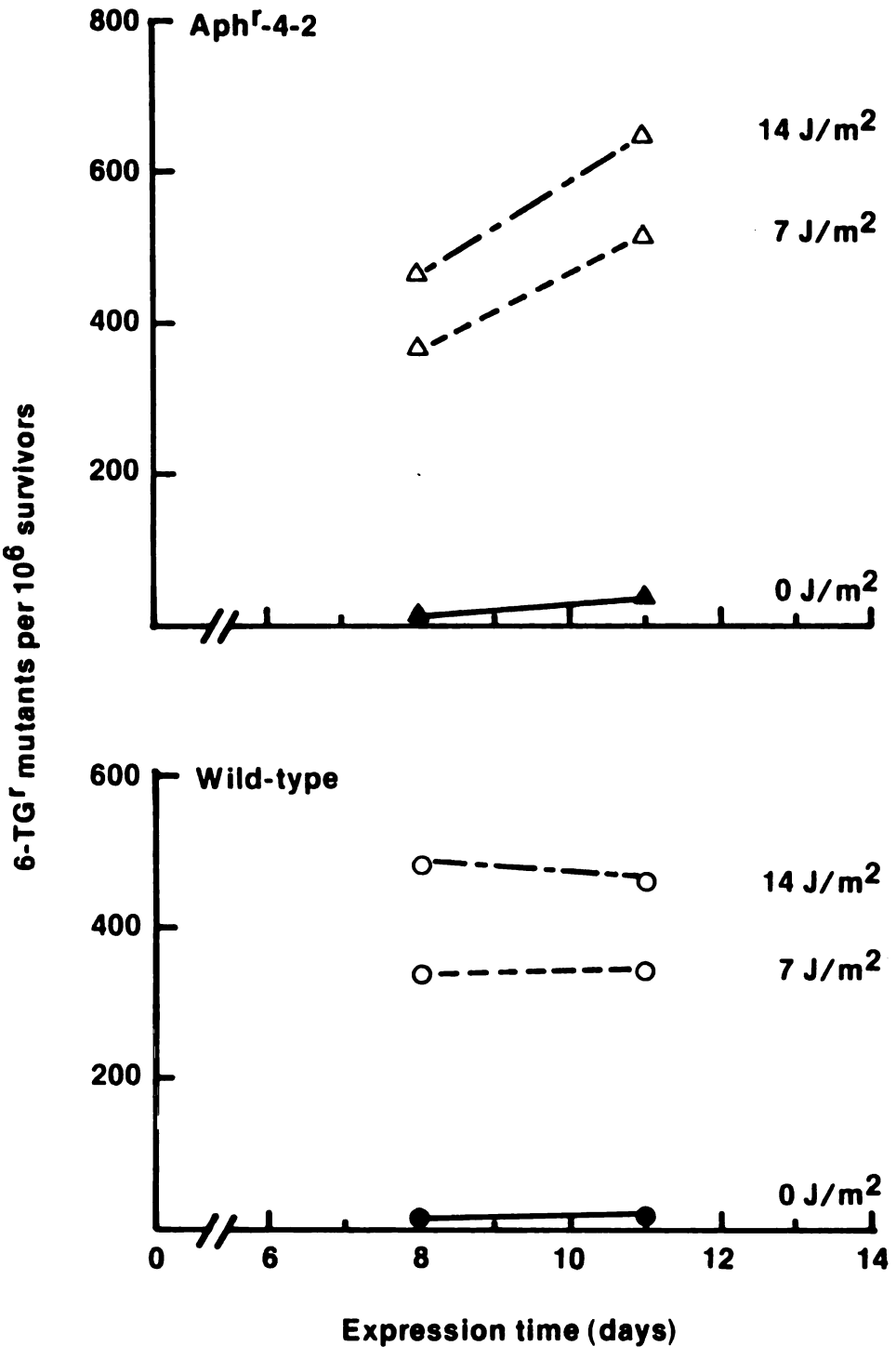


Figure 14. Expressions of UV-induced 6TG^r mutants in aph^r-4-2 (△) and V79 (○) cells.

aph^r-4-2, continued to increase 8 days after UV-irradiation. This is exemplified by the result from two experiments shown in Figs. 13 and 14. Because the frequency of fast growing revertants will increase in the population of aph^r-4-2 after an extended period of growth, no assay was done beyond 14 days after UV-irradiation. Therefore, the optimal expression time for aph^r-4-2 cannot be determined from these results. However, when the maximum mutation frequencies of aph^r-4-2 were compared with those of the wild type, aph^r-4-2 clearly had a higher mutation frequency than did V79 for a given dose of UV (Fig. 15, reconstructed from Fig. 13). The revertant, aph^r-4-R2, was similar to the wild type in UV mutability (data not shown). When all the mutation frequencies of the mutant, the revertant and the V79 cells were plotted according to their survival (Fig.16, N=4), a smooth curvilinear response curve, similar to that of a previous report using CHO (305) or V79 cells (304), was obtained. There appears to be no discernible difference between the wild type (V79) and the mutant (aph^r-4-2), or its revertant (aph^r-4-R2).

4. Effect of Thymidine on Mutation Expression.

Both aph^r-4-2 and aph^r-4-R2 are thymidine auxotrophs. They grow in a medium with 5% non-dialyzed FCS, but not with 5% dialyzed FCS. In the present experiments, the cells were grown in medium containing no exogenous nucleosides, but supplemented with 5% non-dialyzed FCS one day after UV-irradiation. In some experiments, exogenous TdR (2-4 μM) with or without AdR (2 μM), was added during expression and selection periods to determine whether exogenous deoxyribonucleosides affect mutation expression. The results shown in Figures 6, 9, 13, and 16 indicate that exogenous TdR did not affect mutation expression for oua^r and 6TG^r mutants. The same results were obtained when aphidicolin (0.5 μM) or exogenous AdR was tested (data not shown). The data, concerning the expression of oua^r, DT^r and 6TG^r mutants induced by UV, are shown in Appendix B.

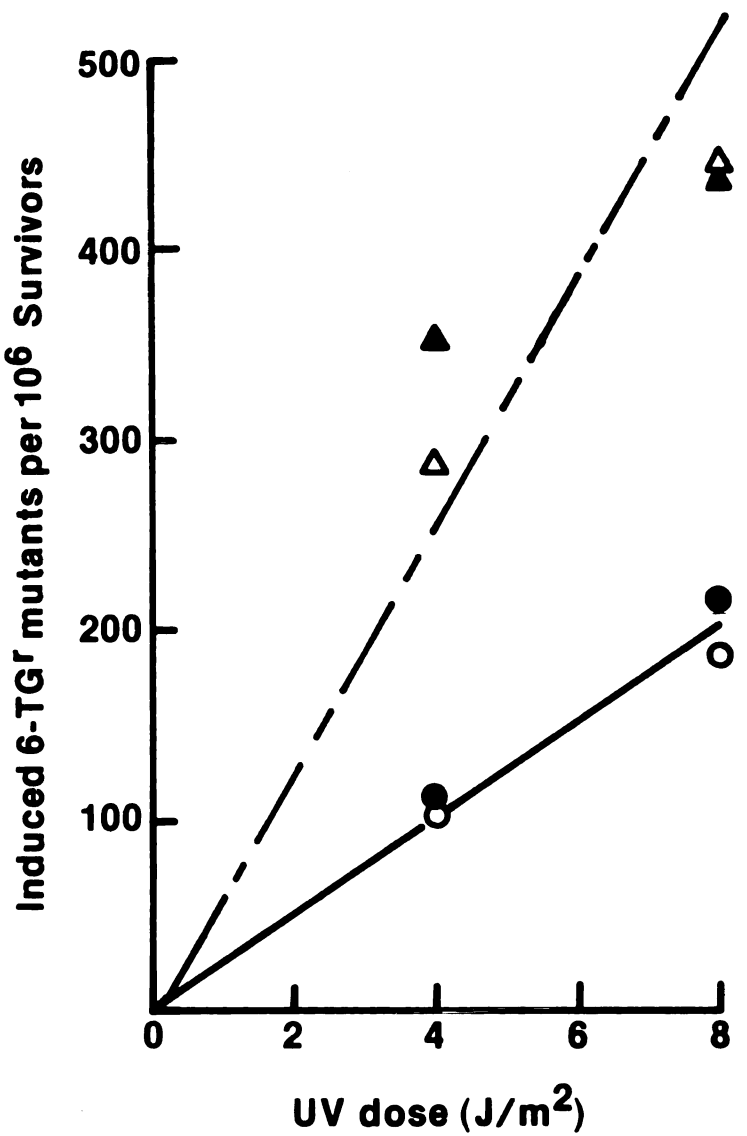


Figure 15. Dose response of UV-induced 6TG^r mutation frequencies in aph^r-4-2 (△, ▲) and V79 (○, ●) cells. Mutagenized cells were grown in medium with (closed symbols) or without (open symbols) 2 μM TdR during expression and selection period.

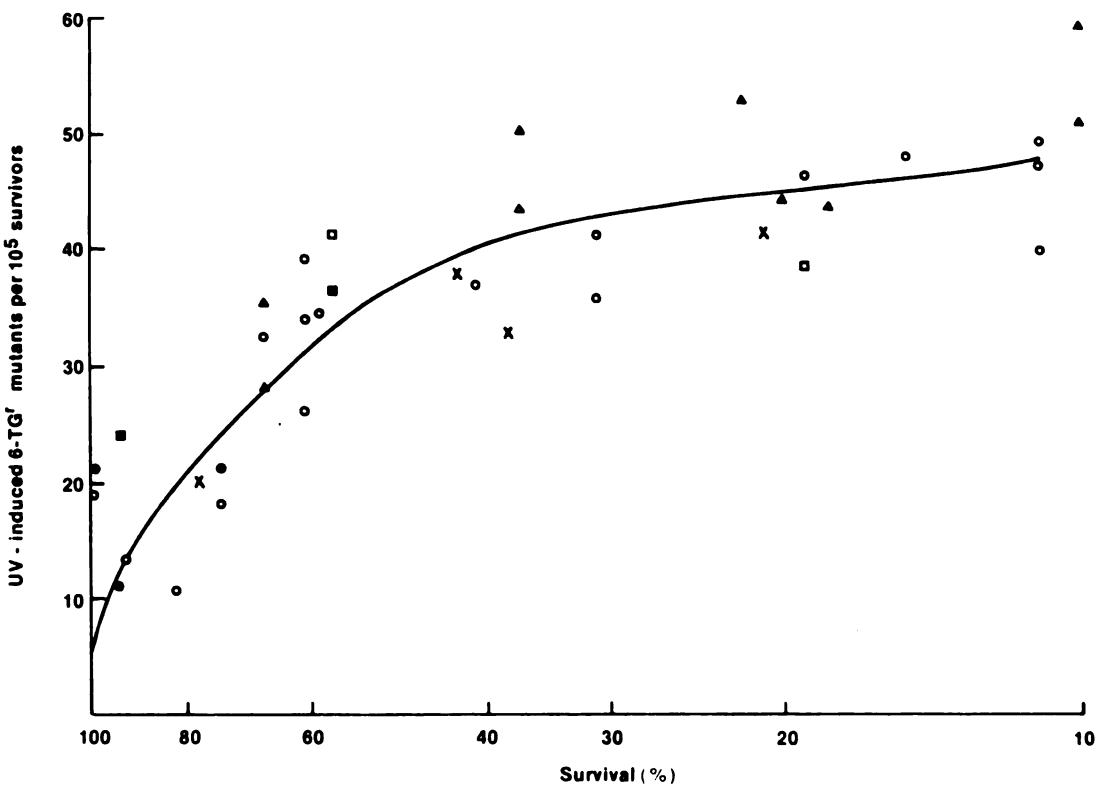


Figure 16. UV-induced 6TG^r mutant frequencies of aph^r-4 (X), aph^r-4-2 (△), aph^r-4-R2 (□) and V79 (○) cells as a function of cytotoxicities of UV damage. Mutagenized cells were grown in medium with (closed symbols) or without (open symbols) 2 μM TdR. The background mutation frequencies have been subtracted from the induced mutation frequencies. Results from 4 experiments are presented.

X-ray Induced Mutation

Table 12 shows the results from two experiments using X-ray as a mutagen. The results show that X-rays can induce mutation at two loci, namely $6TG^r$ and DT^r loci (39). Because the X-ray sensitivity of aph^r-4-2 is the same as that of the V79 cells, similar X-ray induced $6TG^r$ or DT^r mutation frequency in both cell lines was expected. Table 12 shows that at an equal X-ray dose, $6TG^r$ mutation frequencies in aph^r-4-2 cells were slightly lower than those in the V79 cells. On the other hand, DT^r mutation frequencies were either the same for both cell lines, or lower in aph^r-4-2 than in the wild type V79 cells.

Repair Capabilities Measured by Liquid Holding Recoveries and Unscheduled DNA Syntheses

1. Liquid Holding Recoveries by Conditioned Medium.

The mutants and the wild type V79 cells were tested for their ability to repair the UV-induced DNA damage. This was done by studying the reduction in cytotoxicity and mutation frequencies after DNA replication arrest by growing cells in the conditioned medium for various hours (186).

It appears (Table 13) that the conditioned medium exposure reduced the plating efficiency of each cell line receiving no UV-irradiation (these cells being the control group). If plating efficiencies of the control group be taken as 100% survival, then, of the treated group receiving UV-irradiation, the wild type V79 cells showed enhancement of survivals at doses between 4.2-12.6 and at 12-20 hours of liquid holding (Table 13; Fig. 17). On the other hand, aph^r-4-2 and aph^r-4-RP5 cells did not show improved survival at all: rather, the rate of survival decreased whether or not the conditioned medium was toxic. The TdR auxotrophic revertants, aph^r-4-R2, improved survivals as it is in the wild type V79 cells. Figure 17 shows that the UV survivals of V79 and aph^r-4-R2 cells at two doses (8.4, 12.6 J/m^2) were enhanced

Table 12. X-ray-induced Mutation Frequencies in Aph^r-mutants and V79 Cells

Expt. No. (Loci)	Cell lines	X-ray dose (rads)	Survival (%)	Expression time (days)	Mutation Frequency per 10^6 Survivors (No. of Mutants)
1. ($6TG^r$)	V79	0	100	8	26 (27)
		735	7		265 (265)
		0		10	30 (40)
		735			220 (207)
	aph^r-4	0	100	8	5 (7)
		735	9		100 (198)
		0		10	3 (6)
		735			137 (247)
(DT^r)	V79	0		5	15 (23)
		735			89 (138)
		0		8	8 (8)
		735			67 (67)
	aph^r-4	0		5	1 (1)
		735			35 (32)
		0		8	1 (1)
		735			37 (40)
2. ($6TG^r$)	V79	0	100	7	57 (103)
		700	8		135 (353)
		0		9	71 (137)
		700			262 (599)
	aph^r-4-2	0	100	7	2 (5)
		700	8		114 (198)
		0		9	3 (10)
		700			116 (290)
(DT^r)	V79	0		7	1.4 (1)
		700			74 (76)
		0		9	<2 (0)
		700			183 (168)
	aph^r-4-2	0		7	6 (15)
		700			137 (98)
		0		9	3 (3)
		700			172 (163)

Expt. No. 1: low cell-density selection for $6TG^r$ locus; DT=0.2 lf/ml.

Expt. No. 2: high cell-density selection for 6TG locus; DT=0.6 lf/ml.

Table 13. Liquid Holding Recoveries (% Survival) by Conditioned Medium in Aph^r-mutants and V79 Cells

Experiment #1	UV (J/m^2)	0	3	5	9		(hrs)
V79	0	(110)		(92)	(110)		
	0	100		100	100		
	7	41		30	80		
aph^r-4-2	0	(63)		(48)	(18)		
	0	100		100	100		
	7	10		7	15		
Experiment #2		0			10	17	(hrs)
V79	0	(88)			-	-	
	0	100					
	13	11			29	29	
aph^r-4-2	0	(59)					
	0	100					
	13	3			4	4	
aph^r-4-R2	0	(88)					
	0	100					
	13	19			29	24	
Experiment #3		0	4		8	12	(hrs)
V79	0	(47)	(46)		(45)	(29)	
	0	100	100		100	100	
	8.4	50	84		74	80	
	12.6	28	21		10	57	
aph^r-4-2	0	(48)	(59)		(50)	(55)	
	0	100	100		100	100	
	8.4	22	14		13	15	
	12.6	6	3		2	2	
aph^r-4-R2	0	(61)	(57)		(54)	(47)	
	0	100	100		100	100	
	8.4	60	75		98	90	
	12.6	41	52		44	55	
aph^r-4-RP5	0	(103)	(99)		(99)	(110)	
	0	100	100		100	100	
	8.4	46	55		59	56	
	12.6	19	18		20	21	

Table 13. continued

Experiment #4	UV_2 (J/m^2)	0	3	6	9	12	16	20 (hrs)
V79	0	(90)	(92)	(76)	(71)	(78)	(77)	(75)
	0	100	100	100	100	100	100	100
	8.4	87	79	92	93	99	116	99
	16.8	32	21	31	35	35	33	32
aph^r-4-2	0	(85)	(74)	(66)	(66)	(70)	(52)	(52)
	0	100	100	100	100	100	100	100
	4.2	84	82	63	75	81	82	66
	8.4	23	23	20	26	26	24	19

Numbers in parentheses are plating efficiencies (%) of non UV-irradiated cells with the same treatment of conditioned medium; and these cells served as control. Colony-forming abilities were expressed as survival (%) calculated by dividing the plating efficiency of cells with UV-irradiation by that of the control cells.

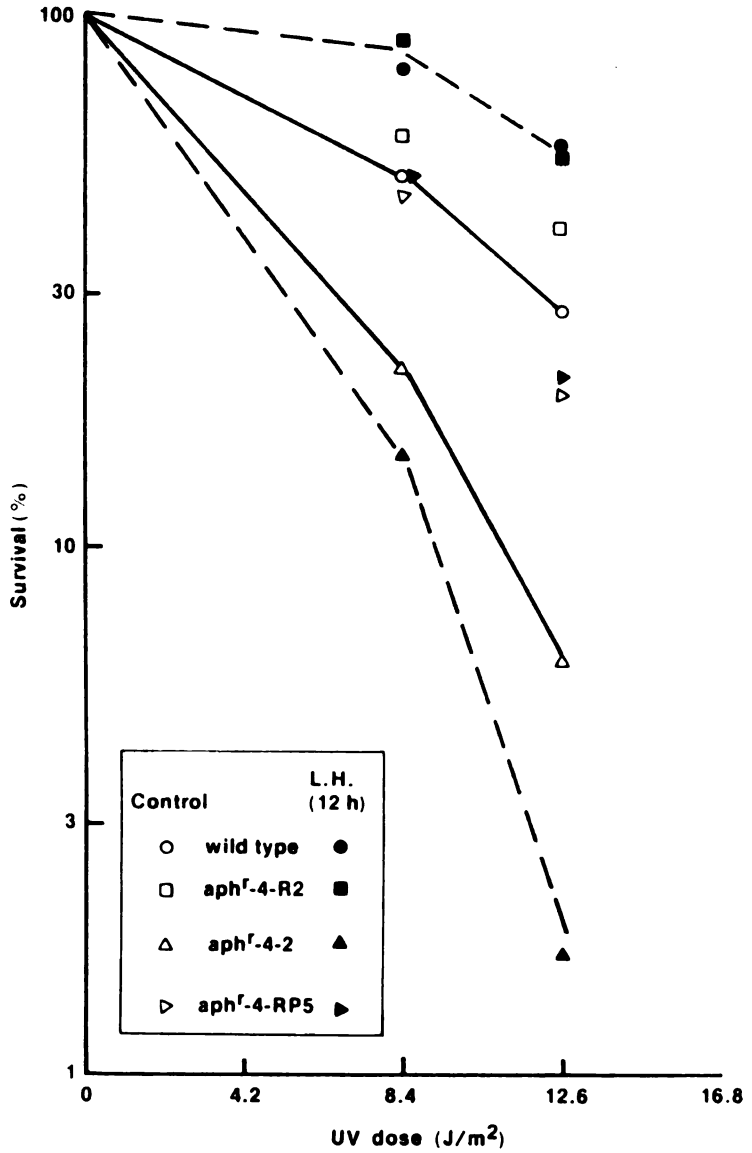


Figure 17. Survival curves of UV-irradiated aph^r-4-2, aph^r-4-R2, aph^r-4-RP5 and V79 cells with (closed) or without (open) liquid holding by conditioned medium for 12 hours.

upon liquid holding for 12 hours, whereas those of aph^r-4-2 or aph^r-4-RP5 were reduced or had no change respectively. Since the UV survival of the wild type increased to 80% after liquid holding for 12 hours from 50% without liquid holding, the mutation frequency of oua^r or DT^r mutants in the wild type V79 and aph^r-4-2 cells was assayed. At a dose of 8.4 J/m^2 (Fig. 18) UV induced 50 (oua^r) or 80 (DT^r) mutants per 10^6 clonable V79 cells (survivors), as compared to 130 (oua^r) or 225 (DT^r) mutants per 10^6 clonable aph^r-4-2 cells. Upon liquid holding of 12 hours the mutation frequency was reduced to 10 (oua^r) or O(DT^r) per 10^6 clonable V79 cells, whereas the frequency was increased to 225 from 130 (oua^r) or to 375 from 225 (DT^r) per 10^6 clonable aph^r-4-2 cells (Fig. 18). The mutation enhancement after liquid holding in aph^r-4-2 was repeatable (data not shown) in a second experiment.

At a dose of 16.8 J/m^2, the 12-hour-liquid holding did not change UV survival (Table 13, Expt. #4) or mutation frequency in the wild type V79 cells (data not shown). It appears that liquid holding recovery in the V79 cells was dose dependent.

2. Unscheduled DNA Syntheses.

To study the capacity of excision repair in these cell lines, the unscheduled DNA synthesis (UDS) induced by UV was measured in confluent cultures treated with tyrosine-arginine-deficient medium and hydroxyurea to reduce the normal DNA synthesis. The method developed by Trosko and Yagger (273) detects the incision, excision and polymerization steps of excision repair by measuring the incorporation of ^{3}H-thymidine into DNA. Figure 19 indicates that the UDS as measured by ^{3}H-TdR incorporations at two UV doses of aph^r-4-2, aph^r-4-R2, aph^r-4-RP4 and V79 cells was the same whether measured for 1.5 or 3.0 hours of ^{3}H-TdR incorporation after UV irradiation.

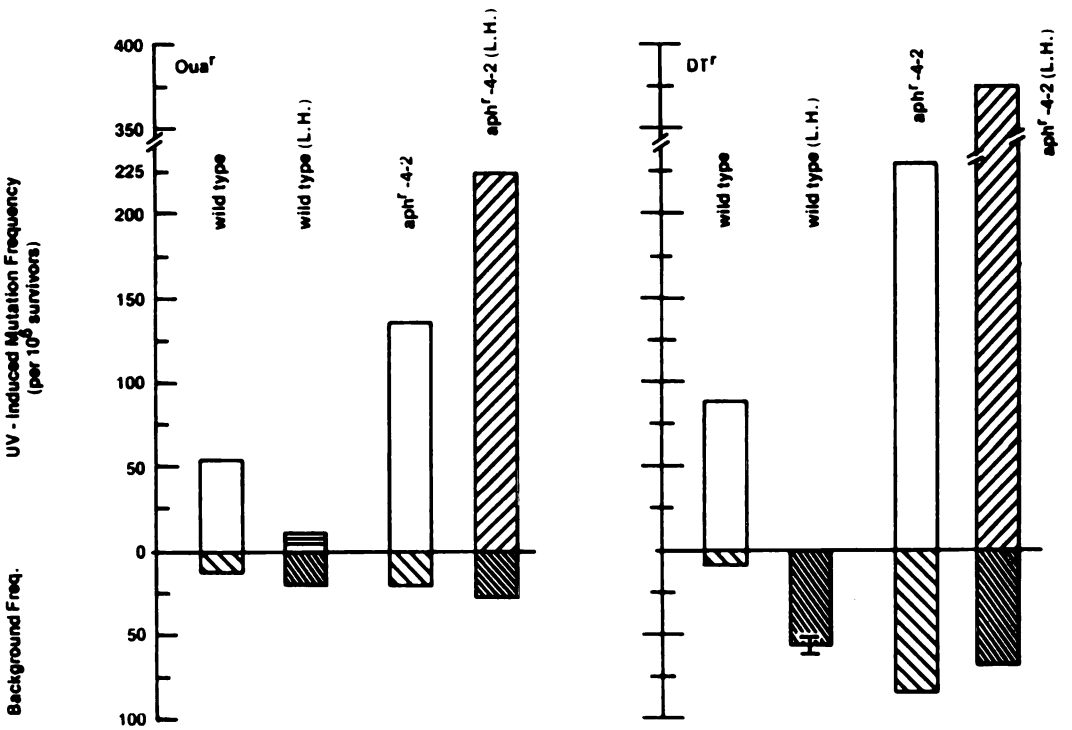


Figure 18. Mutation frequencies for aph^r-4-2 and V79 cells at oua^r (left) or DT^r (right) locus. Mutation frequencies not induced by UV with or without liquid holding (L.H.) by conditioned medium were indicated as the background frequency and were located below the zero line. Mutation frequencies induced by UV without L.H. (open box) served as control. UV-induced mutation frequencies with liquid holding of 12 hours (shaded box) were located above the zero line on the right side of each control. Survivals are presented in experiment No. 3, Table 13, (UV = 8.4 J/m^2).

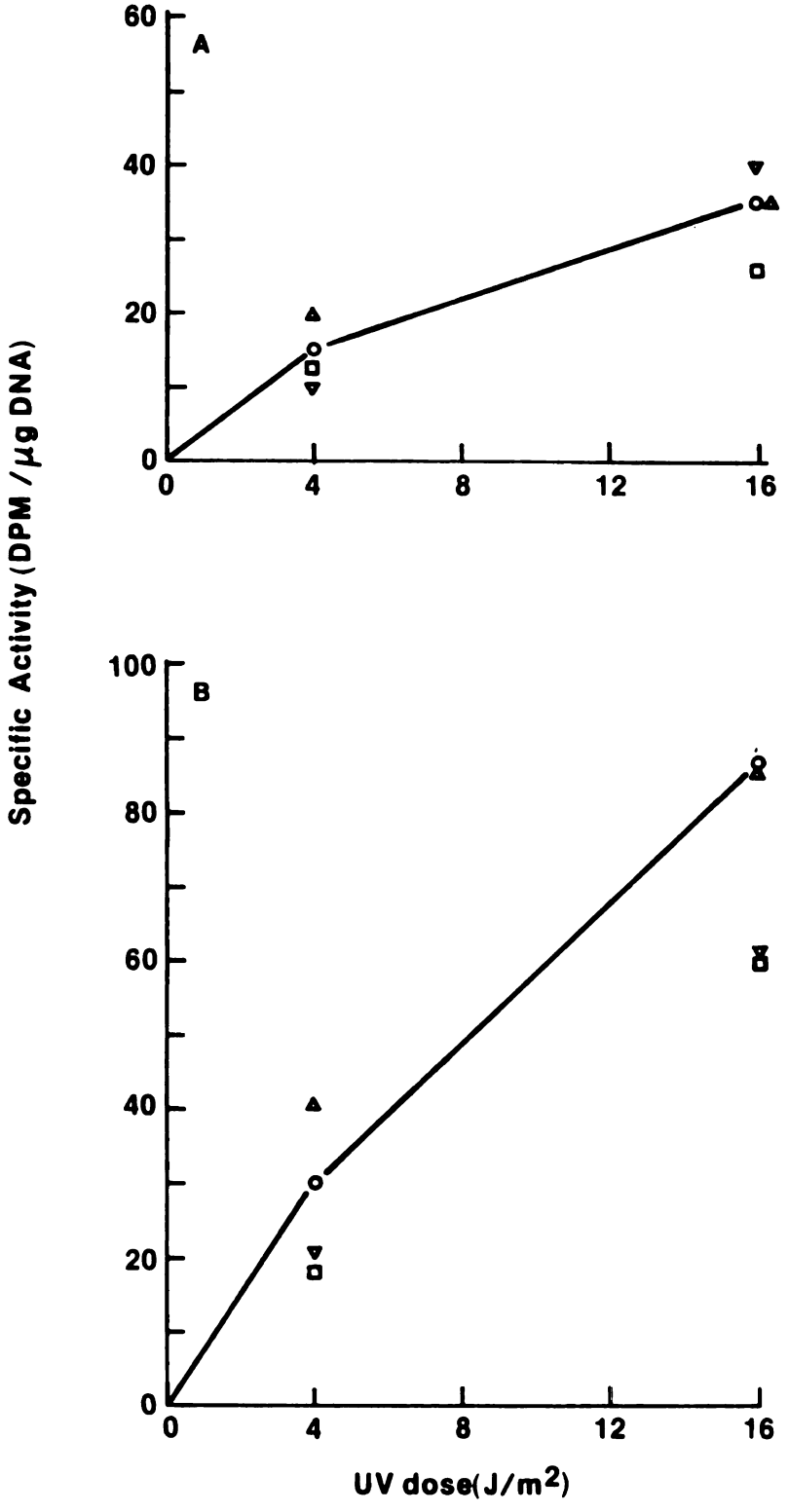


Figure 19. UV-induced unscheduled DNA synthesis as measured by ^{3}H-TdR incorporations for aph^{r}-4-2 (△), aph^{r}-4-R2 (□), aph^{r}-4-RP4 (▽) and V79 (○) cells at 1.5 (A) or 3.0 (B) hours after UV irradiation.

Spontaneous Mutation Rate Determinations

1. Fluctuation Analyses.

Tables 14-16 show the results of fluctuation analyses at oua^r, DT^r, and 6TG^r loci. In experiments involving oua^r mutant selection, the medium was supplemented with 4 μM TdR, although TdR has been shown not to affect the mutation frequency for oua^r locus of V79, aph^r-4-2 and aph^r-4-R2 cells. The results presented in Table 14 show that aph^r-4-2 and aph^r-4-RP5 cells had a higher spontaneous mutation rate than V79 and aph^r-4-R2 cells. Because the growth rates of aph^r-4-2 clones were not homogenous [three groups of clones were separated and their doubling times were approximately 24(F), 36(I), and more than 48 hours (S)], the assay of oua^r mutation rates was performed separately in these three groups of cells. In spite of their growth characteristics, the spontaneous mutation rates in this locus were found to be higher in aph^r-4-2 cells than in V79 or aph^r-4-R2 cells.

Spontaneous mutation rates, determined by fluctuation analysis, at DT^r locus in V79, aph^r-4-2, aph^r-4-R2 and aph^r-4-RP4 cells are shown in Table 15. The mutation rate obtained for the V79 was 2.4 and 8.5 x 15^{-8} per cell per division from two experiments. The UV sensitive aph^r-4-2 and aph^r-4-RP4 cells had a mutation rate 6 to 10 times higher than the wild type cells. As was found in the oua^r locus, the revertant, aph^r-4-R2, had a mutation rate of 8 x 10^{-9}, slightly lower than that of V79 cells. Table 16 shows the results of mutation rate determination at the HGPRT locus. To eliminate metabolic cooperation between 6TG^r and 6TG^s cells, TPA was added to the medium during mutant selection so that more cells per culture could be assayed. This method not only reduces the experimental costs, but also allows for mutation rate determination in more cell divisions. This table shows that aph^r-4-2 and aph^r-4-RP4 had four- to nine-fold higher mutation rates than the wild type V79 cells (see Appendix C for hypothesis on comparisons between mutation rates calculated from Po estimations).

Table 14. Fluctuation Analysis for V79, Aph^r-mutants at Oua^r Locus[1,2]

Experiment #1

Cell line:	V79	aph^r-4-2	aph^r-4-R2	aph^r-4-RP5
Replicate cultures (C):	32	48 (I)[3]	41	40
Cell numbers per culture:				
initial (No)	1	1	1	1
final (N_f) ($\times 10^6$)	2.7	0.9	1.5	6.9
divisions (d) ($\times 10^6$)	3.9	1.3	2.2	9.9
Po	0.88	0.58	0.98	0.075
oua^r (total	(6)	(124)	(1)	(673)
per culture: range	0-3	0-60	0-1	0-125
variance	0.34	77.2	0.02	782
mean	0.19	2.58	0.02	16.8
ln(1/Po)	0.13	0.54	0.02	2.6
CaN_f/C	0.13	0.74	0.04	3.88
Mutation rate per cell per division:	3.3×10^{-8}	41.9×10^{-8}	1.1×10^{-8}	26.2×10^{-8}

Experiment #2

Cell line:	V79	aph^r-4-2		aph^r-4-R2	
Replicate cultures (C):	36	18(F)[3]	22(S)[3]	21(F)[4]	21(I)[4]
Cell division per culture ($\times 10^6$)	3.8	2.1	5.4	2.7	4.2
Po	0.56	0.22	0.14	0.76	0.81
oua^r (total)	(35)	(103)	(391)	(10)	(4)
mean	0.972	5.72	17.8	0.48	0.19
ln(1/Po)	0.58	1.5	1.97	0.27	0.21
CaN_f/C	0.37	1.85	3.97	0.27	0.16
Mutation rate per cell per division:	16×10^{-8}	73×10^{-8}	45×10^{-8}	10×10^{-8}	5×10^{-8}

1. Enough numbers of replicate cultures originating from single cells were isolated from each cell line after 6-10 days of growth. Each culture was then plated to 2 wells (16 mm diameter) in 24-well plates (Costar, Cambridge, Mass.). When each culture reached nearly confluency, sufficient numbers of cultures were trypsinized individually with 1 ml trypsin (0.05%) and distributed equally into 3 plates (9 cm, Corning Glass, Corning, NY) for mutant selection. Another 6-10 cultures were individually counted, plated to 3 plates as above, and were recounted 5 hours later, or at the time of drug additions. Plating efficiency was 100% for the wild type cells and 80% for aph^r-4-2 and its revertants. N_f/C reflects correction by plating efficiency. Mutation rate determinations were calculated from equations formulated by Luria and Delbruck (163) i.e., a = ln(1/Po)/divisions.
2. Selection 1mM ouabain and 4 μM TdR.
3. Doubling time (hrs) = F, 24; I, 36; S, 48.
4. Doubling time (hrs) = F, 16; I, 18.

Tabl

Expe

Ce
Rep

Ce

Po

DT

Pe

In
Ca

Mu

Expe

Ce
Rep

Ce

Po
DT

In
Ca

Mu

1.

2.

3.

Table 15. Fluctuation Analysis for V79, Aph^r-mutants at DT^r Locus[1,2,3].

Experiment #1

Cell line:	V79	aph^r-4-2	aph^r-4-R2	aph^r-4-RP5
Replicate cultures (C):	26	22	37	28
Cell numbers per culture:				
initial (No)	1	1	1	1
final (N_f) ($x10^6$)	2.3	3.8	5	2.5
division (d) ($x10^6$)	3.3	5.4	7.2	3.6
Po	0.923	0.318	0.946	0.07
DT^r (total)	(2)	(416)	(6)	(1797)
Per culture: range	0-1	0-163	0-5	0-575
variance	0.07	1461.5	0.68	15520
mean	0.08	18.91	0.16	64.2
ln(1/Po)	0.08	1.15	0.06	2.64
CaN_f/C	0.07	4.18	0.11	11.16
Mutation rate per cell per division:	$2.4x10^{-8}$	$21.2x10^{-8}$	$7.7x10^{-9}$	$73.9x10^{-8}$

Experiment #2

Cell line:	V79	aph^r-4-2
Replicate cultures (C):	34	34
Cell division per culture ($x10^6$)	3.6	3.6
Po	0.74	0.12
DT^r (total)	(19)	(429)
mean	0.559	12.6
ln(1/Po)	0.307	2.14
CaN_f/C	0.258	2.77
Mutation rate per cell per division:	$8.5x10^{-8}$	$59x10^{-8}$

1. See Table 14.
2. Mutants (Experiment #1) were selected by DT [0.2 lf/ml (3 days)] for the V79 and aph^r-4-R2 cell lines. These mutants were kept in growth medium for additional 6 days without DT until colonies were developed, whereas DT^r from aph^r-4-2 and aph^r-4-RP4 were grown in fresh selective medium (DT = 0.6 lf/ml) for the entire period of colony development.
3. Experiment #2. DT^r mutants were selected by *in situ* method in the presence of DT (0.6 lf/ml) for 4 days.

Table 16. Fluctuation Analysis for V79, Aph^r-mutants at $6TG^r$ Locus[1,2].

Cell lines:	V79	aph^r-4-2		aph^r-4-RP4
Replicate cultures (C):	54	18	26	21
Cell numbers per culture:				
initial (No)	1	2	1	2
final (N_f) ($\times 10^6$)	3	1.7	3.1	2
divisions (d) ($\times 10^6$)	4.3	2.4	4.5	2.9
Po	0.91	0.778	0.385	0.762
$6TG^r$ (total)	(11)	(86)	(1867)	(114)
Per culture: range	0-4	0-67	0-802	0-45
variance	0.53	234.3	37625.4	165
mean	0.204	4.78	71.8	5.43
ln(1/Po)	0.097	0.251	0.956	0.272
CaN_f/C	0.113	1.46	12.43	1.55
Mutation rate per cell per division:	2.3×10^{-8}	10.5×10^{-8}	21×10^{-8}	9.4×10^{-8}

1. See Table 14.

2. Each replicate culture was originated from one or two single cells. Mutants were selected at a cell density of 1-1.5 x 10^6 cells per plate (9 cm). TPA (10 ng/ml) was present for four days, 6TG (6 µm) and TdR (2 µM) were present for the entire period of colony development.

2. Mu

than t

rates

type (

single

locus

than 1

divisio

numbe

aph^r-4

higher

reverta

10^9 ce

When

time-p

rate (1

Table

locus i

indicate

this loc

than th

Si

In this

cell-divi

cells ga

2. Multiple Replatings.

Because the expression time of $6TG^r$ and DT^r mutations is longer (5-8 days) than that of oua^r mutation, multiple replatings were also used to obtain mutation rates in $6TG^r$ and DT^r loci.

To avoid a high frequency of fast growing revertants, such as the aph^r-4-R2 type (cell doubling time 16-18 hours), a population derived from two slow growing single cells of aph^r or V79 cell line was used. The mutation frequency in the $6TG^r$ locus of V79 and aph^r-4-R2 (Fig. 20) rose linearly when the cell divisions were less than 10^{14}. An equilibrium in mutation frequencies was reached beyond 10^{14} cell-divisions in these two cell lines. This phenomenon is commonly observed when the number of initial cells is small (256). The mutation frequency in single cell clones of aph^r-4-2 reached this equilibrium earlier (approximately at 10^9 divisions), but was higher than the wild type V79 and aph^r-4-R2 cells. Because the fast growing revertants in the aph^r-4-2 population rose from 1% at 10^7 cell-divisions to 10% at 10^9 cell-divisions, no mutation frequency determinations was made thereafter. When mutation rates, calculated from two mutation frequencies determined at two time-points in early cell-divisions, were compared, aph^r-4-2 had a higher mutation rate (10×10^{-6} per cell per generation) than the V79 or aph^r-4-R2 (1.0×10^{-6}) cells. Table 17 summarizes the spontaneous mutation rate determinations at the HGPRT locus in aph^r-4-R2, aph^r-4-2 and the wild type V79 cells. The paired t-test (249) indicates that there was no significant difference in spontaneous mutation rates at this locus for aph^r-4-R2 and V79 cells, and that the rates are significantly lower than those of the UV-sensitive mutants aph^r-4-2.

Similar results were also obtained in DT^r locus for aph^r-4-2 cells (Figure 21). In this experiment, only one DT^r mutant from the wild type V79 cells (at 5×10^{10} cell-divisions) appeared in 5 mutation frequency determinations, whereas aph^r-4-2 cells gave mutation frequencies ranging between 10^{-6} to 10^{-5} at 10^7-10^9 cell-

SPONTANEOUS 6TG' MUTANTS PER 10^6 SURVIVORS

Figure

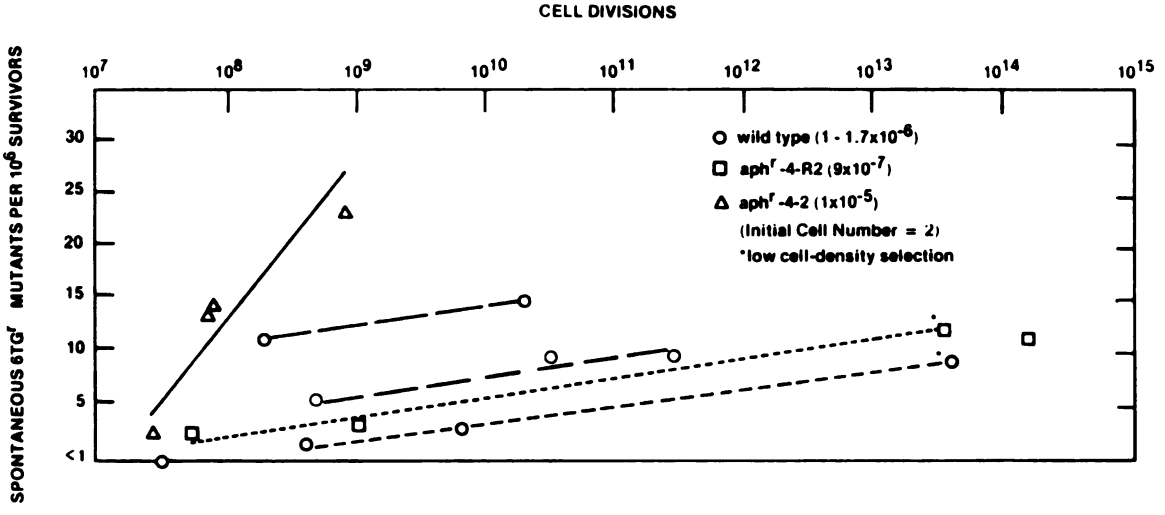


Figure 20. Spontaneous mutation rate determinations for aph^r-4-2 (Δ), aph^r-4-R2 (□) and V79 (O) cells at the 6TG^r locus by multiple replating technique. Mutation frequencies were determined at low cell-density (*, 5×10^4, 60-72 plates) without TPA (Experiment No. 1, Table 17) or at high cell-density (1×10^6, 3 plates) with TPA (Experiment No. 2, Table 17).

Table 17. Spontaneous Mutation Rate Determinations (SMR-6TG^r) Using Multiple Replating Technique

Table 17. Spontaneous Mutation Rate Determinations (SMR-6TG^r) Using Multiple Replating Technique

Cell lines	Experiment No. 1 (low cell-density selections, 5×10^4 cells per plate)[1,3]				
	N_1	MF_1	N_2	MF_2	SMR-6TG^r
V79	2	-	22×10^6	$<9.2 \times 10^{-7}$	-
	7×10^6	$<9.2 \times 10^{-7}$	101×10^6	1.06×10^{-6}	-
	7×10^6	1.06×10^{-6}	105×10^6	2.65×10^{-6}	8.1×10^{-7}
	7×10^6	2.65×10^{-6}	423×10^8	9.15×10^{-6}	1.0×10^{-6}
				mean =	9.05×10^{-7}
aph^r-4-R2	2	-	486×10^5	1.91×10^{-6}	-
	7×10^6	1.91×10^{-6}	105×10^6	3.03×10^{-6}	5.7×10^{-7}
	7×10^6	3.03×10^{-6}	197×10^8	12.12×10^{-6}	1.5×10^{-6}
	7×10^6	3.03×10^{-6}	109×10^{10}	11.10×10^{-6}	9.4×10^{-7}
				mean =	1.0×10^{-6}

Table 17. continued

Cell lines	Experiment No. 2 (high cell-density selections with TPA)[2,3]				
	N_1	MF_1	N_2	MF_2	SMR-6TG^r
V79	2	-	1.4×10^8	1.15×10^{-5}	-
	1.4×10^8	1.15×10^{-5}	1.4×10^{10}	1.47×10^{-5}	9.63×10^{-7}
	2	-	5.9×10^8	6.15×10^{-6}	-
	5.9×10^8	6.15×10^{-6}	3.5×10^{10}	9.95×10^{-6}	1.29×10^{-6}
	5.9×10^8	6.15×10^{-6}	3.3×10^{11}	8.93×10^{-6}	6.09×10^{-7}
				mean =	9.54×10^{-7}
aph^r-4-2	2	-	1.98×10^7	1.94×10^{-6}	-
	1.98×10^7	1.94×10^{-6}	5.6×10^7	1.4×10^{-5}	1.61×10^{-5}
	5.6×10^7	1.4×10^{-5}	5.7×10^8	2.3×10^{-5}	5.38×10^{-6}
	1.98×10^7	1.94×10^{-6}	5.7×10^8	2.3×10^{-5}	8.69×10^{-6}
				mean =	10.1×10^{-6}

[1]Cells resistant to 6TG were selected at low cell densities (5×10^4 per plate, 60 or 72 plates for each mutation frequency determination) without TPA. The mutation rates were expressed as mutations per cell per generation and are not statistically different ($p < 0.05$) in both cell lines by paired *t*-test.

[2]High cell-density selections were employed with TPA (0.01 µg/ml) for four days at a cell density of 1×10^6 per plate, 3 plates for each MF determination.

[3]N_1 & N_2 are cell numbers, MF & MF_2 are mutation frequencies at the first and second mutation frequency determinations. And mutation rates were calculated according to the equation in the text: $\ln 2[2(MF_2-MF_1)/\ln(N_2/N_1)]$.

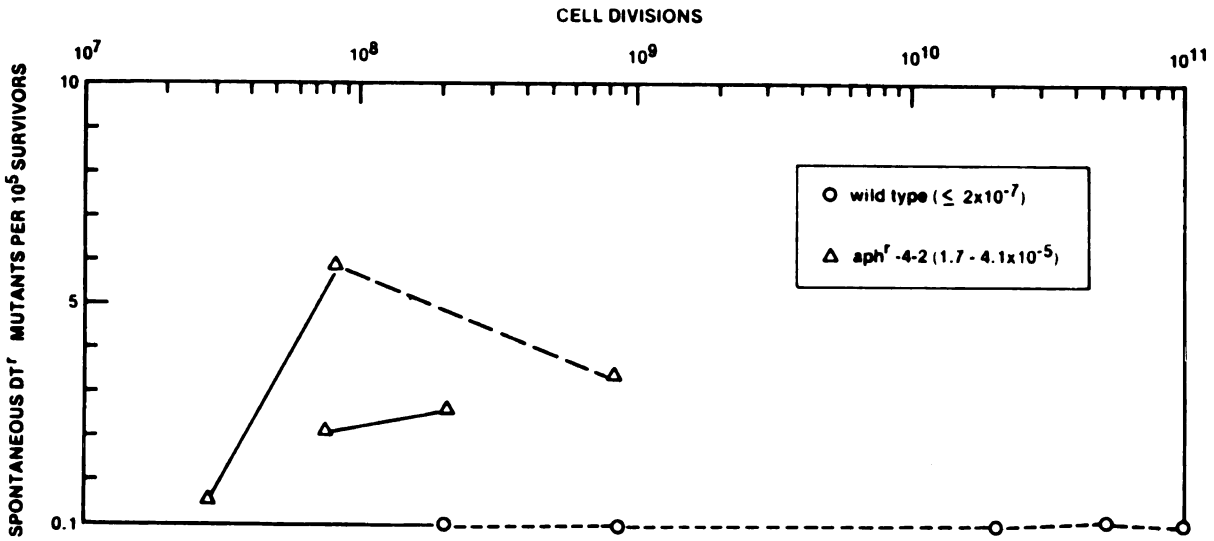


Figure 21. Spontaneous mutation rate determinations for aph^r-4-2 (Δ) and V79 cells (O) at the DT^r locus. The cell-density for each determination was 4×10^5, 6 plates.

divisions. The spontaneous mutation rate at this locus, calculated for aph^r-4-2, was $2\text{-}4 \times 10^{-5}$ per cell per generation compared to $< 2 \times 10^{-7}$ for V79 cells.

The results from both loci suggest that mutations in aph^r-4-2 cells occurred at a higher rate in earlier cell-divisions than those in V79 or aph^r-4-R2 cells. This is reasonable in light of the appearance of fast-growing revertants in the mutant population. The spontaneous hypermutability of the UV-sensitive aph^r-4-2 cells detected by this method was consistent with that observed using fluctuation analyses. Together they showed the hypermutability of aph^r-4-2 is not loci specific as reported in a different mutant (175) of CHO cells.

DISCUSSION

Induction of Aphidicolin-resistant Mutants

Aphidicolin-resistant mutants were isolated from a BrdU/black-light and UV mutagenized population with a frequency of 4.5×10^{-7} (40). One of these mutants is UV-sensitive (UV^S). The frequency for this UV^S mutant is then 1.1×10^{-7}. The latter frequency is similar to that of MNNG-induced diaminopurine resistant (dap^r, $APRT^-$) mutants of CHO cells (41). In a cell line derived from a transitional cell cancer epithelium (253J, 2n=60-80 with two active X chromosomes and chromosome 16, Dr. C-c. Chang, unpublished results and see Refs. 77,96), $6TG^r$ or dap^r mutants were isolated at a frequency of 2×10^{-7} from a BrdU/black-light and UV mutagenized population (Liu, unpublished results). Because $6TG^r$ or dap^r is recessive, it takes two mutations for the expression of the mutant. The similarity in mutation induction frequency of the mutants mentioned above indicates that the aphidicolin resistance of aph^r-4 might be recessive and that it takes at least two mutations to create such a mutant. Mutagen sensitive (UV^S) repair deficient mutants reported by Thompson et al., (264,265) are also recessive, though the phenotypes of these mutants are clearly different. This UV-sensitive aph^r mutant appears to be the first mutagen-sensitive mutant isolated from mammalian cells by a selective agent.

Characterization of Aphidicolin-resistant Mutants

Characterization of aph^r-4 and its thymidine auxotrophic revertant has been reported (40). This dissertation carried out further characterization and confirmation. The results are presented in Table 18.

Table 18. Characterizations of Aph^r-mutants and V79 Cells at 37°C[a]

	Cell Lines	V79	Aph^r-Mutants[b] 4-2	4-R2	4-RP4	4-RP5
1)	AraC (0.5 μM)	S	S	S	S	S
2)	Aphidicolin	S	R	R	R	R
	LD_{50} (μM)	(0.24)	(0.6)	(0.44)	(0.8)	(0.5)
3)	Thymidine	P	A	A	P	P
4)	Thymidine (100 μM)	R	S	S	S	S
5)	Cytidine (4 mM) (with 4 μM TdR)	N	S	N	S	N
6)	BrdU (100 μM)	S	S	S	S	-
7)	GdR (100 μM) (with 2 μM TdR)	N	S	S	S	S
8)	Mutagen Sensitivities (+ Benzamide)[c]					
	UV	N(N)	S(N)	N(N)	S(-)	S(-)
	X-ray	N(N)	N(S)	N(S)	-	-
	DMS	N(S)	N(S)	N(S)	-	-
	MNNG	N(S)	S(S)	S(SS)	-	-
	NAcAAF	N(N)	S(NC)	S(NC)	-	-
9)	Mutability					
	UV	N	H	N	-	-
	X-rays	N	N	-	-	-
10)	UDS (UV)	N	N	N	N	-
11)	Liquid holding (12 hrs., UV) Recoveries:					
	(Survival)	E	D	E	-	NC
	(Mutation)	D	E	-	-	-
12)	Spontaneous Mutation Rate	N	H	N	H	H

[a]A, Auxotroph; araC, Cytosine Arabinoside; BrdU, Bromodeoxyuridine; D, Decreased; E, Enhanced; GdR, Deoxyguanosine; H, High; N, Normal; R, Resistant; S, Sensitive; SS, Very Sensitive; NC, No Change in Sensitivity; -, Not Tested.

[b]Subclones of aph^r-4; results were compared to those of V79 cells.

[c]Compared to those without benzamide.

Results of cytidine toxicity with or without thymidine (TdR), as suggested by Chang et al. (40), demonstrated that a) the wild type V79 cells used in this study are probably deficient in deoxycytidine monophosphate (dCMP) deaminase, because in the presence of cytidine, wild type V79 cells become TdR auxotrophs as they do in a dCMP deaminase deficient mutant of CCL39 cell lines (278); b) the wild type V79 cells, due to deficiency in dCMP deaminase, possibly synthesize dTTP through the uridine diphosphate (UDP) reductase pathway. The fact that the wild type becomes a TdR auxotroph in the presence of cytidine, which elevates cytidine triphosphate and inhibits UDP reductase, strongly suggests that this UDP reductase pathway is the only one used by V79 cells to synthesize dTTP (Fig. 22). Aph^r-4-2 and aph^r-4-RP4 cells were not able to grow in medium containing a high concentration of cytidine and a low concentration of thymidine. The mechanism for this cytidine toxicity is still unknown. Since thymidine was present in the medium, it is not likely due to dTTP deficiency. On the other hand, a high concentration of cytidine could increase dTTP or dCTP to a toxic concentration in these mutant cells, as postulated previously. This hypothesis can be tested by direct measurement of dNTP pools after the addition of cytidine. The difference in responses between these mutants and V79 cells cannot be due to thymidine sensitivity, since aph^r-4-2 and aph^r-4-R2 are similar in thymidine sensitivity but different in cytidine sensitivity. Revertants aph^r-4-R2 and aph^r-4-RP5 are similar to V79 cells in cytidine sensitivity but different in aphidicolin sensitivity. Therefore, thymidine auxotrophy and aphidicolin sensitivity are not necessarily associated with cytidine sensitivity. In fact, two subgroups of aph^r-4 can be classified according to different nucleoside sensitities: namely, TdR auxotrophs (aph^r-4-2 and aph^r-4-R2) and TdR prototrophs (aph^r-4-RP); and, in the presence of thymidine, cytidine sensitive (aph^r-4-2 and aph^r-4-RP4) and cytidine resistant (aph^r-4-R2 and aph^r-4-RP5). Because in the presence of TdR (2 μM) the aph^r-4-2 cells have small numbers of surviving clones in the presence of

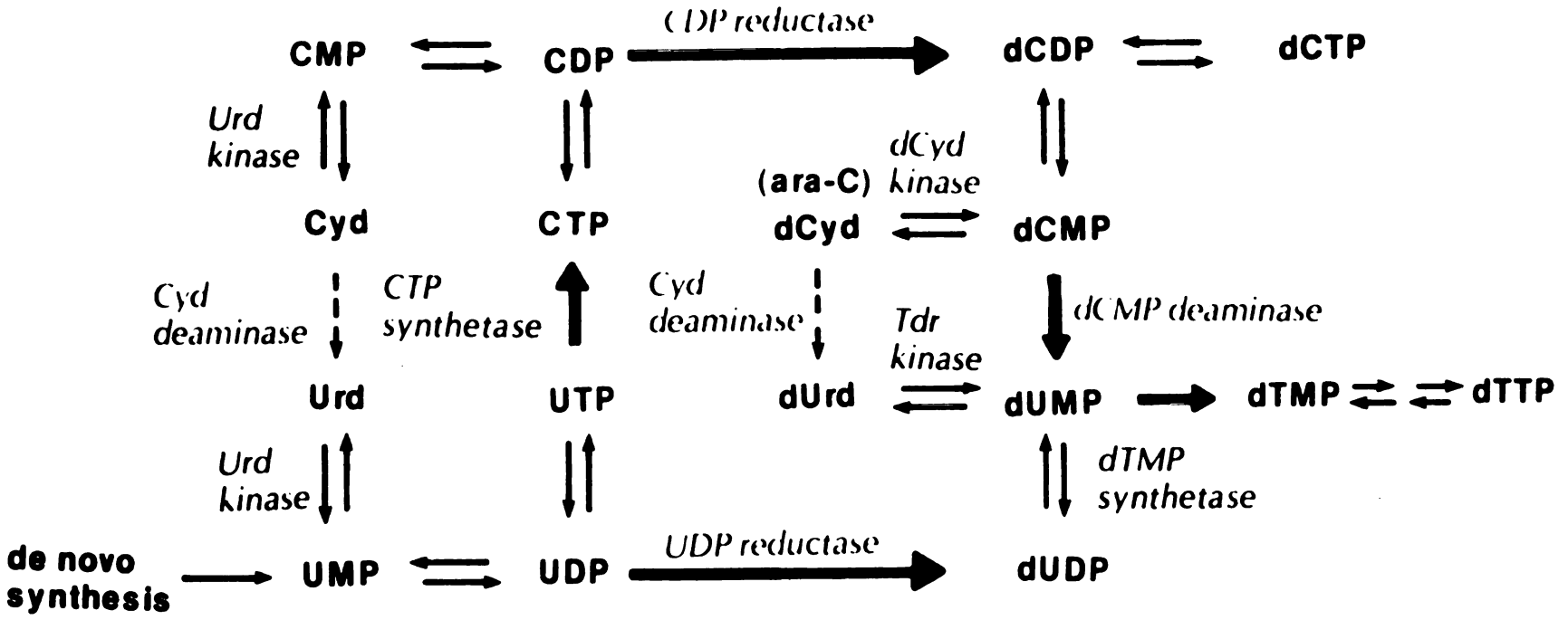


Figure 22. **Pyrimidine metabolism in mammalian cells**

TdR, AdR or GdR at 100 μM, it is possible that revertants resistant to GdR, AdR or TdR can also be isolated.

In the present studies, TdR auxotrophic phenotype and cytidine toxicity are not reversible at a lower temperature in aph^r-4-2 and in its thymidine auxotrophic revertants, aph^r-4-R2. It appears that the mutated enzyme for TdR auxotrophs is not temperature sensitive, as are other aph^r mutants of mouse FM3A cells (8). In fact, cytotoxicity of cytidine for aph^r-4-R2 is even more drastic at 34^{o}C in the presence of TdR than at 37^{o}C. It is possible that UDP reductase of aph^r-4-R2 becomes leaky at 34^{o}C, or that the sensitivity resulting from the feedback inhibition of dCTP is higher at 34^{o}C than at 37^{o}C.

Guanine- or adenine-deoxynucleoside (GdR or AdR respectively) and thymidine sensitivities of the aph^r mutant and its revertants are possibly a result of defects in the subunits of deoxyribonucleotide reductase. Mutants of mouse cells with sensitivities toward exogenous GdR, AdR or TdR have been reported to have a defective deoxyribonucleotide reductase (288). It has been suggested that this reductase is an enzyme complex of four subunits. If the original mutant, aph^r-4-2, contains 2 mutations in the gene(s) coding for these subunits, these mutations may be unstable and easily give rise to revertants. Alternatively, if aph^r-4-2 resulted from one mutation but is a non-locus-specific mutator mutant as shown in this study, it can introduce additional mutations which suppress the mutant phenotype such as growth rate, cytidine- or mutagen-sensitivities. From results of the present studies, it is not possible to distinguish between these two alternatives, although the suppressor mutation may be a favorable explanation.

Deoxycytidine triphosphate (dCTP) pools of aph^r-4-2 and aph^r-4-R2 found in this research are different from those reported previously (40). The dCTP pools of both aph^r-4-2 and aph^r-4-R2 were reported to be 6 to 8 times higher than those of wild type V79 cells (40). In the present studies, the procedure of biochemical assay

is the same as in previous experiments except that during the collection of cell extracts: a) three cell lines were partially synchronized by confluency, twenty-four hours before harvesting; b) the methanol extracts were treated with 0.5 N perchloric acid to eliminate enzymatic activities which have been reported to interfere with dNTP measurements (192); and c) the dNTP markers externally added to wild type cell extracts survived treatments of 0.5 N perchloric acid. Because intracellular dNTP levels are finely tuned according to cell cycles, the possibility exists that cell extracts were collected at different cell cycles and thus led to the discrepancy. Nevertheless, there seems to be no difference in dCTP pools between the mutant and revertant. The discrepancy of dCTP levels between the values reported (40) and the values found here in both aph^r-4-2 and aph^r-4-R2 cells, does not affect the hypothesis on the mechanism of mutabilities in aph^r-4-2 cells, that will be presented later.

Although aph^r-4-2 cells have twofold higher dCTP pools than the wild type V79 cells, this result could be interpreted as due to a difference in growth rate. The result could explain: a) why there is the same araC sensitivity of aph^r and V79 cells; and b) why aph^r-4-2 and aph^r-4-R2 are not more resistant to MNNG or DMS than V79 cells. An elevated endogenous dCTP level would reduce the toxicity of MNNG, DMS or araC, and enhance the survival (40,170,176,278) in the presence of these chemicals.

Mutagen-sensitivity and Its Modification by Various Chemicals

Preliminary studies on mutagens other than UV irradiation suggest that aph^r-4-2 and aph^r-4-R2 are sensitive toward specific mutagens. As compared to the wild type V79 cells, these two aph^r mutants are not more sensitive to DMS. Aph^r-4-2 and aph^r-4-R2 are sensitive to MNNG and NAcAAF. The difference in X-ray

sensitivity of the V79, aph^r-4-2 and aph^r-4-R2 may not be significant. It is premature to draw any conclusion from these studies, but several speculative possibilities will be discussed.

Dimethyl sulfate produces methylated adenine or guanine as does MNNG, and the repair mode is base excision repair [short patch repair of X-ray-like mutagens (111,112,114,200,220,224,262)]. The DNA lesions caused by NAcAAF are generally bulky and are repaired by long patch nucleotide excision repair (UV-like mutagens) (111,112,200,220), though AAF adducts on DNA may cause inhibition to methylation (209). It appears that aph^r-4-2 and aph^r-4-R2 are not nucleotide repair deficient, because UV-induced unscheduled DNA synthesis (UDS) in these two mutants is the same as in the V79 cells. Aph^r-4-2 is possibly not a repair mutant but rather it may possess a defective nucleotide excision repair pathway which is rendered error-prone by a certain mechanism.

In the case when chemical mutagens are used, the DNA lesions are different with different mutagens. Other than DNA adducts and single-strand breaks, ionizing radiation cuases free radicals which interact with cellular protein; MNNG reacts with the thio group of proteins and DMS reacts with the oxygen atom of the macromolecules (154). These adducts can possibly produce lethal effects. On the other hand, when DNA methylation adducts are compared, MNNG causes more O^{6}MeG (7% of total methylated bases) than DMS does of O^{6}MeG (0.3% of total methylated bases) at the same level of survival (154,173,188). It is believed that O^{6}MeG is involved in mispairings which are responsible for lethal and mutagenic effects. Extracellular thymidine has been shown to enhance cytotoxicity of MNNG by increasing mispairings with O^{6}MeG (170). Because both aph^r-4-2 and aph^r-4-R2 are TdR auxotrophs, and dTTP pools of these cells in medium supplemented with 5% FCS are similar to the wild type V79 cells (40, and this report), the hypersensitivity of aph^r-4-2 and aph^r-4-R2 to MNNG cannot be due to endogenous dTTP pools.

The higher sensitivity of aph^r-4-2 and aph^r-4-R2 to MNNG could be due to the mispairing properties of O^6MeG, or to corrections of this mispairing. Because O^6MeG is considered to be responsible for mutagenicity (188), and if the observed hypersensivity in the present study is due to O^6MeG, aph^r-mutants would have a MNNG-induced hypermutability. Indeed, they are hypermutable at oua^r and DT^r loci (data not shown).

While aph^r-4-2 is sensitive to both UV and NAcAAF, aph^r-4-R2 has different sensitivities toward UV and NAcAAF. No clear conclusion can be made from this observation. Repair of UV and NAcAAF cells is not additive in V79 Chinese hamster cells (1) indicating different repair enzymes are necessary to repair these two types of damage. The DNA repair mutants of V79 cells reported by Schultz et al., (233-235) show different sensitivities to UV, X-ray, NAcAAF or MNNG. Compared to wild type V79 cells, the mutant strain UV^{s}7 (deficient in UV-induced UDS) is UV sensitive and hypermutable for UV-induced mutations, but not sensitive to NAcAAF, or to X-ray; strain UV^{r}23 is UV resistant, but normal in NAcAAF or X-ray sensitivity. Other strains, UV^{s}40 and UV^{s}44, are sensitive to UV, X-ray, MNNG and NAcAAF (generalized mutagen-sensitivity). Data from the mutagen sensitivity in aph^r-4-2 and aph^r-4-R2 further suggest that they are different from those nucleotide excision repair mutants (e.g., UV^{s}7) reported by Schultz et al.

In an attempt to further investigate possible mechanisms of genetic defects in aph^r mutants, the role of benzamide, a potent inhibitor of poly(ADP-ribose) polymerase, was tested during DNA repair period on cell survivals. The enhancement of cytotoxicity of N-methyl-N-nitrosourea (187), DMS (70,237) and ionizing radiation (187) by inhibitors of poly(ADP-ribose) polymerase (212) has been shown to result from the inhibition of poly(ADP-ribose) synthesis.

In the present studies, benzamide significantly enhances the cytotoxicity of MNNG and DMS in wild type V79 cells; and this agrees with the results of Jacobson

et al. (134), who reported that the rejoining of MNNG-induced strand breaks is defective in nicotinamide adenine dinucleotide (NAD) depleted mouse 3T3 cells. It also agrees with the results of Durkacz et al., (70,71) and Shall et al., (237), who reported that 3-aminobenzamide enhances cytotoxicity of DMS and prevents strand rejoining in mouse L1210 leukaemia lymphoblast cells. Benzamide slightly enhances lethality of X-ray (less than 1 krad)-irradiated V79 wild type cells and this is in agreement with Nduka et al., (187) who observed that 3-aminobenzamide also slightly reduces survival of γ radiation (less than 1 krad)-treated L1210 cells.

Benzamide inhibits poly(ADP-ribose) polymerase which can be stimulated by single-strand breaks in a dose-responsive manner (14,15). Excision repair of UV- and NAcAAF-induced damage, and ionizing radiation at low doses might not sufficiently activate poly(ADP-ribose) synthesis; therefore, any inhibition may not be detectable by measuring cell survival. The difference in cytotoxicity enhancements by benzamide in these two alkylating agents and low dose ionizing radiation treated V79 cells, may also be due to specific DNA lesions caused by an alkylating agent that activate poly(ADP-ribose) polymerase.

The reason for benzamide's greater enhancement of MNNG- or X-ray-induced cytotoxicity in aph^r-4-2 and aph^r-4-R2 may be twofold: a) poly(ADP-ribose) polymerase inhibitors have a synergistic effect on cytotoxicity in partially NAD depleted cells (134,237); and b) alternatively, benzamide has been shown to inhibit break rejoining in DMS- (70,71), or MNU- (237) induced gaps. If enzyme(s) involved in repair of single-strand breaks for aph^r-4-2 or aph^r-4-R2 is (are) defective, gap closure (break rejoining) could proceed more slowly in the presence of benzamide and thus reduce cell survival. Perhaps ADP-ribosylation of nuclear protein by poly(ADP-ribose) polymerase may have a functional role in the cellular repair mechanism, either by modification of the chromatin structure or by specific modulation of the DNA repair step (17,18,70,71,73,94,165,213,274).

Caffeine, at the two doses tested, seems to potentiate the cytotoxic effect of UV in these three cell lines. Caffeine is proposed to be inhibitory to "post-replication" repair; it reduces cell survival, and it enhances or has no effect on mutations in V79 cells (36,37). It seems that the caffeine-sensitive repair step(s) may not be different in these three cell lines. Alternativey, caffeine is a weak inhibitor of poly(ADP-ribose) polymerase, but has a greater UV-cytotoxicity enhancement than benzamide does. This suggests that the caffeine sensitive DNA repair step is possibly different from the benzamide sensitive step. Furthermore, caffeine has been shown to reduce DNA-chain growth (239,263) and to enhance the number of replicons, or to alter purine pools (287). Because aph^r-4-2 cells exhibit slightly greater caffeine sensitivity than V79 or aph^r-4-R2 cells do, the increased number of replicons or altered purine pools in aph^r-4-2 cells may increase their cytotoxicity (or mutation) by inhibiting the repair or by increasing mispairing during DNA repair. Further investigations are required to elucidate the roles of benzamide and caffeine in repair pathways.

Mutability of the UV-sensitive Aphidicolin-resistant Mutant

The results in UV-induced mutagenesis showed that: a) aph^r-4-2 is hypermutable for oua^r, DT^r and $6TG^r$ mutations when its maximum mutation frequencies were compared with those of V79 cells at the same UV dose; b) the thymidine auxotrophic revertant, aph^r-4-R2 is similar to V79 cells in UV-sensitivity and mutability; and c) when UV-induced mutation frequencies were compared at the same survival rate, the aph^r-4-2 mutant showed a higher frequency than both V79 and its revertant at the oua^r and DT^r loci, but not at the $6TG^r$ locus.

Of the three mutation assay systems used in our experiments, the $6TG^r$ mutants are considered to be the results of nonsense, deletion, frameshift or point mutations at the HGPRT locus; whereas oua^r mutants reflect missense (point)

mutations at the Na^+/K^+-ATPase locus. The DT^r mutants of V79 cells are not very well characterized. In Chinese hamster ovary (CHO-K1) cells two types of DT^r mutants have been described: permeability-mutants with impaired uptakes of DT and translation-mutants with altered elongation factor 2 (EF-2) of protein synthesis (68). The latter type of mutants can be selected with a high concentration of DT, such as the one used in the present studies. Similar to $6TG^r$ mutants, but unlike oua^r mutants, DT^r mutants, which are resistant to high concentrations of DT, can be induced by X-rays. This is contrary to the prediction that the DT^r mutant (EF-2 mutants) may be similar to oua^r mutants, since the gene products of both loci are essential for the survival of cells. The fact that they are different may indicate that small deletions as well as missense mutations can cause DT^r mutations. The observation that aph^r-4-2 had a higher mutation frequency per unit of UV dose and per unit of survival at the Na^+/K^+-ATPase and EF-2 loci, may be considered as evidence that aph^r-4-2 cells have a higher frequency of UV-induced missense mutations.

Because the expression times for 6TG mutations are different for aph^r-4-2 and wild type V79 cells, it is difficult to draw a clear conclusion from the results. The delayed expression of UV-induced $6TG^r$ mutation in aph^r-4-2 may be a real phenomenon, or it may be due to an indirect effect of some other mechanisms not related to mutation expression, such as selection advantage for $6TG^r$ mutants. If the phenomenon is real, the aph^r-4-2 can be considered as hypermutable per UV dose, also, at the HGPRT locus. If it is an indirect effect, then aph^r-4-2 apparently exhibits differential mutability at different loci.

In aph^r-4-2 cells, X-ray induced $6TG^r$ mutation frequency does not show delay expression. Therefore, the delayed UV-induced $6TG^r$ mutation appears to be mutagen specific. Because X-ray radiation generates deletion type mutations, it appears that aph^r-4-2 cells are not sensitive to deletion type mutagens (X-rays). As

shown in the DT^r mutation locus, aph^r-4-2 is not hypermutable after X-ray irradiation. The hypermutability of aph^r-4-2 after UV irradiation may be specific in point mutation. Elkind (75,76) reported that radiation survivors repair most (if not all) of the strand breaks. It can be assumed that X-ray-induced mutations are fixed at the subsequent DNA replication in the survivors. On the other hand, UV-induced pyrimidine dimers are not totally removed and may be transferred to the daughter strand (60). These dimers served as mutation "substrates" for the next generations and may show a delayed-fixation in aph^r-4-2, whose excision repair may be rendered error-prone. In prokaryotes, a mutation (mfd) in B/r strains of Escherichia coli may cause deficiency in UV-induced mutation frequency decline (85). This phenomenon may be similar to that observed in aph^r-4-2, but the genetic basis may be different.

Pyrimidine auxotrophs have been reported in mutants of fungi, N. crassa, S. cerevisiae and U. maydis (181), and of CHO cells (175). In the case of U. maydis, the UV, X-ray or nitrosoguanidine sensitivity is a direct result of pyrimidine auxotrophic mutation (181,182). A mutant of U. maydis, pyr1-1, has a reduced level of thymidine nucleotides, but has no significant change in any other deoxyribonucleo tides. Photoreactivation did not alter this sensitivity. The pyr1-1 mutant does have UV-induced hypermutability (as measured by caesium-resistance), and is deficient in UV-induced mitotic recombination (due to inviability of recombinants). A diploid heteroallelic at the pyr1 locus was UV sensitive but not deficient in UV-induced mitotic recombination. This UV-sensitivity of pyr1-1 appears to be a result of failure in a repair DNA polymerase to fill post-excision single-strand gaps in the DNA.

Mammalian repair deficient mutants with hypermutability of V79 Chinese hamster cells reported by Schultz (233-235) are not TdR auxotrophs and are not resistant to aphidicolin. They also show a reduced UDS after UV-irradiation. The

aph^r-4-2 hypermutability appears not to be associatd with a reduced UDS capacity as shown in Fig. 19.

In the present studies, the thymidine auxotrophic revertant of aph^r-4 regained not only its normal UV sensitivity, but also its normal mutability. From these results it is concluded that UV-sensitivity and mutability in aph^r-4-2 are correlated and possibly controlled by a single gene. The fact that both aph^r-4-2 and aph^r-4-R2 are thymidine auxotrophic and have similar levels of dCTP, but that only the former is UV-sensitive and hypermutable, may also be considered as evidence that the nucleotide pool in aph^r-4-2 does not contribute to its UV-sensitivity and hypermutability. The exogenous TdR or AdR (2-4 μM) was also found not to affect UV-induced mutation expression.

The basis for the UV-sensitivity and hypermutability of aph^r-4-2 is not known. Our results indicate that it is not defective in excision repair, because UV-induced unscheduled DNA synthesis is normal in all three cell lines. In addition, aph^r-4-2 is not hypermutable in X-ray-induced mutagenesis. Base excision repair may be as efficient as in the V79 cells. Unlike V79, the UV induced cytotoxic and mutagenic effects on aph^r-4-2 appear not to be reduced by conditioned medium, which has been shown (186) to arrest V79 cell replication and enhance DNA excision repair following UV irradiation.

Induced mutations in mammalian cells are considered results from unrepaired (by-pass repair) and/or misrepaired (excision repair) DNA damage. In normal diploid human fibroblasts and V79 cells, unrepaired damage can be overcome by liquid holding with conditioned medium so that the error-free excision repair is enhanced (168,186). Personal observations indicate that single cells (V79 and aph^r-4-2) grown in the conditioned medium do not divide (data not shown). Together with the result reported by Nakano et al. (186), it is assumed that the excision repair pathway is enhanced in both cell lines during liquid holding. The fact that the UV-induced

mutation frequency and cytotoxicity of V79 cells are reduced upon liquid holding (positive recoveries) indicates the excision repair process in V79 cells is error free. Furthermore, the fact that aph^r-4-2 cells grow slower than V79 cells in the growth medium and show the negative liquid holding recoveries, i.e., both UV-induced mutation and cytotoxicity are enhanced, indicates the excision repair of aph^r-4-2 cells is not error free, or this repair pathway has been rendered error-prone or mutagenic by certain mechanism(s).

The possibility that aph^r-4-2 could be an α-polymerase mutant has not been ruled out (40). If the mutant is, in fact, defective in α-polymerase, our data could be considered as evidence that α-polymerase is involved in the DNA repair process as indicated by previous reports (16,47,110,248). The DNA repair in aph^r-4-2 must have been rendered error-prone (e.g., defective base selectivity) by the mutation, despite its slow growth, which normally favors error-free excision repair.

The mutator activities of UV-sensitive aph^r-4-2, aph^r-4-RP4 or aph^r-4-RP5 in three genetic loci (oua^r, DT^r and 6TG^r) are not locus specific and do not associate with the TdR auxotrophic phenotype. These observations are similar to those of UV-induced hypermutability of aph^r-4-2. Therefore, the UV-sensitivity and mutability are possibly controlled by a single gene, as discussed previously.

Mammalian variants that have an elevated mutation rate reported thus far, are from CHO TdR$^-$ mutants (175), mouse cells (288) and fibroblasts from patients with Bloom's syndrome (97,282). In human cells, the mechanism for the elevated mutation rate may be due to the presence of clastogenic material, which can be detected in the culture medium of Bloom's fibroblasts (78,283). In the rodents, the mutator activities are reported to be associated with either TdR auxotrophic phenotype (175) and/or an elevated dCTP pool (288). These mutator activities are locus specific. Both mutator activities can be abolished by reduction of the ratio of dCTP to dTTP through additions of exogenous TdR. It appears that the mechanism

for this mutator activity is through a mass action of DNA precursor substrates and/or a deficiency in proofreading enzyme activity in CHO or mouse cells. Furthermore, the UV-induced cytotoxicity and mutagenicity have not been reported in these mutator mutants from the rodents. In the present studies, TPA was used to eliminate metabolic cooperation commonly observed in quantitative mutation assay at $6TG^r$ locus. The results from $6TG^r$ mutation rate determination in the wild type V79 cells are similar to results reported by other investigators (see Table 2).

The results clearly indicate that the mutator activities cannot be explained by the dNTP pools alone. Firstly, the aph^r-4-R2 cells have dCTP pools similar to those of aph^r-4-2 cells, but the former cells are not hypermutable. Secondly, the mutator activities are not associated with TdR auxotrophic phenotype, because the UV sensitive TdR prototrophic revertants, aph^r-4-RP4 and -RP5, are also hypermutable. It appears that the mutator activities are associated with UV-sensitivities and UV-induced hypermutability.

These aph^r-4 variants were selected by a specific polymerase α inhibitor-aphidicolin. This inhibition is competitive with dCTP in the case of purified polymerase α (106,193,194), or with each of the dNTPs in isolated nuclei (194). A stimulatory factor of polymerase α has been isolated from mammalian cells (136). This stimulatory factor cannot reverse the inhibiting effect of aphidicolin on polymerase α. Aphidicolin has been used to isolate dNTP pool mutants in mammalian cells (7,8,10,226) or polymerase mutants in the fruit fly (259) and in mouse FM3A cells (190). However, none of them is mutagen sensitive or has an induced hypermutability. Because mutator phenotypes of UV-sensitive aph^r mutants are not locus specific at three loci and continuously give rise to "revertants" of different phenotypes (cytidine sensitive vs. resistant in the presence of TdR; TdR auxotrophs vs. prototrophs), it appears that the mutation "gene(s)" is (are) a generalized (non-locus-specific) mutator. In this regard, the gene(s) is (are) similar

to the mutD gene of E. coli (55,56,62,246,247). The mutator phenotype of mutD gene is stimulated by TdR (2 μM). Whether or not aph^r UV^s mutator gene(s) is (are) also stimulated by TdR is unknown, because aph^r-4-2 is TdR^- and the growth medium must contain TdR. Nevertheless, in one experiment when TdR (4 μM) was not added to ouabain medium, the spontaneous mutation rate was lower than for that of the wild type (data not shown). On the other hand, due to possible interference of TdR with toxin cytotoxicity, TdR was not added to selective medium during DT^r mutation rate estimation, and aph^r-4-2 is hypermutable at DT^r locus. Further analysis using UV^s TdR^+ revertants is warranted. Even though aph^r-4-2 mutants and the wild type V79 cells (40) have a polymerization activity which is as sensitive to the inhibition of aphidicolin, one could not eliminate a possible existence of defective enzymatic activities which are responsible for the observed mutator activities, i.e., site specific chromosomal aberration on the long arm of chromosome one (40) and UV-induced cytotoxicity and hypermutability.

Eukaryotic mutator mutants that are sensitive to raditions (UV light) and chemical mutagens (methyl methanesulfonate) have been reported in yeast (115), and a mutagenic repair pathway seems to be responsible for the observed hypermutability in these mutants (mut-3,4,5). Mammalian polymerase α differs from prokaryotic polymerase or yeast polymerase II in that the purified eukaryotic polymerase does not have an associated 3'-5' exonuclease activity which is involved in correction of mispaired bases. Nevertheless, polymerization error in a DNA polymerase from acute lymphoblastic leukemic cells is tenfold higher than that in a polymerase from normal cells (254). It has been suggested that this defect is in the base-selectivity of the polymerase. The UV-sensitive mutator aph^r-4-2 cells could be defective in this selectivity, whereas a "reversion" or second mutation could have occurred in aph^r-4-R2 so that the selectivity is normal. The fact that aph^r-4-2 cells gave rise to different kinds of reverents, and have an elevated non-locus specific

mutation rate favors the notion that revertants may be results of suppressor mutation in aph^r-4-2 cells during normal semi-conservative DNA replication. This altered base-selectivity could also be responsible for a repair process which is "error-prone" or mutagenic in aph^r-4-2 cells (301). The resulting mutation from this altered base selectivity would be a missense mutation such as that observed in oua^r locus.

The mechanism for the elevated spontaneous mutation rate in the UV-sensitive aphdicolin-resistant mutants remains unknown. The data indicate: a) aph^r-4-2 is generalized mutator mutant; b) the mutator activities are associated with UV sensitivities but not with dNTP pools; c) UV-induced hypermutability of aph^r-4-2 does not seem to be due to a defect in DNA repair; rather, it seems that the excision repair has been rendered error-prone; d) mutagen sensitivities of aph^r-4-2 seem to be specific to mutagens whose adducts require long patch repair pathway; e) it appears that polymerase base-selectivity is involved in the hypermutability of aph^r-4-2; and f) aph^r-4-2 cells give rise to revertants which may allow in-depth characterization of the mutator gene(s).

If the base-selectivity of polymerase in aph^r-4-2 cells is indeed defective, these mutants provide new biological and biochemical means to study processes of DNA replication and mutagenesis in mammalian somatic cells.

SUMMARY

Isolation of mutagen-sensitive mutants in mammalian cells has been reported by several laboratories. Most of these mutants were selected from mutagenized cells by methods similar to replica plating technique. Recently a UV-sensitive mutant has been isolated by selection with aphidicolin, a specific inhibitor of DNA polymerase α. This mutant also exhibits other pleiotrophic effects such as thymidine auxotrophy, sensitivity to cytidine, slow growth and BrdU-dependent site-specific chromosomal aberrations. The present dissertation reports characteriztions and a detailed study of spontaneous and induced mutability of this mutant and its revertants. Compared to V79 cells, this mutant (aph^r-4-2) was more sensitive to UV and NAcAAF whose DNA adducts are believed to be repaired by the "long-patch" nucleotide excision pathway, but was not more sensitive to X-rays or MNNG whose lesions are repaired by the "short-patch" nucleotide excision pathway. The thymidine auxotrophic revertant and the mutant were sensitive to MNNG. In the previous report (40), the mutant was shown to contain no α-polymerase that is resistant, in crude extract measurement, to aphidicolin. In this study, the mutant was found to be normal in unscheduled DNA synthesis. However, unlike V79 cells, the mutant appears to increase mutation and cytotoxicity following UV damage by conditioned medium which arrests cell replication (186).

The mutant was hypermutable at 3 loci ($6TG^r$, oua^r and DT^r) when UV-induced mutability are compared with V79 cells. Spontaneous mutation rates measured by two different methods also indicate that the mutant had an elevated spontaneous mutation rate at 3 loci. The thymidine auxotrophic revertant was not only normal in

UV-sensitivity but also normal in spontaneous and induced mutability. Therefore, it appears that a single gene controls both increased mutability and mutagen sensitivity. The results also indicate that nucleotide pools or thymidine auxotrophy are not correlated with UV-sensitivity and spontaneous or induced mutability. Furthermore, preliminary data showed that UV-induced mutability in the mutant was not reduced after conditioned medium treatment.

This study thus identified an aphidicolin-resistant mutant that is mutagen sensitive and has elevated spontaneous and induced mutability, a phenomenon never reported before for any mammalian cell mutant. The molecular basis for the defect of this mutant is not known. We speculate that it is defective in certain enzyme related to both DNA replication and repair, the defect of the enzyme apparently rendered both replication and repair error prone.

Further biochemical analysis of the properties of aph^r-4-2 polymerase to define their relationship to the observed mutator activities is warranted. The significance of the results presented in this dissertation seems to be: a) mutator mutants can be isolated by aphidicolin; b) aph^r-4-2 cells exhibit a generalized mutator mutation; c) the existence of mutator activity in this instance is not related to pyrimidine metabolism; d) ultraviolet light sensitivity and mutator activity in aph^r mutants are possibly controlled by a single gene; e) evidence supporting the existence of an error-prone repair pathway; and f) new biochemical and biological means to elucidate the role of DNA polymerase(s) in somatic cell mutagenesis.

APPENDICES

APPENDIX A

DEOXYRIBONUCLEOSIDE TRIPHOSPHATE EXTRACTIONS AND MEASUREMENTS

APPENDIX A

DEOXYRIBONUCLEOSIDE TRIPHOSPHATE EXTRACTIONS AND MEASUREMENTS

Extractions

Deoxyribonucleoside triphosphates (dNTP) were extracted from partially synchronized Chinese hamster V79 fibroblasts according to North et al., (192) and Warren (283). Fibroblasts from aph^r-4-2, aph^r-4-R2 and wild type V79 cell lines were grown in flasks (75 cm^2, Corning Glass Work) until confluency. Twenty-four hours before cell harvesting, cells were released from confluency with 0.01% trypsin, counted and 3-4 x 10^6 cells were plated on to 3 plates (9 cm, Corning Glass Works) for each cell strain in duplicate sets. After 24 hours of incubation at 37°C in growth medium supplemented with 5% FCS, the cells were rinsed twice with ice-cold phosphate-buffered saline (PBS) and were harvested with a rubber policeman in ice-cold 60% methanol. The cells in 3 plates of each strain were pooled into a test tube and the volume was adjusted to approximately 10 ml with 60% methanol, then were placed in -20°C overnight. Another set of wild type cells served as a control by additions of dCTP/dTTP or dGTP/dATP (concentrations were adjusted to 25-50 pmole per tube) to each pooled methanol-cell suspension immediately following cell harvesting. This group served as the control to test whether endogenous dNTP survived the extraction procedures. The methanol extracts were centrifuged (29,000 x G, Survall) and the supernatant was separated from the precipitate. The precipitate was saved for determination of DNA contents and this supernatant was

lyophilized to dryness in a lyophilizer (Virtis Co.). Each dry material was treated with 3 ml of 0.5 N perchloric acid to allow for enzyme inactivation at $4^{o}C$ for half hour. The content in each tube was neutralized to pH=7 with 1.5N potassium hydroxide (0.705 ml) at $4^{o}C$ for another half hour. The potassium chloride in the solution was discarded after centrifugation and the supernatant was again lyophilized to dryness. The dry powder was dissolved in 1 ml distilled water, centrifuged and the resulting supernatant was stored in 4 aliquots at $-20^{o}C$ for future dNTP measurements.

Measurements

The amounts of dNTP in each extract of 1 ml sample were measured by incorporations of the complementary ^{3}H-dNTP into the defined copolymer of poly(dA-dT)-poly(dA-dT) or poly(dI-dC)-poly(dI-dC) and E. coli DNA polymerase I.

1. Reaction Reagents

a. dATP and dTTP Assays.

The poly(dA-dT)-poly(dA-dT) copolymer of 1515 µM (10 optical density unit per ml) was diluted to 200 µM in 10 mM Tris-HCl (pH 8.0) and 20 mM KCl solution. For each sample of dATP assay, a total of 0.09 ml of the reaction reagents was prepared by mixing 0.04 ml of 50 mM glycine-KOH (pH 9.2), 0.02 ml of 75 mM $MgCl_2$, 0.02 ml of 200 µM poly(dA-dT)-poly(dA-dT) copolymer and 0.01 ml of 10 µM ^{3}H-dTTP (2.5 µci/ml). For dTTP assay, 0.01 ml of 10 µM ^{3}H-dATP (7.5 µci/ml) was used instead of ^{3}H-dTTP.

b. dGTP and dCTP Assay.

The poly(dI-dC)-poly(dI-dC) copolymer of 10 O.D./ml was diluted to 71.6 μM in the same solution as previously described. For each sample of dGTP assay, a total of 0.09 ml of the reaction reagents was prepared with 0.04 ml of 50 mM Tris-HCl (pH 8.0), 0.01 ml of 100 mM $MgCl_2$, 0.02 ml of 71.6 μM of poly(dI-dC)-poly(dI-dC) and 0.01 ml of 10 μM ^{3}H-dCTP (25 μci/ml). For dCTP assay, 0.01 ml of 10 μM ^{3}H-dGTP (7.5 μci/ml) was used.

2. Escherichia coli DNA-polymerase I

One part of the DNA-polymerase I (4545 units/ml) was diluted to 9.09 units/ml in a solution containing 25 parts of 1 M Tris-HCl (pH 8.0), 5 parts of 100 mM dithiothreitol, 5 parts of 10 mg/ml bovia serum albumin and 419 parts of distilled water. Generally, 0.5 ml of the above solution was made at 4°C immediately before dNTP assay.

3. Assay Procedures

For each set of assays, a standard curve was obtained from known amounts of the dNTP in question. Each sample (0.01-0.08 ml) and reaction components (0.09 ml of reaction reagents and 0.01 ml of DNA polymerase solution) were mixed at 4°C and the final volume was adjusted to 0.2 ml with distilled water. The reaction tubes were then transfered to a 37°C waterbath. After incubation for 35 (for dATP, dTTP assays) or 40 (for dGTP, dCTP assays) minutes, the reaction was stopped by transferring the tube to an ice bath and additions of 3 ml of ice-cold 10% trichloroacetic acid with 1% sodium pyrophosphate (TCA-NaPPi) solution to each

tube. Thirty minutes later, each mixture was filtered through TCA-NaPPi-presoaked Whatman GF/C filters (2.4 cm, Whatman Ltd., England) under sampling-manifold vacuum (Millipore, Model 3025). Each tube and filter were rinsed 4 times with TCA-NaPPi solution, and twice with ice-cold 95% ethanol. The filters were then dried under a heat lamp. The dried filters were placed in scintillation vials containing 8 ml of a toluene-based counting fluid [12 gm of PPO-2,5diphenyloxozole, 0.3 gm of POPOP-1,4-bis-[2-(5-phenyloxazolyl)]-benzene and 3.0 L of toluene]. The radioactivity was measured in a Beckman LS 9000 liquid scintillation spectrophotometer. The amount of dNTP was calculated from the slope of the standard curve and the results were expressed as pmoles dNTP per µg DNA.

DNA Measurements

The DNA content of methanol precipitates was determined according to the procedure described by Giles and Myers (87). The precipitates were suspended in 1 ml of distilled water and sonicated with a Branson sonifier (Sonifier Cell Disruptor, Heat Systems Co., New York) at a power level of 25 for 3-5 seconds. Equal volumes (0.5 ml) of the suspended sample and 20% perchloric acid were mixed and to this 1 ml mixture, 1 ml of 4% diphenylamine in glacial acetic acid (freshly prepared) and 0.05 ml of acetaldehyde (1.6 mg/ml) were added. The reaction mixture was further mixed with a vortex-genie (Model K-500-G Scientific Industries, Inc., Massachusetts) and incubated overnight at room temperature. The optical density of each sample was measured at 595 nm against a reagent blank on a spectrophotometer (Stasar II, Gilford Instrument Laboratory, Inc., Ohio). The results were calculated from a standard curve determined simultaneously by using calf thymus DNA (between 5-40 µg). The results were expressed as µg DNA.

APPENDIX B

EXPRESSIONS OF OUA^r, DT^r, AND 6TG^r MUTANTS

APPENDIX B EXPRESSIONS OF OUA^r, DT^r AND 6TG^r MUTANTS

Expt. No.	Cell lines	UV J/m^2	Survival (%)	Expression time (days)	Resistant Cells/10^6 Survivors (No. of Mutants) Oua^r	DT^r	6TG^r	Induced 6TG^r
1	V79	0	100	4	0 (0)			
	aph^r-4	0	100		1 (4)			
	V79	14	20		17 (31)			
	aph^r-4	14	12		105 (189)			
	V79	0		6	0 (0)	5 (11)		
	aph^r-4	0			0.4 (1)	9 (17)		
	V79	14			36 (50)	151 (284)		
	aph^r-4	14			191 (358)	348 (515)		
	V79	0		10	0 (0)		5 (16)	0
	aph^r-4	0			1 (3)		3 (14)	0
	V79	14			25 (50)		148 (395)	143
	aph^r-4	14			56 (111)		313 (772)	310
2	V79	0	100	8			99 (115)	0
	aph^r-4	0	100				6.5 (7)	0
	V79	4.2	92				230 (270)	131
	aph^r-4	4.2	78				134 (204)	127
	V79	8.4	61				401 (490)	302
	aph^r-4	8.4	43				293 (302)	286
	V79	12.6	31				540 (616)	441
	aph^r-4	12.6	21				347 (277)	341
	V79	16.8	11				679 (696)	580
	aph^r-4	16.8	7				494 (455)	488
	V79	0		10			92 (136)	0
	aph^r-4	0					5.3 (7)	0
	V79	4.2					209 (298)	117
	aph^r-4	4.2					136 (151)	131
	V79	8.4					298 (538)	206
	aph^r-4	8.4					232 (274)	227
	V79	12.6					532 (464)	440
	aph^r-4	12.6					290 (326)	285

Expt. No.	Cell lines	UV J/m^2	Survival (%)	Expression time (days)	Resistant Cells/10^6 Survivors (No. of Mutants)					
					Oua^r		DT^r		6TG^r	
2	V79	16.8							592	(673)
	aph^r-4	16.8							426	(409)
	V79	0		14					119	(211)
	aph^r-4	0							13	(17)
	V79	4.2							253	(445)
	aph^r-4	4.2							218	(261)
	V79	8.4							383	(713)
	aph^r-4	8.4							391	(456)
	V79	12.6							477	(794)
	aph^r-4	12.6							426	(512)
	V79	16.8							520	(898)
	aph^r-4	16.8							505	(755)
3	in situ		oua^r data were deleted due to no UV sensitivities in aph^r-4 (reversion)							
4	V79	0	100	10					22	(17)
	aph^r-4	0	100						8	(18)
	V79	7	61						369	(310)
	aph^r-4	7	38						380	(476)
	V79	14	15						514	(379)
	aph^r-4	14	6						522	(479)
	V79	0		12					11	(6)
	aph^r-4	0							5	(7)
	V79	7							238	(133)
	aph^r-4	7							269	(450)
	V79	14							430	(229)
	aph^r-4	14							479	(719)
	V79	0		14					17	(13)
	aph^r-4	0							4	(11)
	V79	7							376	(245)
	aph^r-4	7							359	(551)
	V79	14							495	(321)
	aph^r-4	14							562	(746)

Induced 6TG^r	
503	
	421
0	
	0
134	
	205
264	
	378
358	
	413
401	
	492
0	
	0
347	
	372
492	
	514
0	
	0
227	
	264
419	
	474
0	
	0
359	
	355
478	
	558

Expt. No.	Cell lines	UV J/m^2	Survival (%)	Expression time (days)	Resistant Cells/10^6 Survivors (No. of Mutants)							
					Oua^r		DT^r		$6TG^r$		Induced	$6TG^r$
5	V79	0	100	4	15	(17)						
	aph^r-4-2	0	100		11	(23)						
	V79	7	67		39	(78)						
	aph^r-4-2	7	37		281	(297)						
	V79	14	19		110	(97)						
	aph^r-4-2	14	5		392	(227)						
	V79	0		6	13	(18)						
	aph^r-4-2	0			24	(37)						
	V79	7			79	(158)						
	aph^r-4-2				222	(457)						
	V79	14			124	(262)						
	aph^r-4-2	14			286	(443)						
	V79	0		8	11	(27)			10	(20)	0	
	aph^r-4-2	0			22	(45)			6	(10)		0
	V79	7			81	(209)			335	(540)	325	
	aph^r-4-2	7			269	(761)			369	(685)		363
	V79	14			124	(342)			480	(828)	470	
	aph^r-4-2	14			347	(996)			471	(845)		465
	V79	0		11					13	(24)	0	
	aph^r-4-2	0							15	(24)		0
	V79	7							336	(596)	323	
	aph^r-4-2	7							518	(729)		503
	V79	14							471	(815)	458	
	aph^r-4-2	14							646	(867)		631
6	V79	0	100	3			13	(8)				
	aph^r-4-2	0	100				59	(41)				
	V79	8.4	41				67	(25)				
	aph^r-4-2	8.4	22				215	(68)				
	aph^r-4-2*	8.4	21				208	(52)				
	V79	0		5			5	(2)				
	aph^r-4-2	0					88	(54)				

Expt. No.	Cell lines	UV J/m^2	Survival (%)	Expression time (days)	Resistant Cells/10^6 Survivors (No. of Mutants) Oua^r		DT^r		6TG^r		Induced 6TG^r
6	V79	8.4					64	(24)			
	aph^r-4-2	8.4					305	(122)			
	aph^r-4-2*	8.4					432	(144)			
	V79	0		7			6	(2)	150	(185)	0
	aph^r-4-2	0					78	(62)	30	(40)	0
	V79	8.4					40	(10)	570	(469)	420
	aph^r-4-2	8.4					252	(182)	500	(601)	470
	aph^r-4-2*	8.4					483	(150)	270	(138)	240
	V79	0		9					150	(197)	0
	aph^r-4-2	0							30	(39)	0
	V79	8.4							490	(639)	340
	aph^r-4-2	8.4							320	(752)	290
	aph^r-4-2*	8.4							320	(232)	290
	V79	0		13					120	(128)	0
	aph^r-4-2	0							30	(33)	0
	V79	8.4							490	(499)	370
	aph^r-4-2	8.4							560	(599)	530
	aph^r-4-2*	8.4							340	(94)	310
	*with ½ µm aphidicolin 2 days after UV irradiation.										
7	V79	0	100	8	4	(9)	10	(11)	40	(78)	0
	aph^r-4-R2	0	100		1	(1)	4	(3)	26	(33)	0
	aph^r-4-2	0	100		4	(7)	55	(48)	19	(27)	0
	V79	12.6	11		41	(70)	107	(92)	517	(738)	477
	aph^r-4-R2	12.6	19		69	(59)	89	(36)	110	(277)	384
	aph^r-4-2	12.6	3		192	(303)	625	(494)	533	(703)	514
	aph^r-4-2*	12.6	-		131	(140)	535	(285)	329	(307)	310
	V79	0		10	4	(7)	9	(12)	39	(51)	0
	aph^r-4-R2	0			<1	(0)	2	(3)	15	(19)	0
	aph^r-4-2	0			10	(17)	53	(34)	13	(19)	0

Expt. No.	Cell lines	UV J/m^2	Survival (%)	Expression time (days)	Resistant Cells/10^6 Survivors (No. of Mutants) Oua^r	DT^r	6TG^r	Induced 6TG^r
7	V79	12.6			62 (99)	45 (60)	528 (700)	489
	aph^r-4-R2	12.6			119 (168)	42 (50)	449 (530)	434
	aph^r-4-2	12.6			208 (328)	389 (512)	523 (688)	510
	aph^r-4-2*	12.6			121 (103)	165 (141)	335 (286)	322
	V79	0		17	3 (8)	16 (19)	36 (72)	0
	aph^r-4-R2	0			<1 (1)	5 (4)	32 (44)	0
	aph^r-4-2	0			5 (11)	20 (24)	17 (29)	0
	V79	12.6			30 (54)	70 (57)	447 (665)	411
	aph^r-4-R2	12.6			118 (169)	46 (33)	587 (702)	555
	aph^r-4-2	12.6			157 (259)	435 (354)	513 (695)	496
	aph^r-4-2*	12.6			33 (8)	245 (30)	279 (57)	262

*with ½ μM Aphidicolin 3 days after UV irradiation.

8 delete - no linear induction of mutants v.s. UV dose

9					Oua^r -TdR	Oua^r +TdR(4 μM)	DT^r -TdR	DT^r +TdR(4 μM)
	V79	0	100	4	3(4)	2(2)	25(15)	9(5)
	aph^r-4-R2	0	100		1(2)	1(2)	6(4)	7(6)
	aph^r-4-2	0	100		8(8)	2(2)	142(95)	191(161)
	V79	4.2	99		15(21)	12(16)	57(39)	57(39)
	aph^r-4-R2	4.2	93		44(75)	33(43)	63(47)	85(56)
	aph^r-4-2	4.2	67		78(48)	59(69)	790(489)	735(427)
	V79	8.4	59		20(31)	20(28)	92(72)	-
	aph^r-R2	8.4	57		55(75)	51(59)	101(68)	186(107)
	aph^r-4-2	8.4	17		-	-	-	-

Expt. No.	Cell lines	UV_2 J/m^2	Survival (%)	Expression time (days)	TdR 2μM	Resistant Cells/10^6 Survivors (No. of Mutants) Oua^r	Induced Oua^r	6TG^r	Induced 6TG^r
10	V79	0	100	6	-	8 (12)	0	54 (42)	0
	aph^r-4-2	0	100		-	13 (18)	0	2 (16)	0
	V79	4.2	82		-	12 (18)	4	169 (126)	112
	aph^r-4-2	4.2	67		-	110 (136)	97	189 (117)	187
	V79	8.4	74		-	32 (48)	24	244 (184)	187
	aph^r-4-2	8.4	20		-	149 (167)	136	323 (181)	321
	V79	0	100		+	3 (2)	0	66 (22)	0
	aph^r-4-2	0	100		+	8 (16)	0	2 (20)	0
	V79	4.2	94		+	22 (23)	19	185 (95)	119
	aph^r-4-2	4.2	67		+	101 (137)	23	218 (148)	216
	V79	8.4	74		+	31 (29)	27	236 (112)	170
	aph^r-4-2	8.4	18		+	127 (164)	119	272 (175)	270
	V79	0		8	-			41 (16)	0
	aph^r-4-2	0			-			9 (7)	0
	V79	4.2			-			144 (79)	103
	aph^r-4-2	4.2			-			290 (180)	281
	V79	8.4			-			223 (79)	182
	aph^r-4-2	8.4			-			453 (211)	444
	V79	0			+			52 (20)	0
	aph^r-4-2	0			+			16 (13)	0
	V79	4.2			+			163 (108)	111
	aph^r-4-2	4.2			+			371 (204)	355
	V79	8.4			+			265 (143)	213
	aph^r-4-2	8.4			+			454 (301)	438

Expt. No.	Cell lines	UV_2 J/m	Survival (%)	Expression time (days)	Resistant Cells/10^6 Survivors (No. of Mutants)			
					Oua^r (4 μM TdR)	DT^r	Induced Oua^r	Induced DT^r
11	V79	0	100	6	6(3)	8(2)	0	0
(Liquid	aph^r-4-2	0	100		6(4)	96(8)	0	0
Holding)	V79	8.4	50		44(8)	85(31)	38	77
	aph^r-4-2	8.4	22		70(49)	316(111)	62	220
	V79	0		10	11(2)		0	
	aph^r-4-2	0			21(7)		0	
	V79	8.4			66(13)		55	
	aph^r-4-2	8.4			158(65)		137	
	With Liquid Holding (12 hours)							
	V79	0	61	6	38(6)	63(5)	0	0
	aph^r-4-2	0	100		9(8)	71(31)	0	0
	V79	8.4	81		52(14)	52(7)	14	0
	aph^r-4-2	8.4	15		78(39)	445(111)	69	374
	V79	0		10	19(4)		0	
	aph^r-4-2	0			27(14)		0	
	V79	8.4			30(7)		11	
	aph^r-4-2	8.4			252(139)		224	
12	V79	0	100	6	1(2)	1(1)	0	0
	aph^r-4-2	0	100		31(23)	112(42)	0	0
	V79	16.8	32		72(40)	148(41)	71	147
	aph^r-4-2	8.4	23		144(138)	608(240)	113	496
	Liquid Holding (12 Hours)							
	V79	0	87	6	2(2)	13(9)	0	0
	aph^r-4-2	0	82		24(19)	56(22)	0	0
	V79	16.8	36		65(26)	117(23)	63	104
	aph^r-4-2	8.4	26		250(165)	896(270)	226	840

APPENDIX C

A HYPOTHESIS ON COMPARISONS OF SPONTANEOUS MUTATION RATES OBTAINED FROM $\underline{P}o$ ESTIMATIONS

APPENDIX C

A HYPOTHESIS ON COMPARISONS OF SPONTANEOUS MUTATION RATES OBTAINED FROM Po ESTIMATIONS

Mutations in mammalian somatic cells are rare events and are cell-division dependent. Mutation rates estimated from the fluctuation analysis vary from laboratories to laboratories and from experiments to experiments in the same cell line (Tables 2 and 19). Table 19 shows results from two experiments in which the $6TG^r$ mutation rate was estimated from Po calculation in V79 cells. In experiment No. 1, the mutation rate was calculated when $6TG^r$ mutants were selected at low cell-divisions per replicate culture. It had a Po of 0.9. In experiment No. 2, the $6TG^r$ mutants were selected at high cell-divisions. It also had the same value of Po as the previous experiment. However, the mutations rates estimated from the Po are different. Because the cell line was the same in both experiments, it can be assumed that the expression of $6TG^r$ mutants was the same in both experiments, and the expression time contributed a similar effect in Po calculation. Furthermore, because the values of variance/mean in both experiments were not the same and were greater than one, the mutations were from random events. The differences in both experiments were the numbers of replicate cultures (C) and of cell divisions. To account for the difference in the observed mutation rates, a hypothesis is formulated and is described here.

The limitations of mutation rate estimations using the Po calculation in the fluctuation analysis are the Po value and the numbers of cell divisions. The limits of

Table 19. Spontaneous Mutation Rates ($6TG^r$) in Chinese Hamster V79 Cells.[1]

		Experiment No. 1	Experiment No. 2
Replicate cultures (C):		20	54
Cell numbers per culture:			
initial (No)		2	1
final (N_f) ($x10^6$)		0.63	3
division (d) ($x10^6$)		0.91	4.3
$\underline{P}o$		0.9	0.91
$6TG^r$ (total)		$(7)^2$	$(11)^3$
Per culture:	range	0-5	0-4
	variance	1.33	.53
	mean	0.35	.204
	var./mean	4	2.6
$\ln(1/\underline{P}o)$		0.1	0.097
CaN_f/C		0.23	0.113
Mutation rate per cell per division:		1.1×10^{-7}	2.3×10^{-8}
R^4		0.016	0.019

1. See Table 14.
2. Low cell-density (1×10^5 per plate) selections without TPA.
3. High cell-density ($1\text{-}1.5 \times 10^6$ per plate) selections with TPA. See footnote 2 on Table 16.
4. R = [(Mut. rate - AL)/(AH - AL)], where AL and AH are low and high limits of mutation rate estimation respectively. See APPENDIX C.

Po are 1/C and (C-1)/C, where C is the number of replicate cultures. No Po calculation can be made when all the replicate cultures contain either at least one mutant (Po = 0) or no mutants (Po = 1). For a given Po value, the calculated mutation rate will be high if cell-division is low. Therefore, the mutation rate calculation is then limited by the experimental design, i.e., the numbers of replicate cultures and of cell divisions. For example, an experiment with replicate cultures of 20 and a cell division of 9×10^5/culture (Table 19) allows the Po mutation rate estimation of 3.3×10^{-6} to 5.6×10^{-8} [see Appendix D, in which N = number of replicate cultures; D = cell divisions; MH and ML are high and low mean: ln(1/Po), respectively, when Po = 1/N and (N-1)/N; and AH and AL are high and low limits of mutation rates calculated from MH and ML respectively using A = ln(1/Po)/D]. Thus, these two numbers are the limits for a mutation rate estimation using N = 20 and $D = 9 \times 10^5$. In experiments using N = 54 and $D = 4.3 \times 10^6$, limits of the mutation rate estimation would be 9.5×10^{-7} and 5×10^{-9}.

If we further assume the mutation (non-mutator mutation) is a rare event and arises from a similar mechanism in V79 cells, the mutation rate calculated should be in the lower range of the experimental limits. If we then define the R is the relative range and is the ratio of the difference between the observed mutation rate and the low limit to the experiment mutation rate limits, the R value should have a low and constant value (exceptions are when D is a very large number). For example, the R value in Expt. No. 1 is 0.016 $[(1.1 \times 10^{-7}-5.6 \times 10^{-8})/(3.3 \times 10^{-6}-5.6 \times 10^{-8})]$ and it is similar to that of 0.019 in Expt. No. 2 $[(2.3 \times 10^{-8}-5 \times 10^{-9})/9.5 \times 10^{-7}-5 \times 10^{-9})]$. Although the mutation rate obtained in Expt. No. 1 was 1.2×10^{-7} and was higher than that of Expt. No. 2 (2.3×10^{-8}), the R value suggests that these two mutation rates represent a similar mutation.

If the mutation is not a rare event, such as a mutator mutation, the R value should be higher than that of "wild-type" V79 cells (Table 20). Conversely, an

Table 20. The Relative Mutation Rate in Aph^r-Mutants and V79 Cells[a]

Locus		Wild Type V79	Wild Type CHO	aph^r-4-2	aph^r-4-R2	aph^r-4-RP4	aph^r-4-RP5
Oua^r	Experiment No. 1	0.027	0.11	0.286(I)	0	-	0.713
	Experiment No. 2	0.164	-	0.512(F), 0.786(S)	0.076(F), 0.05(I)	-	-
DT^r	Experiment No. 1	0.012	-	0.362	0.008	-	0.801
	Experiment No. 2	0.079	-	0.599	-	-	-
6TG^r[b]	Experiment No. 1	0.016[c.d.]	0.04[c]	0.09[d]	-	0.078[d]	-
	Experiment No.2	0.019	-	0.261	-	-	-

a. Numbers presented in this table are ratio (R) = (observed spon. mutation rate - the low experimental limit)/(the experimental limits). The observed mutation rates are from Tables 14,15,16, except those of CHO cell line are from Ref. 175. Mutation rate = ln(1/Po)/d. For the experimental limits, see APPENDIX D, where N = replicate cultures (C); D = cell divisions per culture (d); MH and ML are high and low means [M = ln(1/po)] at Po = 1/C and Po = (C-1)/C respectively; AH and AL are high and low theoretical mutation rates respectively. The experimental limits are the difference between AH and AL.

b. High cell-density selection with TPA.

c. Low cell-density selection without TPA.

d. Initial cell per replicate culture is 2.

antimutator mutation should have a lower value than that of the wild-type V79 cells. The R values calculated from mutation rate estimations in aph^r-4-2 and aph^r-4-R2 generally agree with this hypothesis (Tables 14-16,20). A series of experiments has been carried out to verify this hypothesis.

The hypothesis postulated that simple comparisons between the values of mutation rates obtained through the Po estimation may be misled. The significance of this hypothesis would be that a) it includes experimental limits in comparisons of mutation rates; b) the R values allow a statistical analysis (e.g., paired t test) for the significance of the observed mutation rate.

APPENDIX D

EXPERIMENTAL LIMITS OF THE FLUCTUATION ANALYSIS USING $\underline{P}o$ ESTIMATIONS

APPENDIX D

EXPERIMENTAL LIMITS OF THE FLUCTUATION ANALYSIS USING Po ESTIMATIONS

#	D	SB	AR	AL	AL
15	0.1E 05	2.66	3.27E-33	0.37	3.73E-05
15	0.2E 05	2.66	3.13E-33	0.37	3.35E-35
15	0.3E 05	2.66	3.69E-34	0.37	3.23E-35
15	0.4E 35	2.55	3.56E-34	0.37	3.17E-35
15	0.5E 05	2.65	3.53E-34	0.37	3.14E-35
15	0.6E 05	2.66	3.44E-34	0.37	3.12E-35
15	0.7E 05	2.66	3.39E-34	0.37	3.13E-35
15	0.8E 35	2.66	3.33E-34	0.37	3.87E-36
15	0.9E 05	2.66	3.30E-34	0.37	3.79E-36
15	0.1E 36	2.65	3.27E-34	0.37	3.73E-36
15	0.2E 36	2.66	3.13E-34	0.37	3.35E-36
15	0.3E 36	2.65	3.89E-35	0.37	3.23E-36
15	0.4E 35	2.66	3.56E-35	0.37	3.17E-36
15	0.5E 36	2.65	3.53E-35	0.37	3.14E-36
15	0.6E 36	2.66	3.44E-35	0.37	3.12E-36
15	0.7E 36	2.65	3.39E-35	0.37	3.13E-36
15	0.9E 36	2.65	3.33E-05	0.37	3.87E-37
15	0.9E 36	2.66	3.30E-35	0.37	3.79E-37
15	0.1E 37	2.66	3.27E-05	0.37	3.73E-37
15	0.2E 37	2.66	3.13E-35	0.37	3.35E-37
15	0.3E 37	2.66	3.89E-36	0.37	3.23E-37
15	0.4E 37	2.65	3.56E-36	0.37	3.17E-37
15	0.5E 07	2.65	3.53E-36	0.37	3.14E-37
15	0.6E 37	2.65	3.44E-06	0.07	3.12E-37
15	0.7E 07	2.65	3.39E-36	0.37	3.13E-37
15	0.9E 37	2.56	3.33E-36	0.07	3.87E-38
15	0.9E 37	2.66	3.30E-36	0.37	3.79E-39
15	0.1E 38	2.65	3.27E-36	0.37	3.73E-38
15	0.2E 38	2.65	3.13E-36	0.37	3.35E-38
15	0.3E 08	2.66	3.69E-37	0.07	3.23E-38
15	0.4E 38	2.55	3.55E-37	0.37	3.17E-39
15	0.5E 38	2.66	3.53E-07	0.07	3.14E-38
15	0.6E 38	2.55	3.44E-37	0.07	3.12E-38
15	0.7E 38	2.65	3.39E-37	0.37	3.13E-38
15	0.9E 38	2.65	3.33E-37	0.37	3.87E-39
15	0.9E 38	2.66	3.30E-37	0.37	3.79E-39
20	0.1E 35	3.00	3.30E-03	0.05	3.53E-35
20	0.2E 35	3.00	3.15E-33	0.05	3.25E-35
20	0.3E 35	3.00	3.13E-33	0.05	3.17E-35
20	0.4E 35	3.00	3.75E-34	0.05	3.12E-35
20	0.5E 35	3.00	3.53E-34	0.05	3.13E-35
20	0.6E 35	3.00	3.53E-34	0.05	3.83E-36
20	0.7E 35	3.00	3.43E-34	0.05	3.71E-36
20	0.8E 35	3.00	3.37E-34	0.05	3.62E-36
20	0.9E 35	3.00	3.33E-04	0.05	3.55E-36
20	0.1E 36	3.00	3.33E-34	0.05	3.53E-36
20	0.2E 36	3.00	3.15E-04	0.05	3.25E-36
20	0.3E 36	3.00	3.13E-34	0.05	3.17E-36
20	0.4E 36	3.00	3.75E-05	0.05	3.12E-36
20	0.5E 36	3.00	3.63E-05	0.05	3.13E-36
20	0.6E 36	3.00	3.53E-05	0.05	3.83E-37
20	0.7E 36	3.00	3.43E-35	0.05	3.71E-37
20	0.9E 36	3.00	3.37E-35	0.05	3.62E-37
20	0.9E 36	3.00	3.33E-05	0.05	3.55E-37
20	0.1E 37	3.00	3.33E-05	0.05	3.53E-37
20	0.2E 37	3.00	3.15E-35	0.05	3.25E-37
20	0.3E 37	3.00	3.13E-35	0.05	3.17E-37
20	0.4E 37	3.00	3.75E-36	0.05	3.12E-37
20	0.5E 37	3.00	3.50E-36	0.05	3.13E-37
20	0.5E 37	3.00	3.53E-36	0.05	3.83E-39

20	3.7E 37	3.00	3.63E-36	0.35	3.71E-38
20	3.8E 37	3.00	3.37E-36	0.35	3.62E-38
20	3.9E 37	3.00	3.13E-36	0.35	3.55E-38
20	0.1E 38	3.00	3.30E-36	0.35	3.50E-38
20	0.2E 38	3.00	3.15E-36	0.35	3.25E-38
20	0.3E 38	3.00	3.10E-36	0.35	3.17E-38
20	0.4E 38	3.00	3.75E-37	0.35	3.12E-38
20	0.5E 38	3.00	3.53E-37	0.35	3.13E-38
20	0.6E 38	3.00	3.50E-37	0.35	3.83E-39
20	0.7E 38	3.00	3.63E-37	0.35	3.71E-39
20	0.8E 38	3.00	3.37E-37	0.35	3.62E-39
20	0.9E 38	3.00	3.33E-37	0.35	3.56E-39
25	0.1E 35	3.22	3.32E-33	0.33	3.03E-35
25	0.2E 35	3.22	3.16E-33	0.33	3.23E-35
25	0.3E 35	3.22	3.11E-33	0.33	3.13E-35
25	0.4E 35	3.22	3.80E-34	0.33	3.10E-35
25	0.5E 35	3.22	3.69E-34	0.33	3.83E-36
25	0.6E 35	3.22	3.59E-34	0.33	3.67E-36
25	0.7E 35	3.22	3.48E-34	0.33	3.57E-36
25	0.8E 35	3.22	3.40E-34	0.33	3.53E-36
25	0.9E 35	3.22	3.36E-34	0.33	3.48E-36
25	0.1E 36	3.22	3.34E-34	0.33	3.43E-36
25	0.2E 36	3.22	3.16E-34	0.33	3.23E-36
25	0.3E 36	3.22	3.11E-34	0.33	3.13E-36
25	0.4E 36	3.22	3.80E-35	0.33	3.13E-36
25	0.5E 36	3.22	3.69E-35	0.33	3.83E-37
25	0.6E 36	3.22	3.59E-05	0.33	3.67E-37
25	0.7E 36	3.22	3.48E-35	0.33	3.57E-37
25	0.8E 36	3.22	3.40E-05	0.33	3.53E-37
25	0.9E 36	3.22	3.36E-35	0.33	3.48E-37
25	0.1E 37	3.22	3.32E-35	0.33	3.43E-07
25	0.2E 37	3.22	3.16E-35	0.33	3.23E-37
25	0.3E 37	3.22	3.11E-35	0.33	3.13E-37
25	0.4E 37	3.22	3.80E-36	0.33	3.13E-37
25	0.5E 37	3.22	3.69E-36	0.33	3.83E-38
25	0.6E 37	3.22	3.59E-36	0.33	3.67E-38
25	0.7E 37	3.22	3.48E-36	0.33	3.57E-38
25	0.8E 37	3.22	3.40E-36	0.33	3.53E-38
25	0.9E 37	3.22	3.36E-36	0.33	3.48E-38
25	0.1E 38	3.22	3.32E-36	0.33	3.43E-38
25	0.2E 38	3.22	3.16E-36	0.33	3.23E-38
25	0.3E 38	3.22	3.11E-36	0.33	3.13E-38
25	0.4E 38	3.22	3.80E-37	0.33	3.13E-38
25	0.5E 38	3.22	3.64E-37	0.33	3.83E-39
25	0.6E 38	3.22	3.54E-37	0.33	3.67E-39
25	0.7E 38	3.22	3.46E-37	0.33	3.57E-39
25	0.8E 38	3.22	3.40E-37	0.33	3.53E-39
25	0.9E 38	3.22	3.36E-37	0.33	3.48E-39
30	0.1E 35	3.03	3.34E-33	0.33	3.33E-35
30	0.2E 35	3.03	3.17E-33	0.33	3.15E-35
30	0.3E 35	3.03	3.11E-33	0.33	3.13E-35
30	0.4E 35	3.03	3.85E-34	0.33	3.75E-36
30	0.5E 35	3.03	3.63E-34	0.33	3.63E-36
30	0.6E 35	3.03	3.57E-34	0.33	3.53E-36
30	0.7E 35	3.03	3.49E-34	0.33	3.43E-36
30	0.8E 35	3.03	3.42E-34	0.33	3.37E-36
30	0.9E 35	3.03	3.36E-34	0.33	3.33E-36
30	0.1E 36	3.03	3.34E-34	0.33	3.33E-36
30	0.2E 36	3.03	3.17E-34	0.33	3.15E-36
30	0.3E 36	3.03	3.11E-34	0.33	3.13E-36
30	0.4E 36	3.03	3.85E-05	0.33	3.75E-37

30	0.5E 06	3.40	3.38E-35	0.33	3.63E-37
30	0.6E 06	3.40	3.57E-35	0.33	3.53E-37
30	0.7E 06	3.40	3.49E-35	0.33	3.43E-37
30	0.8E 06	3.40	3.42E-35	0.33	3.37E-37
30	0.9E 06	3.40	3.38E-35	0.33	3.33E-37
30	0.1E 07	3.40	3.33E-35	0.33	3.33E-37
30	0.2E 07	3.40	3.17E-05	0.33	3.15E-37
30	0.3E 07	3.40	3.11E-35	0.33	3.13E-37
30	0.4E 07	3.40	3.05E-36	0.33	3.75E-38
30	0.5E 07	3.40	3.59E-36	0.03	3.63E-38
30	0.5E 07	3.40	3.37E-36	0.33	3.53E-38
30	0.7E 07	3.40	3.49E-36	0.33	3.43E-38
30	0.8E 07	3.40	3.42E-36	0.33	3.37E-38
30	0.9E 07	3.40	3.38E-36	0.33	3.33E-38
30	0.1E 08	3.40	3.34E-36	0.33	3.33E-38
30	0.2E 08	3.40	3.17E-36	0.33	3.15E-38
30	0.3E 08	3.40	3.11E-36	0.03	3.13E-38
30	0.4E 08	3.40	3.05E-07	0.33	3.75E-39
30	0.5E 08	3.40	3.68E-37	0.33	3.63E-39
30	0.6E 08	3.40	3.57E-37	0.33	3.53E-39
30	0.7E 08	3.40	3.49E-37	0.33	3.43E-39
30	0.8E 08	3.40	3.42E-37	0.33	3.37E-39
30	0.9E 08	3.40	3.38E-37	0.33	3.33E-39
35	0.1E 05	3.56	3.36E-33	0.33	3.33E-35
35	0.2E 05	3.55	3.18E-33	0.33	3.15E-35
35	0.3E 05	3.56	3.12E-33	0.33	3.13E-35
35	0.4E 05	3.56	3.09E-34	0.33	3.75E-36
35	0.5E 05	3.55	3.71E-34	0.33	3.63E-36
35	0.6E 05	3.56	3.59E-34	0.33	3.53E-36
35	0.7E 05	3.56	3.51E-34	0.33	3.43E-36
35	0.8E 05	3.56	3.44E-34	0.33	3.37E-36
35	0.9E 05	3.56	3.40E-34	0.33	3.33E-36
35	0.1E 06	3.55	3.36E-34	0.33	3.33E-36
35	0.2E 06	3.55	3.18E-34	0.33	3.15E-36
35	0.3E 06	3.56	3.12E-34	0.33	3.13E-36
35	0.4E 06	3.55	3.09E-35	0.33	3.75E-37
35	0.5E 06	3.56	3.71E-05	0.03	3.63E-37
35	0.6E 06	3.56	3.59E-35	0.33	3.50E-37
35	0.7E 06	3.56	3.51E-05	0.33	3.43E-37
35	0.8E 06	3.56	3.44E-35	0.33	3.37E-37
35	0.9E 06	3.56	3.40E-35	0.33	3.33E-37
35	0.1E 07	3.56	3.36E-05	0.33	3.30E-37
35	0.2E 07	3.56	3.18E-35	0.33	3.15E-37
35	0.3E 07	3.55	3.12E-35	0.33	3.13E-37
35	0.4E 07	3.56	3.09E-36	0.03	3.75E-38
35	0.5E 07	3.55	3.71E-36	0.33	3.63E-38
35	0.6E 07	3.56	3.59E-36	0.33	3.50E-38
35	0.7E 07	3.56	3.51E-06	0.03	3.43E-38
35	0.8E 07	3.56	3.44E-36	0.33	3.37E-38
35	0.9E 07	3.56	3.40E-36	0.33	3.33E-38
35	0.1E 08	3.56	3.36E-36	0.33	3.30E-38
35	0.2E 08	3.55	3.18E-36	0.33	3.15E-38
35	0.3E 08	3.56	3.12E-36	0.33	3.13E-38
35	0.4E 08	3.56	3.09E-07	0.33	3.75E-39
35	0.5E 08	3.56	3.71E-37	0.33	3.63E-39
35	0.6E 08	3.56	3.59E-37	0.33	3.53E-39
35	0.7E 08	3.55	3.51E-37	0.33	3.43E-39
35	0.8E 08	3.55	3.44E-07	0.33	3.37E-39
35	0.9E 08	3.56	3.40E-07	0.33	3.33E-39
40	0.1E 05	3.69	3.37E-33	0.33	3.33E-35
40	0.2E 05	3.69	3.18E-33	0.03	3.15E-35

40	0.3E 35	3.69	3.12E-33	0.33	3.13E-35
40	0.4E 35	3.69	3.92E-34	0.33	3.75E-36
40	0.5E 35	3.69	3.74E-34	0.33	3.63E-36
40	0.6E 35	3.69	3.51E-34	0.33	3.53E-36
40	0.7E 35	3.69	3.53E-34	0.33	3.43E-36
40	0.8E 35	3.69	3.46E-34	0.33	3.37E-36
40	0.9E 35	3.69	3.41E-34	0.33	3.33E-36
40	0.1E 35	3.69	3.37E-34	0.33	3.33E-36
40	0.2E 35	3.69	3.10E-34	0.33	3.15E-36
40	0.3E 35	3.69	3.12E-34	0.33	3.13E-36
40	0.4E 35	3.69	3.92E-35	0.33	3.75E-37
40	0.5E 35	3.69	3.74E-05	0.33	3.63E-37
40	0.6E 35	3.69	3.51E-35	0.33	3.53E-37
40	0.7E 35	3.69	3.53E-35	0.33	3.43E-37
40	0.8E 35	3.69	3.46E-35	0.33	3.37E-37
40	0.9E 35	3.69	3.41E-35	0.33	3.33E-37
40	0.1E 37	3.69	3.37E-35	0.33	3.33E-37
40	0.2E 37	3.69	3.10E-35	0.33	3.15E-37
40	0.3E 37	3.69	3.12E-35	0.33	3.13E-37
40	0.4E 37	3.69	3.92E-36	0.33	3.75E-38
40	0.5E 37	3.69	3.74E-36	0.33	3.63E-38
40	0.6E 37	3.69	3.51E-36	0.33	3.53E-38
40	0.7E 37	3.69	3.53E-36	0.33	3.43E-38
40	0.8E 37	3.69	3.46E-36	0.33	3.37E-38
40	0.9E 37	3.69	3.41E-36	0.33	3.33E-38
40	0.1E 38	3.69	3.37E-36	0.33	3.33E-38
40	0.2E 38	3.69	3.10E-36	0.33	3.15E-38
40	0.3E 38	3.69	3.12E-36	0.33	3.13E-08
40	0.4E 38	3.69	3.92E-37	0.33	3.75E-39
40	0.5E 38	3.69	3.74E-37	0.33	3.63E-39
40	0.6E 38	3.69	3.51E-37	0.33	3.53E-39
40	0.7E 38	3.69	3.53E-37	0.33	3.43E-39
40	0.8E 38	3.69	3.46E-37	0.33	3.37E-39
40	0.9E 38	3.69	3.41E-37	0.33	3.33E-39
45	0.1E 35	3.81	3.39E-33	0.32	3.23E-35
45	0.2E 35	3.81	3.13E-33	0.32	3.13E-35
45	0.3E 35	3.81	3.13E-33	0.32	3.67E-36
45	0.4E 35	3.81	3.95E-34	0.32	3.53E-36
45	0.5E 35	3.81	3.75E-34	0.32	3.43E-36
45	0.6E 35	3.81	3.53E-34	0.32	3.33E-36
45	0.7E 35	3.81	3.54E-34	0.32	3.29E-36
45	0.8E 35	3.81	3.48E-34	0.32	3.25E-36
45	0.9E 35	3.81	3.42E-34	0.32	3.22E-36
45	0.1E 36	3.81	3.38E-34	0.32	3.23E-36
45	0.2E 35	3.81	3.19E-34	0.32	3.13E-36
45	0.3E 35	3.81	3.13E-34	0.32	3.67E-37
45	0.4E 36	3.81	3.95E-35	0.32	3.53E-37
45	0.5E 35	3.81	3.76E-05	0.32	3.43E-37
45	0.6E 35	3.81	3.53E-35	0.32	3.33E-37
45	0.7E 35	3.81	3.54E-35	0.32	3.29E-37
45	0.8E 35	3.81	3.48E-35	0.32	3.25E-37
45	0.9E 35	3.81	3.42E-35	0.32	3.22E-37
45	0.1E 37	3.81	3.38E-35	0.32	3.23E-37
45	0.2E 37	3.81	3.19E-35	0.32	3.13E-37
45	0.3E 37	3.81	3.13E-35	0.32	3.67E-38
45	0.4E 37	3.81	3.95E-36	0.32	3.53E-38
45	0.5E 37	3.81	3.76E-36	0.32	3.43E-38
45	0.6E 37	3.81	3.53E-36	0.32	3.33E-38
45	0.7E 37	3.81	3.54E-36	0.32	3.29E-38
45	0.8E 37	3.81	3.48E-36	0.32	3.25E-38
45	0.9E 37	3.81	3.42E-36	0.32	3.22E-38

45	0.1E 33	3.81	3.33E-36	0.32	3.23E-38
45	0.2E 33	3.81	3.13E-36	0.32	3.13E-38
45	0.3E 33	3.81	3.13E-36	0.32	3.67E-39
45	0.4E 33	3.81	3.95E-37	0.32	3.53E-39
45	0.5E 33	3.81	3.75E-37	0.32	3.43E-39
45	0.6E 33	3.81	3.63E-37	0.32	3.33E-39
45	0.7E 33	3.81	3.54E-37	0.32	3.23E-39
45	0.8E 33	3.81	3.49E-37	0.32	3.25E-39
45	0.9E 33	3.81	3.47E-37	0.32	3.22E-39
50	0.1E 35	3.91	3.37E-33	0.32	3.23E-35
50	0.2E 35	3.91	3.23E-33	0.32	3.13E-35
50	0.3E 35	3.91	3.13E-33	0.32	3.67E-36
50	0.4E 35	3.91	3.94E-36	0.32	3.53E-36
50	0.5E 35	3.91	3.78E-36	0.32	3.43E-36
50	0.6E 35	3.91	3.55E-36	0.32	3.33E-36
50	0.7E 35	3.91	3.56E-36	0.32	3.23E-36
50	0.8E 35	3.91	3.49E-36	0.32	3.25E-36
50	0.9E 35	3.91	3.43E-36	0.32	3.22E-36
50	0.1E 35	3.91	3.39E-36	0.32	3.23E-36
50	0.2E 35	3.91	3.23E-36	0.32	3.13E-36
50	0.3E 35	3.91	3.13E-36	0.32	3.67E-37
50	0.4E 35	3.91	3.98E-35	0.32	3.53E-37
50	0.5E 35	3.91	3.73E-35	0.32	3.43E-37
50	0.6E 35	3.91	3.55E-35	0.32	3.33E-37
50	0.7E 35	3.91	3.56E-05	0.32	3.23E-37
50	0.8E 35	3.91	3.49E-35	0.32	3.25E-37
50	0.9E 36	3.91	3.43E-05	0.32	3.22E-37
50	0.1E 37	3.91	3.39E-35	0.32	3.23E-37
50	0.2E 37	3.91	3.23E-05	0.32	3.13E-37
50	0.3E 37	3.91	3.13E-05	0.32	3.67E-38
50	0.4E 37	3.91	3.98E-36	0.32	3.53E-38
50	0.5E 37	3.91	3.78E-36	0.32	3.43E-38
50	0.6E 37	3.91	3.65E-36	0.32	3.33E-38
50	0.7E 37	3.91	3.56E-36	0.32	3.23E-38
50	0.8E 37	3.91	3.49E-36	0.32	3.25E-38
50	0.9E 37	3.91	3.43E-36	0.32	3.22E-38
50	0.1E 38	3.91	3.39E-36	0.32	3.23E-38
50	0.2E 38	3.91	3.23E-36	0.32	3.13E-38
50	0.3E 38	3.91	3.13E-36	0.32	3.67E-39
50	0.4E 38	3.91	3.98E-37	0.32	3.53E-39
50	0.5E 38	3.91	3.78E-37	0.32	3.43E-39
50	0.6E 38	3.91	3.65E-07	0.32	3.33E-39
50	0.7E 38	3.91	3.56E-37	0.32	3.23E-39
50	0.8E 38	3.91	3.49E-37	0.32	3.25E-39
50	0.9E 38	3.91	3.43E-37	0.32	3.22E-39
55	0.1E 35	4.01	3.43E-33	0.32	3.23E-35
55	0.2E 35	4.01	3.23E-33	0.32	3.13E-35
55	0.3E 35	4.01	3.13E-33	0.32	3.67E-36
55	0.4E 35	4.01	3.13E-33	0.32	3.53E-36
55	0.5E 35	4.01	3.98E-36	0.32	3.43E-36
55	0.6E 35	4.01	3.67E-36	0.32	3.33E-36
55	0.7E 35	4.01	3.57E-36	0.32	3.29E-36
55	0.8E 35	4.01	3.53E-36	0.32	3.25E-36
55	0.9E 35	4.01	3.45E-36	0.32	3.22E-36
55	0.1E 36	4.01	3.43E-36	0.32	3.23E-36
55	0.2E 35	4.01	3.23E-36	0.32	3.13E-36
55	0.3E 35	4.01	3.13E-36	0.32	3.67E-37
55	0.4E 35	4.01	3.13E-36	0.32	3.53E-37
55	0.5E 35	4.01	3.80E-35	0.32	3.43E-37
55	0.6E 35	4.01	3.67E-35	0.32	3.33E-37
55	0.7E 36	4.01	3.57E-35	0.32	3.29E-37

55	0.3E 35	0.31	0.53E-35	0.32	0.25E-37
55	0.9E 35	0.31	0.45E-35	0.32	0.22E-37
55	0.1E 37	0.31	0.40E-35	0.02	0.20E-37
55	0.2E 37	0.31	0.23E-05	0.32	0.13E-37
55	0.3E 37	0.31	0.13E-05	0.02	0.67E-39
55	0.4E 37	0.31	0.10E-05	0.02	0.53E-39
55	0.5E 37	0.31	0.30E-36	0.32	0.43E-39
55	0.6E 37	0.31	0.57E-36	0.02	0.33E-39
55	0.7E 37	0.31	0.57E-36	0.02	0.29E-39
55	0.8E 37	0.31	0.50E-36	0.02	0.25E-39
55	0.9E 37	0.01	0.45E-36	0.02	0.22E-39
55	0.1E 38	0.31	0.80E-36	0.32	0.23E-39
55	0.2E 38	0.31	0.20E-36	0.32	0.13E-39
55	0.3E 38	0.01	0.13E-36	0.32	0.67E-39
55	0.4E 38	0.31	0.13E-36	0.32	0.53E-39
55	0.5E 38	0.31	0.33E-37	0.32	0.43E-39
55	0.6E 38	0.31	0.67E-37	0.32	0.33E-39
55	0.7E 38	0.31	0.57E-37	0.32	0.29E-39
55	0.8E 38	0.01	0.50E-37	0.32	0.25E-39
55	0.9E 38	0.01	0.45E-37	0.32	0.22E-39
60	0.1E 35	0.09	0.41E-33	0.32	0.20E-35
60	0.2E 35	0.09	0.20E-33	0.32	0.13E-35
60	0.3E 35	0.39	0.14E-33	0.32	0.67E-36
60	0.4E 35	0.39	0.10E-33	0.32	0.53E-36
60	0.5E 35	0.39	0.32E-34	0.32	0.43E-36
60	0.6E 35	0.39	0.63E-34	0.32	0.33E-36
60	0.7E 35	0.39	0.58E-34	0.02	0.29E-36
60	0.8E 35	0.39	0.31E-34	0.32	0.25E-36
60	0.9E 35	0.39	0.85E-34	0.32	0.22E-36
60	0.1E 36	0.39	0.41E-34	0.32	0.43E-36
60	0.2E 36	0.39	0.25E-34	0.32	0.13E-36
60	0.3E 36	0.39	0.14E-34	0.32	0.67E-37
60	0.4E 36	0.39	0.13E-34	0.32	0.53E-37
60	0.5E 36	0.39	0.82E-35	0.32	0.43E-37
60	0.6E 36	0.09	0.58E-05	0.32	0.33E-37
60	0.7E 36	0.09	0.53E-35	0.32	0.29E-37
60	0.8E 36	0.03	0.51E-35	0.32	0.25E-37
60	0.9E 36	0.39	0.45E-05	0.32	0.22E-07
60	0.1E 37	0.39	0.41E-35	0.32	0.20E-37
60	0.2E 37	0.03	0.20E-05	0.32	0.10E-07
60	0.3E 37	0.39	0.14E-05	0.32	0.67E-08
60	0.4E 37	0.39	0.13E-35	0.32	0.53E-08
60	0.5E 37	0.39	0.92E-36	0.32	0.43E-38
60	0.6E 37	0.39	0.63E-36	0.32	0.33E-38
60	0.7E 37	0.09	0.58E-36	0.32	0.29E-38
60	0.8E 37	0.39	0.51E-36	0.32	0.25E-38
60	0.9E 37	0.39	0.45E-36	0.32	0.22E-38
60	0.1E 38	0.39	0.41E-36	0.32	0.23E-38
60	0.2E 38	0.09	0.20E-36	0.02	0.13E-38
60	0.3E 38	0.39	0.14E-36	0.32	0.67E-39
60	0.4E 38	0.39	0.13E-36	0.32	0.53E-39
60	0.5E 38	0.09	0.92E-37	0.32	0.43E-39
60	0.6E 38	0.39	0.63E-37	0.32	0.33E-39
60	0.7E 38	0.39	0.58E-37	0.32	0.29E-39
60	0.8E 38	0.39	0.51E-37	0.32	0.25E-39
60	0.9E 38	0.39	0.45E-37	0.32	0.22E-39
65	0.1E 35	0.17	0.44E-33	0.32	0.20E-35
65	0.2E 35	0.17	0.41E-33	0.32	0.13E-35
65	0.3E 35	0.17	0.14E-33	0.32	0.67E-36
65	0.4E 35	0.17	0.13E-33	0.32	0.53E-36
65	0.5E 35	0.17	0.83E-34	0.32	0.43E-36

65	3.5E 35	0.17	3.53E-36	0.32	3.33E-36
65	3.7E 35	0.17	3.63E-36	0.32	3.29E-36
65	3.8E 35	0.17	3.52E-36	0.32	3.25E-36
65	0.9E 35	0.17	3.46E-36	0.32	3.22E-36
65	3.1E 35	0.17	3.42E-36	0.32	3.23E-36
65	3.2E 35	0.17	3.21E-36	0.32	3.13E-36
65	3.3E 35	0.17	3.19E-36	0.32	3.67E-37
65	0.4E 35	0.17	3.13E-36	0.32	3.53E-37
65	3.5E 35	0.17	3.33E-35	0.32	3.43E-37
65	0.6E 35	0.17	3.69E-35	0.32	3.33E-37
65	3.7E 35	0.17	3.63E-35	0.32	3.29E-37
65	3.8E 35	0.17	3.52E-35	0.32	3.25E-37
65	3.9E 35	0.17	3.46E-35	0.32	3.22E-37
65	3.1E 37	0.17	3.42E-35	0.32	3.23E-37
65	3.2E 37	0.17	3.21E-35	0.32	3.13E-37
65	3.3E 37	0.17	3.19E-35	0.32	3.67E-38
65	3.4E 37	0.17	3.13E-35	0.32	3.53E-38
65	3.5E 37	0.17	3.33E-36	0.32	3.43E-38
65	3.6E 37	0.17	3.69E-36	0.32	3.33E-38
65	3.7E 37	0.17	3.60E-36	0.32	3.29E-38
65	0.8E 37	0.17	0.52E-36	0.02	3.25E-38
65	3.9E 37	0.17	3.46E-36	0.02	3.22E-38
65	3.1E 38	0.17	3.42E-36	0.02	3.23E-38
65	0.2E 38	0.17	3.21E-36	0.02	3.13E-38
65	3.3E 38	0.17	3.19E-36	0.32	3.67E-39
65	3.4E 38	0.17	3.13E-36	0.32	3.53E-39
65	0.5E 38	0.17	3.33E-07	0.02	3.43E-39
65	0.6E 38	0.17	3.59E-37	0.32	3.33E-39
65	0.7E 38	0.17	3.63E-37	0.02	3.29E-39
65	0.8E 38	0.17	3.52E-37	0.02	3.25E-39
65	0.9E 38	0.17	3.46E-37	0.32	3.22E-39
70	0.1E 35	0.25	3.42E-03	0.31	3.13E-35
70	0.2E 35	0.25	3.21E-03	0.01	3.53E-36
70	0.3E 35	0.25	3.19E-03	0.31	3.33E-36
70	0.4E 35	0.25	3.11E-03	0.31	3.25E-36
70	3.5E 35	0.25	3.85E-36	0.01	3.22E-36
70	3.6E 35	0.25	3.71E-36	0.31	3.17E-36
70	3.7E 35	0.25	3.61E-36	0.31	3.14E-36
70	0.8E 35	0.25	3.53E-36	0.01	3.12E-36
70	0.9E 35	0.25	3.47E-36	0.01	3.11E-36
70	0.1E 35	0.25	3.42E-36	0.01	3.13E-36
70	0.2E 35	0.25	3.21E-36	0.01	3.53E-37
70	3.3E 35	0.25	3.19E-36	0.01	3.33E-37
70	0.4E 35	0.25	3.11E-36	0.01	3.25E-37
70	0.5E 36	0.25	3.85E-35	0.01	3.22E-37
70	3.6E 35	0.25	3.71E-35	0.31	3.17E-37
70	3.7E 35	0.25	3.61E-35	0.31	3.14E-37
70	3.9E 35	0.25	3.53E-35	0.01	3.12E-37
70	3.9E 35	0.25	3.47E-35	0.31	3.11E-37
70	3.1E 37	0.25	3.42E-35	0.31	3.10E-37
70	3.2E 37	0.25	3.21E-35	0.31	3.53E-33
70	3.3E 37	0.25	3.19E-35	0.31	3.33E-33
70	3.4E 37	0.25	3.11E-35	0.31	3.25E-38
70	3.5E 37	0.25	3.85E-36	0.31	3.23E-38
70	3.6E 37	0.25	3.71E-36	0.31	3.17E-33
70	0.7E 37	0.25	3.61E-36	0.31	3.14E-38
70	3.8E 37	0.25	3.53E-36	0.31	3.12E-33
70	3.9E 37	0.25	3.47E-36	0.31	3.11E-38
70	3.1E 38	0.25	3.42E-36	0.31	3.13E-38
70	3.2E 38	0.25	3.21E-36	0.31	3.53E-39
70	3.3E 38	0.25	3.19E-36	0.31	3.33E-39

70	3.4E 33	0.25	3.11E-36	0.31	3.25E-33
70	3.5E 33	0.25	3.35E-37	0.31	3.23E-33
70	3.6E 33	0.25	3.71E-37	0.31	3.17E-33
70	3.7E 33	0.25	3.91E-37	0.31	3.14E-33
70	3.8E 33	0.25	3.53E-37	0.31	3.12E-33
70	3.9E 33	0.25	3.07E-37	0.31	3.11E-33
75	3.1E 35	0.32	3.63E-33	0.31	3.13E-35
75	3.2E 35	0.32	3.22E-33	0.31	3.53E-36
75	3.3E 35	0.32	3.19E-33	0.31	3.33E-36
75	3.4E 35	0.32	3.11E-33	0.31	3.25E-36
75	0.5E 35	0.32	3.36E-36	0.31	3.23E-36
75	0.6E 35	0.32	3.72E-36	0.01	3.17E-36
75	0.7E 35	0.32	3.62E-36	0.31	3.16E-36
75	0.8E 35	0.32	3.54E-36	0.31	3.12E-36
75	0.9E 35	0.32	3.43E-36	0.31	3.11E-36
75	0.1E 36	0.32	3.43E-36	0.01	3.13E-36
75	0.2E 36	0.32	3.22E-36	0.01	3.53E-37
75	0.3E 36	0.32	3.19E-36	0.01	3.33E-37
75	0.4E 36	0.32	3.11E-36	0.01	3.25E-37
75	3.5E 35	0.32	3.36E-35	0.31	3.23E-37
75	3.6E 35	0.32	3.72E-35	0.31	3.17E-37
75	0.7E 35	0.32	3.62E-35	0.31	3.16E-37
75	3.9E 36	0.32	3.54E-35	0.01	3.12E-37
75	3.9E 35	0.32	3.40E-05	0.01	3.11E-37
75	3.1E 37	0.32	3.43E-05	0.01	3.13E-37
75	3.2E 37	0.32	3.22E-35	0.31	3.53E-39
75	3.3E 37	0.32	3.19E-35	0.31	3.33E-38
75	3.4E 37	0.32	3.11E-35	0.31	3.25E-38
75	0.5E 37	0.32	3.86E-36	0.01	3.23E-38
75	3.6E 37	0.32	3.72E-36	0.01	3.17E-38
75	3.7E 37	0.32	3.62E-36	0.01	3.16E-38
75	3.8E 37	0.32	3.54E-36	0.01	3.12E-38
75	3.9E 37	0.32	3.43E-36	0.31	3.11E-38
75	3.1E 33	0.32	3.43E-36	0.31	3.13E-38
75	3.2E 33	0.32	3.22E-36	0.31	3.53E-39
75	3.3E 33	0.32	3.19E-36	0.31	3.33E-39
75	3.4E 03	0.32	3.11E-36	0.01	3.25E-39
75	3.5E 03	0.32	3.36E-37	0.01	3.23E-39
75	3.6E 03	0.32	3.72E-37	0.01	3.17E-39
75	3.7E 33	0.32	3.62E-37	0.01	3.16E-39
75	3.8E 33	0.32	3.54E-37	0.01	3.12E-39
75	3.9E 33	0.32	3.40E-37	0.01	3.11E-39
80	3.1E 35	0.33	3.44E-33	0.01	3.13E-35
80	3.2E 35	0.33	3.22E-03	0.31	3.50E-36
80	3.3E 35	0.33	3.15E-03	0.31	3.33E-36
80	3.4E 35	0.33	3.11E-03	0.01	3.25E-36
80	3.5E 35	0.33	3.88E-36	0.01	3.23E-36
80	3.6E 35	0.33	3.73E-36	0.01	3.17E-36
80	3.7E 35	0.33	3.63E-36	0.01	3.16E-36
80	3.8E 35	0.33	3.55E-36	0.01	3.12E-36
80	3.9E 35	0.33	3.69E-36	0.31	3.11E-36
80	0.1E 36	0.33	3.44E-36	0.01	3.13E-36
80	0.2E 36	0.33	3.22E-36	0.01	3.53E-37
80	0.3E 35	0.33	3.15E-36	0.31	3.33E-37
80	0.4E 35	0.33	3.11E-36	0.31	3.25E-37
80	3.5E 35	0.33	3.38E-35	0.31	3.23E-37
80	3.6E 35	0.33	3.73E-05	0.31	3.17E-37
80	3.7E 35	0.33	3.63E-35	0.31	3.16E-37
80	3.8E 36	0.33	3.55E-05	0.31	3.12E-37
80	3.9E 35	0.33	3.49E-35	0.31	3.11E-37
80	0.1E 37	0.33	3.44E-35	0.01	3.13E-37

80	3.2E 37	0.38	3.22E-35	0.31	3.53E-38
80	3.3E 37	0.38	3.15E-05	0.31	3.33E-38
80	3.4E 37	0.38	3.11E-35	0.31	3.25E-38
80	3.5E 37	0.38	3.08E-36	0.31	3.23E-38
80	3.6E 07	0.38	3.73E-36	0.01	3.17E-38
80	3.7E 37	0.38	3.53E-36	0.31	3.14E-38
80	3.8E 37	0.38	3.55E-36	0.01	3.12E-38
80	3.9E 37	0.38	3.49E-36	0.31	3.11E-38
80	3.1E 38	0.38	3.44E-36	0.31	3.13E-38
80	3.2E 38	0.38	3.22E-36	0.01	3.50E-39
80	3.3E 38	0.38	3.15E-06	0.31	3.33E-39
80	3.4E 38	0.38	3.11E-06	0.01	3.25E-39
80	3.5E 38	0.38	3.08E-37	0.01	3.23E-39
80	3.6E 38	0.38	3.73E-07	0.01	3.17E-39
80	3.7E 38	0.38	3.63E-37	0.01	3.14E-39
80	3.8E 38	0.38	3.55E-37	0.31	3.12E-39
80	3.9E 38	0.38	3.49E-37	0.01	3.11E-39
85	3.1E 35	0.44	3.44E-33	0.31	3.13E-35
85	3.2E 35	0.44	3.22E-33	0.31	3.53E-36
85	3.3E 35	0.44	3.15E-33	0.31	3.33E-36
85	3.4E 35	0.44	3.11E-33	0.31	3.25E-36
85	3.5E 05	0.44	3.39E-34	0.31	3.23E-36
85	3.6E 35	0.44	3.74E-34	0.31	3.17E-06
85	3.7E 35	0.44	3.53E-34	0.01	3.14E-36
85	3.8E 35	0.44	3.55E-34	0.31	3.12E-36
85	3.9E 35	0.44	3.49E-34	0.31	3.11E-36
85	3.1E 36	0.44	3.44E-34	0.31	3.13E-36
85	3.2E 36	0.44	3.22E-04	0.31	3.53E-37
85	3.3E 05	0.44	3.15E-04	0.01	3.33E-37
85	3.4E 35	0.44	3.11E-34	0.31	3.25E-37
85	3.5E 35	0.44	3.89E-05	0.31	3.23E-37
85	3.6E 35	0.44	3.74E-35	0.31	3.17E-37
85	3.7E 35	0.44	3.53E-05	0.31	3.14E-37
85	3.8E 36	0.44	3.55E-05	0.01	3.12E-37
85	3.9E 35	0.44	3.49E-05	0.31	3.11E-37
85	3.1E 37	0.44	3.44E-35	0.31	3.13E-37
85	3.2E 37	0.44	3.22E-05	0.01	3.53E-38
85	3.3E 37	0.44	3.15E-35	0.31	3.33E-38
85	3.4E 37	0.44	3.11E-35	0.31	3.25E-38
85	3.5E 37	0.44	3.39E-36	0.31	3.23E-38
85	3.6E 37	0.44	3.74E-36	0.31	3.17E-38
85	3.7E 37	0.44	3.53E-36	0.01	3.14E-38
85	3.8E 37	0.44	3.55E-36	0.31	3.12E-38
85	3.9E 37	0.44	3.49E-36	0.31	3.11E-38
85	3.1E 38	0.44	3.44E-36	0.31	3.13E-38
85	3.2E 38	0.44	3.22E-36	0.31	3.53E-39
85	3.3E 38	0.44	3.15E-36	0.31	3.33E-39
85	3.4E 38	0.44	3.11E-36	0.31	3.25E-39
85	3.5E 38	0.44	3.39E-37	0.31	3.23E-39
85	3.6E 38	0.44	3.74E-37	0.31	3.17E-39
85	3.7E 38	0.44	3.63E-37	0.31	3.14E-39
85	3.8E 38	0.44	3.55E-37	0.31	3.12E-39
85	3.9E 38	0.44	3.49E-37	0.31	3.11E-39
90	3.1E 35	0.53	3.45E-33	0.31	3.13E-35
90	3.2E 35	0.53	3.22E-33	0.31	3.53E-36
90	3.3E 35	0.53	3.15E-33	0.31	3.33E-36
90	3.4E 35	0.53	3.11E-33	0.31	3.45E-36
90	3.5E 35	0.53	3.40E-34	0.31	3.23E-36
90	3.6E 35	0.53	3.75E-34	0.01	3.17E-36
90	3.7E 35	0.53	3.54E-34	0.31	3.14E-36
90	3.8E 35	0.53	3.56E-34	0.31	3.12E-36

90	0.9E 35	0.55	3.53E-34	0.01	3.11E-36
90	0.1E 36	0.55	3.45E-34	0.01	3.13E-36
90	0.2E 36	0.55	3.22E-34	0.01	3.53E-37
90	0.3E 36	0.55	3.15E-34	0.01	3.33E-37
90	0.4E 36	0.55	3.11E-34	0.01	3.25E-37
90	0.5E 06	0.55	3.09E-35	0.01	3.23E-37
90	0.6E 36	0.55	3.75E-35	0.01	3.17E-37
90	0.7E 36	0.55	3.69E-35	0.01	3.19E-37
90	0.8E 36	0.55	3.56E-35	0.01	3.12E-37
90	0.9E 36	0.55	3.53E-35	0.01	3.11E-37
90	0.1E 37	0.55	3.05E-05	0.01	3.13E-37
90	0.2E 37	0.55	3.42E-35	0.01	3.53E-38
90	0.3E 37	0.55	3.15E-35	0.01	3.33E-38
90	0.4E 37	0.55	3.11E-35	0.01	3.25E-38
90	0.5E 37	0.55	3.09E-36	0.01	3.23E-38
90	0.6E 37	0.55	3.75E-36	0.01	3.17E-38
90	0.7E 37	0.55	3.69E-36	0.01	3.19E-38
90	0.8E 37	0.55	3.56E-36	0.01	3.12E-38
90	0.9E 37	0.55	3.53E-36	0.01	3.11E-38
90	0.1E 38	0.55	3.05E-06	0.01	3.13E-38
90	0.2E 38	0.55	3.22E-06	0.01	3.53E-39
90	0.3E 38	0.55	3.15E-36	0.01	3.33E-39
90	0.4E 38	0.55	3.11E-36	0.01	3.25E-39
90	0.5E 38	0.55	3.03E-37	0.01	3.23E-39
90	0.6E 38	0.55	3.75E-37	0.01	3.17E-39
90	0.7E 38	0.55	3.69E-37	0.01	3.19E-39
90	0.8E 38	0.55	3.56E-37	0.01	3.12E-39
90	0.9E 38	0.55	3.53E-37	0.01	3.11E-39
95	0.1E 05	0.55	3.05E-33	0.01	3.13E-35
95	0.2E 05	0.55	3.23E-33	0.01	3.53E-36
95	0.3E 35	0.55	3.15E-33	0.01	3.33E-36
95	0.4E 35	0.55	3.11E-33	0.01	3.25E-36
95	0.5E 35	0.55	3.03E-34	0.01	3.23E-36
95	0.6E 35	0.55	3.76E-34	0.01	3.17E-36
95	0.7E 35	0.55	3.69E-34	0.01	3.19E-36
95	0.8E 35	0.55	3.57E-34	0.01	3.12E-36
95	0.9E 35	0.55	3.53E-34	0.01	3.11E-36
95	0.1E 06	0.55	3.05E-34	0.01	3.13E-36
95	0.2E 35	0.55	3.23E-04	0.01	3.53E-37
95	0.3E 35	0.55	3.15E-34	0.01	3.33E-37
95	0.4E 35	0.55	3.11E-34	0.01	3.25E-37
95	0.5E 06	0.55	3.01E-05	0.01	3.23E-37
95	0.6E 35	0.55	3.76E-35	0.01	3.17E-37
95	0.7E 35	0.55	3.65E-35	0.01	3.19E-37
95	0.8E 35	0.55	3.57E-05	0.01	3.12E-37
95	0.9E 35	0.55	3.51E-35	0.01	3.11E-37
95	0.1E 37	0.55	3.45E-35	0.01	3.13E-37
95	0.2E 07	0.55	3.23E-35	0.01	3.53E-38
95	0.3E 37	0.55	3.15E-05	0.01	3.33E-38
95	0.4E 07	0.55	3.11E-35	0.01	3.25E-38
95	0.5E 37	0.55	3.01E-36	0.01	3.23E-38
95	0.6E 37	0.55	3.76E-36	0.01	3.17E-38
95	0.7E 37	0.55	3.65E-36	0.01	3.19E-38
95	0.8E 37	0.55	3.57E-06	0.01	3.12E-38
95	0.9E 37	0.55	3.51E-36	0.01	3.11E-38
95	0.1E 38	0.55	3.45E-36	0.01	3.13E-38
95	0.2E 38	0.55	3.23E-36	0.01	3.53E-39
95	0.3E 38	0.55	3.15E-36	0.01	3.33E-39
95	0.4E 38	0.55	3.11E-36	0.01	3.25E-39
95	0.5E 08	0.55	3.01E-37	0.01	3.23E-39
95	0.6E 38	0.55	3.76E-37	0.01	3.17E-39

95	0.7E 03	0.55	0.55E-07	0.01	0.11E-09
95	0.8E 03	0.55	0.57E-07	0.01	0.12E-09
95	0.9E 03	0.55	0.51E-07	0.01	0.11E-09
100	0.1E 05	0.61	0.66E-03	0.01	0.13E-05
100	0.2E 05	0.61	0.23E-03	0.01	0.53E-06
100	0.3E 05	0.61	0.15E-03	0.01	0.33E-06
100	0.4E 05	0.61	0.12E-03	0.01	0.25E-06
100	0.5E 05	0.61	0.92E-04	0.01	0.22E-06
100	0.6E 05	0.61	0.77E-04	0.01	0.17E-06
100	0.7E 05	0.61	0.66E-04	0.01	0.14E-06
100	0.8E 05	0.61	0.58E-04	0.01	0.12E-06
100	0.9E 05	0.61	0.51E-04	0.01	0.11E-06
100	0.1E 06	0.61	0.46E-04	0.01	0.13E-06
100	0.2E 06	0.61	0.23E-04	0.01	0.53E-07
100	0.3E 06	0.61	0.15E-04	0.01	0.33E-07
100	0.4E 06	0.61	0.12E-04	0.01	0.25E-07
100	0.5E 05	0.61	0.92E-05	0.01	0.22E-07
100	0.6E 05	0.61	0.77E-05	0.01	0.17E-07
100	0.7E 05	0.61	0.66E-05	0.01	0.14E-07
100	0.8E 06	0.61	0.58E-05	0.01	0.12E-07
100	0.9E 06	0.61	0.51E-05	0.01	0.11E-07
100	0.1E 07	0.61	0.46E-05	0.01	0.10E-07
100	0.2E 07	0.61	0.23E-05	0.01	0.53E-08
100	0.3E 07	0.61	0.15E-05	0.01	0.33E-08
100	0.4E 07	0.61	0.12E-05	0.01	0.25E-08
100	0.5E 07	0.61	0.92E-06	0.01	0.22E-08
100	0.6E 07	0.61	0.77E-06	0.01	0.17E-08
100	0.7E 07	0.61	0.66E-06	0.01	0.14E-08
100	0.8E 07	0.61	0.58E-06	0.01	0.12E-08
100	0.9E 07	0.61	0.51E-06	0.01	0.11E-08
100	0.1E 08	0.61	0.46E-06	0.01	0.10E-08
100	0.2E 08	0.61	0.23E-06	0.01	0.53E-09
100	0.3E 08	0.61	0.15E-06	0.01	0.33E-09
100	0.4E 08	0.61	0.12E-06	0.01	0.25E-09
100	0.5E 08	0.61	0.92E-07	0.01	0.22E-09
100	0.6E 08	0.61	0.77E-07	0.01	0.17E-09
100	0.7E 08	0.61	0.66E-07	0.01	0.14E-09
100	0.8E 08	0.61	0.58E-07	0.01	0.12E-09
100	0.9E 08	0.61	0.51E-07	0.01	0.11E-09

LIST OF REFERENCES

LIST OF REFERENCES

1. Ahmed, F.E., and R.B. Setlow. (1977) DNA repair in V79 cells treated with combination of UV radiation and N-acetoxy-2-acetylaminofluorene. Can. Res., 37:3414-3419.

2. Althaus, F.R., S.D. Lawrence, G.L. Sattler and H.C. Pitot. (1980) The effect of nicotinamide on unscheduled DNA synthesis in cultured hepatocytes. Biochem. Biophys. Res. Commun., 95:1063-1070.

3. Andrews, A.D., S.F. Barrett and J.H. Robbins. (1978) Xeroderma pigmentosum neurological abnormalities correlate with colony-forming ability after ultraviolet irradiation. Proc. Natl. Acad. Sci. USA. 75:1984-1988.

4. Arlett, C.F. (1977) Mutagenicity testing with V79 Chinese hamster cells. In: Handbook of Mutagenicity Test Procedures. Kilbey, Legator, Nichols, Ramel (eds.), pp. 175-192. Elsevier Scientific Pub., New York.

5. Arlett, C.T. and A.R. Lehmann. (1978) Human disorders showing increased sensitivity to the induction of genetic damage. Annu. Rev. Genet., 12:95-115.

6. Auerbach, C. (1976) In: Mutation Research, Problems, Results and Perspectives. Chapman and Hall, London.

7. Ayusawa, D., K. Iwata, T. Kozu, S. Ikegami, and T. Seno. (1979) Increase in dATP pool in aphidicolin-resistant mutants of mouse FM3A cells. Biochem. Biophys. Res. Commun., 91:946-954.

8. Ayusawa, D., K. Iwata and T. Seno. (1980) Isolation of mouse FM3A cell mutants with thermolabile thymidylate synthetase by resistance to aphidicolin. Biochem. Biophys. Res. Commun. 96:1654-1661.

9. Ayusawa, D., K. Iwata, S Ikegami and T. Seno. (1980) Reversal of aphidicolin-directed inhibition of DNA synthesis in vivo and in vitro by deoxyribonucleosides or their 5'-triphosphates. Cell Structure and Function, 5:147-154.

10. Ayusawa, D., K. Iwata, and T. Seno. (1980) Alteration of ribonucleotide reductase in aphidicolin-resistant mutants of mouse FM3A cells with associated resistance to arabinosyladenine and arabinosylcytosine. Somat. Cell Genet., 7, 27-42.

11. Baker, R.M., D.M. Brunette, R. Mankovitz, L. Thompson, G.F. Whitmore, L. Siminovitch and J.E. Till. (1974) Ouabain resistant mutants of mouse and hamster cells in culture. Cell, 1:9-22.

12. Bank, A., J.G. Mears, F. Ramirez. (1980) Disorders of human hemoglobin. Science, 207:486-492.

13. Benditt, E.P. (1977) The origin of atherosclerosis. Sci. Am., 236:74-85.

14. Benjamin, R.C. and D.M. Gill. (1980) ADP-Ribosylation in mammalian cell ghosts, dependence of poly(ADP-ribose) synthesis on strand breakage in DNA. J. Biol. Chem., 255:10493-10501.

15. Benjamin, R.C. and D.M. Gill. (1980) Poly(ADP-ribose) synthesis in vitro programmed by damaged DNA: A comparison of DNA molecules containing different types of strand breaks. J. Biol. Chem., 255:10502-10508.

16. Berger, N.A., K.K. Kurohara, S.J. Petzold and G.W. Sikorski. (1979) Aphidicolin inhibits eukaryotic DNA replication and repair-implications for involvement of DNA polymerase α in both processes. Biochem. Biophys. Res. Commun., 89:218-225.

17. Berger, N., G.W. Sikorski, S.J. Petzold and K.K. Kurohara. (1980) Defective poly(adenosine diphosphoribose) synthesis in xeroderma pigmentosum. Biochem., 2:289-293.

18. Berger, N.A. and G.W. Sikorski. (1980) Nicotinamide stimulates repair of DNA damage in human lymphocytes. Biochem. Biophys. Res. Commun., 95:67-72.

19. Berger, N.A. and G.W. Sikorski. (1981) Poly(adenosine-diphosphoribose) synthesis in ultraviolet-irradiated xeroderma pigmentosum cells reconstituted with micrococcus luteus UV endonuclease. Biochem., 20:3610-3614.

20. Bernstein, H. (1971) Reversion of frameshift mutations stimulated by lesions in early function genes of bacteriophage T4. Virology, 7:460-466.

21. Bessman, M.J., N. Muzyczka, M.F. Goodman and R.L. Schnaar. (1974) Studies on the biochemical basis of spontaneous mutation. II. The incorporation of a base and its analogue into DNA by wild type, mutator and antimutator DNA polymerase. J. Mol. Biol., 88:409-423.

22. Boveri, T. (1929) The origin of malignant tumors. (First published by Gustav Fisher, Jena, 1914.) Williams and Wilkins Co., Baltimore.

23. Bradley, M.O. and N.A. Sharkey. (1978) Mutagenicity of thymidine to cultured Chinese hamster cells. Nature, 274:607-608.

24. Brendel, M. and R.H. Haynes. (1973) Interactions among genes controlling sensitivity to radiation and alkylation in yeast. Molec. Gen. Genet., 125:197-216.

25. Brennard, J. and M. Fox. (1980) Excess thymidine is not mutagenic in Chinese hamster V79 fibroblasts. Cell Biol. Int. Rep., 4:923-932.

26. Bresler, S.E., M.I. Mosevitsky and L.G. Vyacheslavov. (1973) Mutation as possible replication errors in bacteria growing under conditions of thymine deficiency. Mut. Res., 19:281-293.

27. Brown, M., F.J. Bollum and L.M.S. Chang. (1981) Intracellular localization of DNA polymerase α. Proc. Natl. Acad. Sci. USA., 78:3049-3052.

28. Brundret, K.M., W. Dalziel, B. Hesp, J.A.J. Jarvis and S. Neidle. (1972) X-ray crystallographic determination of the structure of the antibiotic aphidicolin: a tetracyclic diterpenoid containing a new ring system. J. Chem. Soc. D. Chem. Commun., 1027-1028.

29. Buchwald, M. (1977) Mutagenesis at the ouabain-resistance locus in human diploid fibroblasts. Mut. Res. 44:401-412.

30. Bucknall, R.A., H. Moores, R. Simms and B. Hesp. (1973) Antiviral effects of aphidicolin, a new antibiotic product by Cephalosporium aphidicola. Antimicrob. Ag. Chemother., 4:294-298.

31. Burnet, F.M. (1974) Intrinsic mutagenesis: A genetic approach to ageing. Medical and Technical Publ. Co., Ltd., Lancaster, U.K.

32. Capizzi, R.L. and J.W. Jameson. (1973) A table for the estimation of the spontaneous mutation rate of cells in culture. Mut. Res., 17:147-148.

33. Carver, J.H., W.C. Dewey and L.E. Hopwood. (1976) X-ray-induced mutants resistant to 8-azaguanine. I. Effects of cell density and expression time. Mut. Res., 34:447-464.

34. Carver, J.H., S.O. Davidson, M.A. Drum, D.A. Hyre and J.P. Crowley. (1981) Sensitivity of a repair-deficient line of CHO-AT3-2 cells to induction of mutation or sister chromatid exchange by mitomycin C. Environ. Mut. Soc., 3:383 (Abstr.).

35. Caskey, C.T. and G.D. Kruth. (1979) The HPRT locus. Cell, 16:1-9.

36. Chang, C-c., C. Philipps, J.E. Trosko and R.W. Hart. (1977) Mutagenic and epigenetic influence of caffeine on the frequencies of UV-induced ouabain-resistant Chinese hamster cells. Mut. Res., 45:125-136.

37. Chang, C-c., S.M. D'Ambrosio, R. Schultz, J.E. Trosko and R.B. Setlow. (1978) Modification of UV-induced mutation frequencies in Chinese hamster cells by dose fractionation, cycloheximide and caffeine treatments. Mut. Res., 52:231-245.

38. Chang, C-c., J.E. Trosko and T. Akera. (1978) Characterization of ultra-violet light-induced ouabain-resistant mutations in Chinese hamster cells. Mut. Res., 51:85-98.

39. Chang, C-c., J.E. Trosko and T.W. Glover. (1979) Characterization of radiation-induced diphtheria toxin-resistant mutation in Chinese hamster V79 cells. Environ. Mut., 1:174 (Abstr.).

40. Chang, C-c., J.A. Boezi, S.T. Warren, C.L.K. Sabourin, P.K. Liu, L. Glatzer, and J.E. Trosko. (1981) Isolation and characterization of an UV-sensitive hypermutable aphidicolin-resistant Chinese hamster cell line. Somat. Cell Genet., 7:235-253.

41. Chasin, L.A. (1974) Mutations affecting adenine phosphoribosyl transferase activity in Chinese hamster cells. Cell, 2:37-42.

42. Chu, H.E.Y. and H.V. Malling. (1968) Mammalian cell genetics. II. Chemical induction of specific locus mutation in Chinese hamster cells in vitro. Proc. Natl. Acad. Sci. USA., 61:1306-1312.

43. Chu, E.H.Y., P. Brimer, K.B. Jacobson and E.V. Merriam. (1968) Mammalian cell genetics: I. Selection and characterization of mutation auxotrophic for L-glutamine or resistant to 8-azaguanine in Chinese hamster cells in vitro. Genetics, 62:359-377.

44. Chu, E.H.Y. (1971) Mammalian cell genetics: III. Characterization of X-ray induced forward mutations in Chinese hamster cell cultures. Mut. Res., 11:23-34.

45. Chu, E.H.Y., N.C. Sun and C-c. Chang. (1972) Induction of auxotrophic mutations by treatment of Chinese hasmter cells with 5 bromodeoxyuridine and black light. Proc. Natl. Acad. Sci. USA., 69:3459-3463.

46. Chu, E.H.Y. and S.S. Powell. (1976) Selective systems in somatic cell genetics. In: Advances in Human Genetics, H. Harris and K. Hirschorn (eds.), Vol. 7, pp. 189-258, Plenum Pub. Corp., New York.

47. Ciarrocchi, G., J.G. Jose and S. Linn. (1979) Further characterization of a cell-free system for measuring replicative and repair DNA synthesis with cultured human fibroblasts and evidence for the involvement of DNA polymerase α in DNA repair. Nucleic Acid Res., 7:1205-1219.

48. Clarkson, J. and D.L. Mitchell. (1979) The recovery of mammalian cells treated with methyl methanesulfonate, nitrogen mustard or UV light. II. The importance of DNA repair prior to the initiation of S phase. Mut. Res., 61:343-351.

49. Cleaver, J.E. and D. Bootsma (1975) Xeroderma pigmentosum: biochemical and genetic characteristics. Annu. Rev. Genet. 9:19-38.

50. Cleaver, J.E., J.I. William, L. Kapp and S.D. Park. (1978) Cell survival, excision repair and DNA replication in eukaryotic cells. In: DNA Repair Mechanisms, P.C. Hanawalt, E.C. Friedberg and C.F. Fox (eds.), Vol. 9, pp. 85-93, Academic Press, New York.

51. Cleaver, J.E. (1978) DNA repair and its coupling to DNA replication in eukaryotic cells. Biochim. et Biophys. Acta., 516:489-516.

52. Collier, R.J. (1975) Diphtheria toxin: mode of action and structure. Bacteriol. Rev., 39:54-85.

53. Comfort, A. (1964) Aging: The biology of senescence. Holt, New York.

54. Comings, D.E. (1973) A general theory of carcinogenesis. Proc. Natl. Acad. Sci. USA., 70:3324-3328.

55. Corran, J. (1969) Analysis of an apparent case of "gene-controlled mutational stability": The auxotrophic preemption of a specific growth requirement. Mut. Res., 7:287-295.

56. Cox, E.C. (1976) Bacterial mutator genes and the control of spontaneous mutation. Annu. Rev. Genet., 10:135-156.

57. Cozzarelli, N.R. (1977) The mechanism of action of inhibitors of DNA synthesis. Annu. Rev. Biochem., 46:641-668.

58. Craddock, V.M. and C.M. Ansley. (1979) Sequential changes in DNA polymerase α and β during diethylnitrosamine-induced carcinogenesis. Biochim. et Biophys. Acta., 564:15-22.

59. Dalziel, W., B. Hesp, K.M. Stevenson, and J.A.J. Jarvis. (1973) The structure and absolute configuration of the antibiotic aphidicolin: A tetracyclic diterpenoid containing a new ring system. J. Chem. Sol. Perkin. Trans., 1:2841-2851.

60. D'Ambrosio, S.M. and R.B. Setlow. (1978) On the presence of UV-endonuclease sensitive sites in daughter DNA of UV-irradiated mammalian cells. In: DNA Repair Mechanisms, P.C. Hanawalt, B.C. Friedberg and C.F. Cox, pp. 499-503, Acad. Press, New York.

61. Davidson, R.L. and E.R. Kaufman. (1978) Bromodeoxyuridine mutagenesis in mammalian cells is stimulated by thymidine and suppressed by deoxycytidine. Nature, 276:722-723.

62. Degnen, G.E. and E.C. Cox. (1974) Conditional mutator gene in Escherichia coli: Isolation, mapping and effector studies. J. Bacteriol., 117:477-487.

63. Doniger, J. (1978) DNA replication in ultraviolet light irradiated Chinese hamster cells: The nature of replicon inhibition and post-replication repair. J. Mol. Biol., 120:433-446.

64. Drake, J.W. (1966) Spontaneous mutations accumulating in bacteriophage T4 in the complete absence of DNA replication. Proc. Natl. Acad. Sci. USA., 55:738-743.

65. Drake, J.W. (1970) Spontaneous mutation. In: The Molecular Basis of Mutation, pp. 183-184, Holden-day, San Francisco.

66. Drake, J.W. (1973) The genetic control of spontaneous and induced mutation rates in bacteriophage T4. Genetics (Suppl.), 73:45-64.

67. Drake, J.W. and R.H. Baltz. (1976) The biochemistry of mutagenesis. Annu. Rev. Biochem., 45:11-37.

68. Draper, R.K., D. Chin, E. Eurey-Owens, I.E. Scheffler and M.I. Simon. (1979) Biochemical and genetic characterization of three hamster cell mutants resistant to diphtheria toxin. J. Cell Biol., 83:116-125.

69. Duncan, B.K. and B. Weiss. (1978) Uracil-DNA glycosylase mutants are mutators. In: DNA Repair Mechanisms, P.C. Hanawalt, E.C. Friedberg, C.F. Cox (eds.), ICN-UCLA Symposia on Molecular and Cellular Biology, Vol. 9, pp. 183-186, Academic Press, New York.

70. Durkacz, B.W., O. Omidiji, D.A. Gray and S. Shall. (1980) (ADP-ribose)n participates in DNA excision repair. Nature, 283:593-596.

71. Durkacz, B.W. and S. Shall. (1981) The step at which (ADP-ribose)n is involved in DNA repair. J. Supramol. Struc. Cellular Biochem. Suppl. 5, 10th Annual ICN-ICLA Symposia for Molecular and Cellular Biology, p. 183, (Abstr.).

72. Eagle, H. (1959) Amino acid metabolism in mammalian cell cultures. Science, 130:432-437.

73. Edwards, M.J. and A.M.R. Taylor. (1980) Unusual levels of (ADP-ribose)n and DNA synthesis in ataxia telangiectasia cells following γ-ray irradiation. Nature, 287:745-747.

74. Ehrlich, M. and R.Y-h. Wang. (1981) 5-Methylcytosine in eukaryotic DNA. Science, 212:1350-1357.

75. Elkind, M.M. (1978) DNA damage and mammalian cell killing. In: DNA Repair Mechanisms. P.C. Hanawalt, E.C. Friedberg and C.F. Cox (eds.), pp. 477-480, Academic Press, New York.

76. Elkind, M.M. and H. Sutton. (1960) Radiation response of mammalian cells grown in culture: I. Repair of X-ray damage in surviving Chinese hamster cells. Radiation Res., 13:556-693.

77. Elliott, A.Y., P. Cleveland, J. Cervenka, A.E. Castro, N. Stein, T.R. Hakala and E.E. Fraley. (1974) Characterization of a cell line from human transitional cell cancer of the urinary tract. J. Natl. Can. Inst., 53:1341-1349.

78. Emerit, I. and P. Cerutti. (1981) Clastogenic activity from Bloom's syndrome fibroblast cultures. Proc. Natl. Acad. Sci. USA, 78:1868-1872.

79 Failla, G. (1958) The aging process and carcinogenesis. Annu. N. Y. Acad. Sci., 71:1124-1140.

80. Ford, D.K. and G. Yerganian (1958) Observation on the chromosomes of Chinese hamster cells in tissue culture. J. Natl. Cancer Inst., 21:393-425.

81. Fowler, R.G., G.E. Degnen and E.C. Cox. (1974) Mutation specificity of a conditional Escherichia coli mutator, mutD5. Molec. Gen. Genet., 133:179-191.

82. Freese, E.B. and E. Freese. (1967) On the specificity of DNA polymerase. Proc. Natl. Acad. Sci. USA., 57:650-657.

83. Fuchs, R.R.P., J.F. Levevre, J. Pouyet and M.P. Daune. (1976) Comparative orientation of the fluorene residue in native DNA modified by N-acetoxy-N-2-acetylaminofluorene and two 7-halogen derivatives. Biochem., 15:3347-3351.

84. Generoso, W.M., M.D. Shelby and F.J. de Serrres (eds.). (1979) DNA Repair and Mutagenesis in Eukaryotes, Vol. 15, Plenum Press, New York.

85. George, D.L. and E.M. Witkin. (1975) Ultraviolet light-induced response of an mfd mutant of Escherichia coli B/r having a slow rate of dimer excision. Mut. Res., 28:347-354.

86. Giannelli, F., P.F. Benson, S.A. Pawsey and P.E. Polani. (1977) Ultraviolet light sensitivity and delayed DNA-chain maturation in Bloom's syndrome fibroblasts. Nature, 265:466-469.

87. Giles, K.W. and A. Myers. (1965) An improved diphenylamine method for the estimation of deoxyribonucleic acid. Nature, 106:93.

88. Gill, D.M. and R. Meren. (1978) ADP-ribosylation of membrane proteins catalyzed by cholera toxin: Basis of the activation of adenlylate cyclase. Proc. Natl. Acad. Sci. USA., 75:3050-3054.

89. Giulotto, E. and C. Mondello. (1981) Aphidicolin does not inhibit the repair synthesis of mitotic chromosomes. Biochem. Biophys. Res. Commun., 99:1287-1294.

90. Glickman, B.W. (1979) Spontaneous mutagenesis in Escherichia coli strains lacking 6-methyl adenine residues in their DNA. Mut. Res., 61:153-162.

91. Glickman, B.W. and M. Radman. (1980) Escherichia coli mutator mutants deficient in methylation-instructed DNA mismatch correction. Proc. Natl. Acad. Sci. USA., 77:1063-1067.

92. Glover, T.W., C. Chang, J.E. Trosko and S.S. Li. (1979) Ultraviolet light induction of diphtheria toxin-resistant mutants in normal and xeroderma pigmentosum human fibroblasts. Proc. Natl. Acad. Sci. USA., 76:3982-3986.

93. Goldstein, R. and R.B. Painter. (1981) Effect of caffeine on killing, mutation induction, DNA repair and DNA synthesis in cells treated with ethylnitrosourea. Environ. Mut. Soc., 3:397 (Abstr.).

94. Goodwin, P.M., P.J. Lewis, M.I. Davies, C.J. Skidmore and S. Shall. (1978) The effect of gamma radiation and neocarzinostatin on NAD and ATP levels in mouse leukemia cells. Biochim. et Biophys. Acta., 543:576-582.

95. Goulian, M., B. Bleile and B.Y. Tseng. (1980) Methotrexate-induced misincorporation of uracil into DNA. Proc. Natl. Acad. Sci. USA., 77:1956-1960.

96. Grouchy, J. and C. Turleau. (1977) Chromosome 16. In: Clinical Atlas of Human Chromosome, pp. 155-156, John Wiley and Sons, New York.

97. Gupta, R.S. and S. Goldstein. (1980) Diphtheria toxin resistance in human fibroblast cell strains from normal and cancer-prone individuals. Mut. Res., 73:331-338.

98. Gupta, R.S. and L. Siminovitch. (1976) The isolation and preliminary characterization of somatic cell mutants resistant to the protein synthesis inhibitor emetine. Cell, 9:213-220.

99. Gupta, R.S. and L. Siminovitch. (1978) The isolation and characterization of mutants of human diploid fibroblasts resistant to diphtheria toxin. Proc. Natl. Acad. Sci., USA, 75:3337-3340.

100. Gupta, R.S. and L. Siminovitch. (1978) Mutants of CHO cells resistant to the protein synthesis inhibitor emetine: genetic and biochemical characterization of second step mutants. Somat. Cell Genet., 4:77-94.

101. Gupta, R.S.,and L. Siminovitch. (1978) An in vitro analysis of the dominance of emetine sensitivity in CHO cells. J. Biol. Chem., 253:3978-3982.

102. Gupta, R.S. D.Y.H. Chan and L. Siminovitch. (1978) Evidence for functional hemizygosity at the emt^r locus in CHO cells through segregation analysis. Cell, 14:1004-1014.

103. Gupta, R.S. and L. Siminovitch. (1978) Genetic and biochemical studies with the adenosine analog toyocamycin and tubercidin: mutation at the adenosine kinanse locus in Chinese hamster cells. Somat. Cell Genet., 4:715-735.

104. Gupta, R.S. and L. Siminovitch. (1978) Diphtheria toxin resistant mutants of CHO cells affected in protein synthesis: A novel phenotype. Somat. Cell Genet., 4:553-572.

105. Gupta, P.S. and L. Siminovitch. (1980) Genetic markers for quantitative mutagenesis studies in Chinese hamster ovary cells; characteristics of some recently developed selective systems. Mut. Res., 69:113-116.

106. Habara, A., K. Kano, H. Nagano, Y. Mano, S. Ikegami and T. Yamashita. (1980) Inhibition of DNA synthesis in the adenovirus DNA replication complex by aphidicolin and 2',3'-dideoxythymidine triphosphate. Biochem. Biophys. Res. Commun., 92:8-12.

107. Hahn, G.M. (1975) Radiation and chemically induced potentially lethal lesions in noncycling mammalian cells: Recovery analysis in terms of X-ray- and ultraviolet-like-systems. Radiation Res., 64:533-545.

108. Hahn, G.M. and J.B. Little. (1972) Plateau-phase cultures of mammalian: An in vitro model for human cancer. In: Current Topics in Radiation Research, Quarterly, 8:39-83.

109. Hahn, G.M., J.R. Stewart, S-j. Yang and V. Parker. (1968) Chinese hamster cell monolyer culture. I. Changes in cell dynamics and modifications of the cell cycle with the period of growth. Exptl. Cell Res., 49:285-292.

110. Hanaoka, F., H. Kato, S. Ikegami, M. Ohashi and M-a. Yamada. (1979) Aphidicolin does inhibit repair replication in HeLa cells. Biochem. Biophys. Res. Commun., 87:575-580.

111. Hanawalt, P.C., P.K. Cooper, A.K. Gansan and C.A. Smith. (1979) DNA repair in bacteria and mammalian cells. Annu. Rev. Biochem., 48:783-836.

112. Hanawalt, P.C., E.C. Friedberg and C.F. Cox (eds.). (1978) DNA Repair Mechanisms, ICN-UCLA Symposia on Molecular and Cellular Biology, Vol. I, Academic Press, New York.

113. Harris, M. (1971) Mutation rates in cells at different ploidy levels. J. Cell. Physiol., 78:177-184.

114. Haseltine, W.A., L.K. Lynn, C.P. Lindan, R.H. Grafstrom, N.L. Shaper and L. Grossman. (1980) Cleavage of pyrimidine dimers in specific DNA sequences by a pyrimidine dimer DNA-glycosylase of M. luteus. Nature, 285:634-641.

115. Hasting, P.J., S-k. Quah and R.C. von Borstel. (1976) Spontaneous mutation by mutagenic repair of spontaneous lesion in DNA. Nature, 264:719-722.

116. Haynes, R.H. (1975) DNA repair and the genetic control of radiosensitivity in yeast. In: Molecular Mechanisms for DNA Repair, P.C. Hanawalt and R.B. Setlow (eds.), pp. 529-540, Plenum Press, New York.

117. Henderson, E.E. and R. Ribecky. (1980) DNA repair in lymphoblastoid cell lines established from human genetic disorders. Chem.-Biol. Interactions., 33:63-81.

118. Hershfield, M.S. and N.G. Nossal. (1973) In vitro characterization of a mutator T4 DNA polymerase. Genetics (Suppl.), 73:131-136.

119. Hibner, U. and B.M. Alberts. (1980) Fidelity of DNA replication catalyzed in vitro on a natural DNA template by the T4 bacteriophage multi-enzyme complex. Nature, 285:300-305.

120. Hirsch, G.P. (1978) Somatic Mutations and Aging. In: The Genetics of Aging, E.L. Schneider (ed.), pp. 91-134, Plenum Pub. Corp., New York.

121. Hollaender, A. (ed.). (1954) Radiation Biology, Vol. 1, McGraw-Hill, New York.

122. Honjo, T., Y. Nishizuka, I. Kato and O. Hayaishi. (1971) Adenosine diphosphate ribosylation of aminoacyl transferase II and inhibition of protein synthesis by diphtheria toxin. J. Biol. Chem., 246:4251-4260.

123. Hopkins, R.L. and M.F. Goodman. (1980) Deoxyribonucleotides pools, base pairing and sequence configuration affecting bromodeoxyuridine- and 2-aminopurine-induced mutagenesis. Proc. Natl. Acad. Sci., USA, 77:1801-1805.

124. Horiuchi, T. and T. Nagata. (1973) Mutation affecting growth of the Escherichia coli cell under a condition of DNA polymerase I-deficiency. Molec. Gen. Genet., 123:89-110.

125. Huberman, J.A. (1981) New views of the biochemistry of eukaryotic DNA replication revealed by aphidicolin, an unusual inhibitor of DNA polymerase α. Cell, 23:647-648.

126. Ichikawa, A., M. Negishi, K. Tomita and S. Ikegami. (1980) Aphidicolin: A specific inhibitor of DNA synthesis in synchronous mastcytoma P-815 cells. Japan J. Pharmacol., 30:301-308.

127. Iglewski, B.H. and D. Kabat. (1975) NAD-Dependent inhibition of protein synthesis of Pseudomonas aeruginosa toxin. Proc. Natl. Acad. Sci. USA., 72:2284-2288.

128. Iglewski, B.H., P.V. Liu and D. Kabat. (1977) Mechanism of action of Pseudomonas aeruginosa exotoxin A: Adenosine diphosphate-ribosylation of mammalian elongation factor-2 in vitro and in vivo. Infection and Immun., 15:138-144.

129. Ikegami, S., Y. Kamiya and H. Shirai. (1976) Characterization and action of meiotic maturation inhibitors in starfish ovary. Exptl. Cell Res., 103:233-239.

130. Ikegami, S., K. Kawada, Y. Kimura and A. Suzuki. (1979) A rapid convenient procedure for the detection of inhibitors of DNA synthesis using starfish oocytes and sea urchin embryos. Agric. Biol. Chem., 43:161-166.

131. Ikegami, S., T. Taguchi, M. Ohashi, M. Oguro, H. Nagano and Y. Mano. (1978) Aphidicolin prevents mitotic cell division by interfering with the activity of DNA polymerase-α. Nature, 275:458-460.

132. Ishii, Y. and M.A. Bender. (1980) Effects of inhibitors of DNA synthesis on spontaneous and ultraviolet light-induced sister chromatid exchanges in Chinese hamster cells. Mut. Res., 79:19-32.

133. Isomura, K., O. Nikaido, M. Horikawa and T. Sugahara. (1973) Repair of DNA damage in ultraviolet-sensitive cells isolated from HeLa S3 cells. Radiation Res., 53d:143-152.

134, Jacobson, E.L., G. Narasimhan and M.K. Jacobson. (1979) Carcinogen-induced DNA repair in normal and NAD-depleted 3T3 cells. Transactions of the Gulf Coast Molecular Biology Conference, Vol. 31, Suppl. No. 5, pp. 85-91.

135. Johnston, L.H., F. Bonhoeffer and P. Symmons. (1979) The molecular principles and the enzymatic machinery of DNA replication. In: Cell Biology: A Comprehensive Treatise, L. Goldstein, D.M. Prescott (eds.), pp. 59-129, Academic Press, New York.

136. Kalf, G.F., R.M. Metrione and T.R. Koezalka. (1981) A protein stimulatory factor for DNA polymerase α in rat giant trophoblast cells. Biochem. Biophys. Res. Commun., 100:566-575.

137. Kantor, G.J., C. Warner and D.R. Hull. (1977) The effect of ultraviolet light on arrested human diploid cell populations. Photochem. and Photobiol., 25:483-489.

138. Kao, F. and T.T. Puck. (1968) Genetics of somatic mammalian cells: VII. Induction and isolation of nutritional mutants in Chinese hamster cells. Proc. Natl. Acad. Sci. USA, 60:1275-1281.

139. Kato, H. (1980) Evidence that the replication point is the site of sister chromatid exchange. Cancer Genet. and Cytogenet., 2:69-77.

140. Kaufman, E.R. and R.L. Davidson. (1978) Bromodeoxyuridine mutagenesis in mammalian cells: Mutagenesis is independent of the amount of bromouracil in DNA. Proc. Natl. Acad. Sci. USA, 75:4982-4986.

141. Kennedy, A.R. and J.B. Little. (1978) Protease inhibitors suppress radiation-induced maligant transformation in vitro. Nature, 276:825-826.

142. Kimball, R.F. (1979) DNA repair and its relationship to mutagenesis, carcinogenesis and cell death. In: Cell Biology: A Comprehensive Treatise, Vol. 2, L. Goldstein and D.M. Prescott (eds.), pp. 439-478, Academic Press, New York.

143. Klimek, M., M. Vlasinova, P. Sevaikova and A. Oltova. (1973) UV-sensitive and UV-resistant mutants produced in HeLa S3 cells and their repair mechanism. In: Abstracts of Workshop on Repair Mechanisms in Mammalian Cells, Section 2, p. 4, Noordwijkerhout., The Netherlands.

144. Knudson, A.G. (1971) Mutation and Cancer: statistical study of retinoblastoma. Proc. Natl. Acad. Sci., USA, 68:820-823.

145. Kolata, G.B. (1980) Thalassemias: Models of genetic disease. Science, 210:300-302.

146. Kolodny, G.M. (ed.). (1980) Eukaryotic Gene Regulation, Vol. II, CRC Press, Inc., Florida.

147. Kondo, S. (1973) Evidence that mutations are induced by errors in repair and replication. Genetics, 73:109-122.

148. Kriek, E. and J.G. Westra. (1979) Metabolic activation of aromatic amines and amides and interactions with nucleic acids. In: Chemical Carcinogens and DNA, Vol. II, P.L. Grover (ed.), pp. 1-28, CRC Press, Inc., Florida.

149. Krokan, H. P. Schaffer and M.L. De Pamphilis. (1979) Involvement of eukaryotic deoxyribonucleic acid polymerase α and γ in the replication of cellular and viral deoxyribonucleic acid. Biochem., 18:4431-4443.

150. Krontiris, T.G. and G.M. Cooper. (1981) Transforming activity of human tumor DNAs. Proc. Natl. Acad. Sci., USA, 78:1181-1184.

151. Kunkel, T.A. and L.A. Loeb. (1979) On the fidelity of DNA replication . J. Biol. Chem., 254:5718-5725.

152. Kuroki, T. and S.Y. Miyashita. (1976) Isolation of UV-sensitive clones from mouse cell lines by Lederberg style replica plating. J. Cell Physiol., 90:79-90.

153. Lankas, G.R., C.S. Baxter and R.T. Christian. (1977) Effect of tumor promoting agents on mutation frequencies in cultured V79 Chinese hamster cell. Mut. Res., 45:153-156.

154. Lawley, P.D. (1979) Approaches to chemical dosimetry in mutagenesis and carcinogenesis: the relevance of reactions of chemical mutagens and carcinogens with DNA. In: Chemical Carcinogens and DNA, Vol. 1, P.L. Grover (ed.), pp. 1-36, CRC Press, Florida.

155. Lewin, B. (1980) In: Gene Expression, Eukaryotic Chromosomes, Vol. 2, John Wiley and Sons, New York.

156. Lewis, C.M. and R. Holliday. (1970) Mistranslation and ageing in neurospora. Nature, 228:877-880.

157. Lindberg, U. and L. Skoog. (1970) A method for the determination of dATP and dTTP in picomole amounts. Analytical Biochem., 34:152-160.

158. Lindgren, D. (1972) The temperature influence on the spontaneous mutation rate. I. Literature Review Hereditas, 70:165-178.

159. Little, J.B. (1973) Factors influencing the repair of potentially lethal radiation damage in growth-inhibited human cells. Radiation Res., 56:320-333.

160. Little, J.B., J. Nove and R. Weichselbaum. (1980) Abnormal sensitivity of diploid skin fibroblasts from a family with Garnder's syndrome to the lethal effects of X-irradiation, ultraviolet light and mitomycin-C. Mut. Res., 70:241-250.

161. Loeb, L.A. (1969) Purification and properties of deoxyribonucleic acid polymerase from nuclei of sea urchin embryos. J. Biol. Chem., 244:1672-1681.

162. Longiaru, M., J.E. Ikeda, Z. Jarkovsky, S.B. Horwitz, and M.S. Horwitz. (1979) The effect of aphidicolin on adenovirus DNA synthesis. Nucleic Acids Res., 6:3369-3386.

163. Luira, S.E. and M. Delbruck. (1943) Mutations of bacteria from virus sensitivity to virus resistance. Genetics, 28:491-511.

164. McClintock, B. (1951) Chromosome organization and genetic expression. Cold Spr. Harb. Symp. Quant. Biol., 16:13-47.

165. McCurry, L.S. and M.K. Jacobson. (1981) Poly(ADP-ribose) synthesis following DNA damage in cells heterozygous or homozygous for the xeroderma pigmentosum genotype. J. Biol. Chem., 256:551-553.

166. McLennan, A. (1980) Effect of aphidicolin and 2',3'-dideoxythymidine on mitochondrial DNA replication. Biochem. Biophys. Res. Commun., 94:116-122.

167. Maher, V.M. and J.J. McCormick. (1976) Effect of DNA repair on the cytotoxicity and mutagenicity of UV irradiation of chemical carcinogens in normal and xeroderma pigmentosum cells. In: Biology of Radiation Carcinogenesis, Y.M. Yuhas, R.W. Tennant and J.D. Regan (eds.), pp. 129-145, Raven Press, New York.

168. Maher, V.M., D.J. Dorney, A.L. Mendrala, B. Konze-Thomas and J.J. McCormick. (1979) DNA excision-repair processes in human cells can eliminate the cytotoxic and mutagenic consequences of ultraviolet irradiation. Mut. Res., 62:311-323.

169. Maher, V.M. and J.J. McCormick. (1979) DNA repair and carcinogenesis. In: Chemical Carcinogens and DNA, P.L. Grover (eds.), Vol. II, pp. 133-158, CRC Press, Florida.

170. Margison, G.P. and P.J. O'Connor. (1979) Nucleic acid modification by N-nitroso compounds. In: Chemical Carcinogens and DNA, P.L. Grover (ed.), Vol. 1, pp. 111-159, CRC Press, Florida.

171. Marinus, M.G. and Morris, N.R. (1974) Biological function for 6-methyladenine residues in the DNA of Escherichia coli K12. J. Mol. Biol., 85:309-322.

172. Mathews, C.K., T.W. North and G.P.V. Reddy. (1979) Multienzyme complexes in DNA precursor biosynthesis. In: Advances in Enzyme Regulations, G. Weber (eds.), Vol. 17, pp. 133-171, Pergamon Press, New York.

173. Medcalf, A.S.C. and P.D. Lawley. (1981) Time course of O^6-methylguanine removal from DNA of N-methyl-N-nitrosourea-treated human fibroblasts. Nature, 289:797-798.

174. Meuth, M., E. Aufreiter and P. Reichard. (1976) Deoxyribonucleotide pools in mouse-fibroblast cell lines with altered ribonucleotide reductase. Eur. J. Biochem., 71:39-43.

175. Meuth, M., N. L'Heureux-Huard and M. Trudel. (1979) Characterization of a mutator gene in Chinese hamster ovary cells. Proc. Natl. Acad. Sci., USA, 76:6505-6509.

176. Meuth, M. (1981) Role of deoxynucleoside triphosphate pools in the cytotoxic and mutagenic effects of DNA alkylating agents. Somat. Cell Genet., 7:89-102.

177. Middlebrook, J.L. and R.B. Dorland. (1978) Protection of mammalian cells from diphtheria toxin by exogenous nucleotides. Can. J. Microbiol., 25:285-290.

178. Miller, R.W. (1977) Relationship between human teratogens and carcinogens. U.S. Natl. Can. Inst. J., 58:471-474.

179. Miwa, M., M. Kanai, T. Kondo, H. Hoshino, K. Ishihara and T. Sugimura. (1981) Inhibitors of poly(ADP-ribose) polymerase enhance unscheduled DNA synthesis in human peripheral lymphocytes. Biochem. Biophys. Res. Commun., 100:463-470.

180. Mohandas, T., R.S. Sparkes and L.J. Shapiro. (1981) Reactivation of an inactive human X chromosome: evidence for X inactivation by DNA methylation. Science, 211:393-396.

181. Moore, P.D. (1975) Radiation-sensitive pyrimidine auxotrophs of Ustilago maydis. I. Isolation and characterization of mutants. Mut. Res., 28:355-366.

182. Moore, P.D. (1975) Radiation-sensitive pyrimidine auxotroph of Ustilago maydis: II. A study of repair mechanism and UV recovery in Pyr I. Mut. Res., 28:367-380.

183. Morrow, J. (1970) Genetic analysis of azaguanine resistance in an established mouse cell line. Genetics, 65:279-287.

184. Morrow, J. (1977) Gene inactivation as a mechanism for the generation of variability in somatic cells cultivated in vitro. Mut. Res., 44:391-400.

185. Morrow, J., D. Stocco and E. Barrow. (1978) Spontaneous mutation rate to thioguanine resistance is decreased in polyploid hamster cells. J. Cell. Physiol., 96:81-85.

186. Nakano, S., H. Yamagami and R. Takaki. (1979) Enhancement of excision repair efficiency by conditioned medium from density inhibited cultures in V79 Chinese hamster cells: Evidence for excision repair as an error-free repair process. Mut. Res., 62:369-381.

187. Nduka, N., C.J. Skidmore and S. Shall. (1980) The enhancement of cytotoxicity of N-methyl-N-nitrosourea and of γ-radiation by inhibitors of poly(ADP-ribose) polymerase. Eur. J. Biochem., 105:525-530.

188. Newbold, R.F., W. Warren, A.S.C. Medcalf and J. Amos. (1980) Mutagenicity of carcinogenic methylating agents is associated with a specific DNA modification. Nature, 283:596-599.

189. Newcome, H.B. (1949) Origin of bacterial variants. Nature, 164:150-151.

190. Nishimura, M., H. Yasuda, S. Ikegami, M. Ohashi and M-a. Yamada. (1979) Aphidicolin resistant mutant of which DNA polymerase α is induced by this drug. Biochem. Biophys. Res. Commun., 91:939-945.

191. Nomura, H., Y. Tanigawa, A. Kitamura, K. Kawakami and M. Shimoyama. (1981) "Poly(ADP-ribose)-sensitive endonuclease" and the repair of DNA damaged by N-methyl-N-nitrosourea. Biochem. Biophys. Res. Commun., 98:806-814.

192. North, T.W., R.K. Bestwick and C.K. Mathews. (1980) Detection of activities that interfere with the enzymatic assay of deoxyribonucleoside 5'-triphosphates. J. Biol. Chem., 255:6640-6645.

193. Oguro, M., C. Suzuk-hori, H. Nagano, Y. Mano, S. Ikegami. (1979) The mode of inhibitory action by aphidicolin on eukaryotic DNA polymerase α. Eur. J. Biochem., 97:603-607.

194. Oguro, M., M. Shioda, H. Nagano and Y. Mano. (1980) The mode of action of aphidicolin on DNA synthesis in isolated nuclei. Biochem. Biophys. Res. Commun., 92:13-19.

195. Ohashi, M., T. Taguchi and S. Ikegami. (1978) Aphidicolin: A specific inhibitor of DNA polymerases in the cytosol of rat liver. Biochem. Biophys. Res. Commun., 82:1084-1090.

196. Oikawa, A., H. Tohda, M. Kanai, M. Miwa and T. Sugimura. (1980) Inhibitors of poly(adenosine diphosphate ribose) polymerase induce sister chromatid exchanges. Biochem. Biophys. Res. Commun., 97:1311-1316.

197. Okazaki, H., C. Niedergang, M. Couppez, A. Martinage, P. Sautiere and P. Mandel. (1980) In vitro ADP-ribosylation of histones by purified calf thymus polyadenosine diphosphate ribose polymerase. FEBS Letters, 11:227-229.

198. O'Neill, P. (1981) SCEs are induced by the replication of BrdU-substituted DNA. Environ. Mut. Soc., 3:337 (Abstr.).

199. Pappenheimer Jr., A.M. (1977) Diphtheria toxin. Annu. Rev. Biochem., 46:69-94.

200. Park, S.D. and S.H. Hong. (1981) Effects of alkylating agents on UV-induced excision repair. Environ. Mut. Soc., 3:395, (Abstr.).

201. Parker, V.P. and M.W. Lieberman. (1977) Levels of DNA polymerase α, β, and γ in control and repair-deficient human diploid fibroblasts. Nucleic Acids Res., 4:2029-2037.

202. Paterson, M.C. and P.J. Smith. (1979) Ataxia telangiectasia: An inherited human disorder involving hypersensitivity to ionizing radiation and related DNA-damaging chemicals. Annu. Rev. Genet., 13:291-318.

203. Pedrali-noy, G., G. Mazza, F. Focher and S. Spadari. (1980) Lack of mutagenicity and metabolic inactivation of aphidicolin by rat liver microsomes. Biochem. Biophys. Res. Commun., 93:1094-1103.

204. Pedrali-noy, G. and S. Spadari. (1979) Effect of aphidicolin on viral and human DNA polymerases. Biochem. Biophys. Res. Commun., 88:1194-1202.

205. Pedarli-noy, G. and S. Spadari. (1980) Aphidicolin allows a rapid and simple evaluation of DNA-repair synthesis in damaged human cells. Mut. Res., 70:389-394.

206. Pedrali-noy, G. and S. Spadari. (1980) Mechanism of inhibition of herpes simplex virus and vaccina virus DNA polymerases by aphidicolin, a highly specific inhibitor of DNA replication in eukaryotes. J. Virol., 36:457-464.

207. Peterson, A.R., J.R. Landolph, H. Peterson and C. Heidelberger. (1978) Mutagenesis of Chinese hamster cells is facilitated by thymidine and deoxycytidine. Nature, 276:508-510.

208. Peterson, A.R. and H. Peterson. (1981) Temporal aspects of the facilitation by nucleosides of cytotoxicity and mutagenesis produced by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in Chinese hamster cells. Environ. Mut. Soc., 3:330 (Abstr.).

209. Pfohl-Leszkowicz, A., C. Salas, R.P.P. Fuchs and G. Dirheimer. (1981) Mechanism of inhibiton of enzymatic deoxyribonucleic acid methylation by 2-acety(amino)fluorene bound to deoxyribonucleic acid. Biochem., 20:3020-3024.

210. Plevani, P., G. Badaracco, E. Ginelli and Silvio sora. (1980) Effect of mechanism of action of aphidicolin on yeast deoxyribonucleic acid polymerases. Antimicrob. Agents Chemother., 18:50-57.

211. Poste, G. and I.J. Fidler. (1980) The pathogenesis of cancer metastasis. Nature, 283:139-146.

212. Purnell, M.R. and W.J.D. Whish. (1980) Novel inhibitors of poly(ADP-ribose) synthetase. Biochem. J., 185:775-777.

213. Purnell, M.R., P.R. Stone and W.J.D. Whish. (1980) ADP-ribosylation of nuclear proteins. Biochem. Soc. Transactions., 8:215-227.

214. Radman, M. and M. Errera. (1970) Enhanced efficiency of excision repair of non-replicated UV-damaged E. coli DNA. Mut. Res., 9:553-560.

215. Radman, M., G. Villani, S. Boiteux, M. Defais, P. Caillet-Fauquet and S. Spadari. (1977) On the mechanism and genetic control of mutagenesis induced by carcinogenic mutagens. In: Origins of Human Cancer, Book B. Mechanisms of Carcinogenesis, H.H. Hiatt, J.D. Watson and J.A. Winstein (eds.), pp. 903-922, Cold Spr. Harb. Labroatory.

216. Rainbow, A.J. and M. Howes. (1977) Decreased repair of gamma ray damaged DNA in progeria. Biochem. Biophys. Res. Commun., 74:714-719.

217. Rainbow, A.J. and M. Howes. (1977) Defective repair of ultraviolet and gamma-ray damaged DNA in Fanconi's anemia. Int. J. Radiat. Biol., 31:191-195.

218. Randtke, A.S., J.R. Williams and J.B. Little. (1972) Selection of mutant human cells whose progression into DNA synthesis is sensitive to UV irradiation. Exptl. Cell Res., 70:360-364.

219. Reichard, P., Z.N. Canellakis, and E.S. Canellakis. (1961) Studies on a possible regulatory mechanism for the biosynthesis of deoxyribonucleic acid. J. Biol. Chem., 236:2514-2519.

220. Roberts, J.J. (1978) The repair of DNA modified by cytotoxic mutagenic and carcinogenic chemicals. In: Advance in Radiation Biology, J.T. Lett and H. Adler (eds.), Vol. 7, pp. 211-436, Academic Press, New York.

221. Roberts, J.J. and K.N. Ward. (1973) Inhibition of post-replication repair of alkylated DNA by caffeine in Chinese hamster cells but not HeLa cells. Chem.-Biol. Interactions., 7:241-264.

222. Ronen, A. (1979) 2-Aminopurine. Mut. Res., 75:1-47.

223. Rosamond, J. (1980) Special sites in genetic recombination. Nature, 286: 202-205.

224. Rossignol, J-m., A. Gentil, J. Lacharpagne and A.m.de Recondo. (1980) Involvement of DNA polymerase β in a N-methyl-N'-nitro-N-nitrosoguanidine (MNNG)-induced DNA repair process. Bioch. Int., 1:253-261.

225. Roufa, D.J., B.N. Sadow and C.T. Caskey. (1973) Derivation of TK^- clones from revertant of TK^+ mammalian cells. Genetics, 75:515-530.

226. Sabourin, C.L.K., P.F. Bates, L. Glatzer, C-c. Chang, J.E. Trosko and J.A. Boezi. (1981) Selection of aphidicolin-resistant CHO cells with altered levels of ribonucleotide reductase. Somat. Cell Genet., 7:255-268.

227. Sato, K. and N. Hieda. (1979) Isolation and characterization of a mutant mouse lymphoma cell sensitive to methyl methansulfonate and X-ray. Rad. Res., 78:167-171.

228. Sax, K. (1938) Induction by X-rays of chromosome aberrations in Tradescantia microspores. Genetics, 23:494-516.

229. San, K. (1941) Types and frequencies of chromosomal aberrations induced by X-rays. Cold Spr. Harb. Symp. Quant. Biol., 9:93-101.

230. Saxan, L. (1976) Mechanisms of teratogenesis. J. Emb. Exp. Morph., 36:1-12.

231. Schendel, P.F. (1981) Inducible repair systems and their implications for toxicology. In: CRC Critical Reviews in Toxicology, J. McCann (ed.), Vol. 8, pp. 311-362, CRC Press Inc., Florida.

232. Schmickel, R.D., E.H.Y. Chu, J.E. Trosko and C-c. Chang. (1977) Cockayne syndrome: A cellular sensitivity to ultraviolet light. Pediatrics, 60:135-139.

233. Schultz, R.A. (1980) The isolation and partial characterization of Chinese hamster fibroblasts with altered UV sensitivity and/or DNA repair. Dissertation submitted to Michigan State University, East Lansing, Michigan, USA.

234. Schultz, R.A., C-c. Chang and J.E. Trosko. (1981) The mutation studies of mutagen-sensitive and DNA repair mutants of Chinese hamster fibroblasts. Environ. Mut., 3:141-150.

235. Schultz, R.A., J.E. Trosko and C-c. Chang. (1981) Isolation and partial characterization of mutagen-sensitive and DNA repair mutants of Chinese hamster fibroblasts. Environ. Mut., 3:53-64.

236. Siki, S., T. Oda and M. Ohashi. (1980) Differential effects of aphidicolin on replicative DNA synthesis and unscheduled DNA synthesis in permeable mouse sarcoma cells. Biochim. et Biophys. Acta, 610:413-420.

237. Shall, S., B. Durkarz and M. Perera. (1981) The participation of (ADP-ribose) in DNA repair. J. Supramol. Struc. Cellular Biochem. Suppl. No. 5, 10th Annual ICN-UCLA Symposia for Molecular and Cellular Biology, p. 152, (Abstr.).

238. Shapiro, N.I., O.M. Petrova and A.E. Khalizev. (1968) Induction of gene mutations in mammalian cells in vitro. Preprint N1782, Kierchatov Inst. of Atomic Energy, Moscow, USSR.

239. Shiraishi, Y. and A.A. Sandberg. (1980) Effects of caffeine-induced defective DNA replication on SCE and chromosome aberrations produced by alkylating agents. Mut. Res., 72:251-256.

240. Siegel, E.C. (1973) Ultraviolet-sensitive mutator mutU4 of Escherichia coli inviable with polA. J. Bacterial., 113:161-166.

241. Simons, J.W.I.M. (1979) Development of a liquid-holding technique for the study of DNA repair in human diploid fibroblasts. Mut. Res., 59:273-283.

242. Sims, J.L. and N.A. Berger. (1981) The effect of poly(ADPR) polymerase inhibitors on intracellular poly(ADP-ribose) levels and on DNA repair in normal human lymphocytes. J. Supramol. Struc. Cellular Biochem. Suppl. 5, 10th Annual ICN-IUCLA Symposia for Molecular and Cellular Biology, p. 183, (Abstr.).

243. Sinex, F.M. (1974) The mutation theory of ageing. In: Theoretical Aspects of Ageing, Rickstein, Sussan and Chesky (eds.), pp. 23-32, Academic Press, New York.

244. Skidmore, C.J., M.I. Davies, P.M. Goodman, H. Halldorsson, P.J. Lewis, S. Shall and A-a. Ziaee. (1979) The involvement of poly(ADP-ribose) polymerase in the degradation of NAD caused by γ-radiation and N-methyl-N-nitrosourea. Eur. J. Biochem., 101:135-142.

245. Skoog, L. (1970) An enzymatic method for the determination of dCTP and dGTP in picomole amounts. Eur. J. Biochem., 17:202-208.

246. Smirnov, G.B., E.V. Filkova and A.G. Skavronskaya. (1972) The mutator property of uvr502 mutation affecting UV sensitivity of Escherichia coli. Molec. Gen. Genet., 118:54-56.

247. Smirnov, G.B., E.V. Filkova and A.G. Skavronskaya. (1973) Loss and restoration of viability of E. coli due to combinations of mutations affecting DNA polymerase I and repair activities. Molec. Gen. Genet., 121:139-150.

248. Snyder, R.D. and J.D. Regan. (1981) Aphidicolin inhibits repair of DNA in UV-irradiated human fibroblasts. Biochem. Biophys. Res. Commun., 99: 1088-1094.

249. Sokal, R.R. and E.J. Rohlf. (1969) Two-way analysis of variance. In: Biometry, the Principles and Practice of Statistics in Biological Research, R. Emerson, D. Kennedy, R.B. Park, G.W. Beadle and D.M. Whitaker (eds.), pp. 331-332, W.H. Freeman and Co., San Francisco.

250. Speyer, J.F. (1965) Mutagenic DNA polymerase. Biochem. Biophys. Res. Commun., 21:6-8.

251. Speyer, J.F. (1969) Base analogues and DNA polymerase mutagenesis. Fed. Proc., 28:348.

252. Speyer, J.F., J.D. Karam, A.B. Lenny. (1966) On the role of DNA polymerase in base selection. Cold Spr. Harb. Symp. Quant. Biol., 31:693-697.

253. Speyer, J.F. and D. Rosenberg. (1968) The function of T4 DNA-polymerase. Cold Spr. Harb. Symp. Quant. Biol., 33:345-350.

254. Springgate, C.F. and L.A. Loeb. (1973) Mutagenic DNA polymerase in human leukemic cells. Proc. Natl. Acad Sci. USA, 70:245-248.

255. Stamato, T.D. and C.A. Waldren. (1977) Isolation of UV-sensitive variants of CHO-K1 by nylon cloth replica plating. Somat. Cell Genet., 3:431-440.

256. Stocker, B.A.D. (1949) Measurements of rate of mutation of flagellar antigenic phase in Salmonella typhimurium. J. Hyg. Camb., 47:398-413.

257. Stone-Wolff, D.S. and T.G. Rossman. (1981) Mutagenicity of deoxynucleosides in Chinese hamster cells. Environ. Mut., 3:357, (Abstr.).

258. Streisinger, G., Y. Okada, J. Emrich, J. Newton, A. Tsugita, E. Terzaghi, and M. Inouye. (1966) Frameshift mutations and the genetic code. Cold Spr. Harb. Symp. Quant. Biol., 31:77-84.

259. Sugino, A. and K. Nakayama. (1981) DNA polymerase α mutants from a Drosophila melanogaster cell line. Proc. Natl. Acad. Sci. USA, 77:7049-7053.

260. Szilard, L. (1959) On the nature of the ageing process. Proc. Natl. Acad. Sci., USA, 45:30-45.

261. Szybalski, W. and M.J. Smith. (1959) Genetics of human cell lines. I. 8-Azaguanine resistance, a selective "single step" marker. Proc. Soc. Exptl. Biol. Med., 101:662-666.

262. Tan, E.L., P.A. Brimer, R.L. Schenley and A.W. Hsie. (1981) Mutagenicity and cytotoxicity of dimethyl and monomethyl sulfates in the CHO/HGPRT system. Environ. Mut., 3:358, (Abstr.).

263. Tatsumi, K. and B. Strauss. (1979) Accumulation of DNA growing points in caffeine-treated human lymphoblastoid cells. J. Mol. Biol., 135:435-449.

264. Thompson, L.H., D.B. Bush, K. Brookman, C.L. Mooney and D.A. Glaser. (1981) Genetic diversity of UV-sensitive DNA repair mutants of Chinese hamster ovary cells. Proc. Natl. Acad. Sci., USA, 78:3734-3737.

265. Thompson, L.H., A.V. Carrano, K.W. Brookman, J.L. Minkler and C.L. Mooney. (1981) A CHO cell mutant having hypersensitivity to aklylating agents and an extraordinary baseline frequency of sister chromatid exchange. Environ. Mut., 3:382, (Abstr.).

266. Tkeshelashvili, L.K., C.W. Shearman, R.A. Zakour, R.M. Koplitz and L.A. Loeb. (1980) Effects of arsenic, selenium and chromium on the fidelity of DNA synthesis. Can. Res., 40:2455-2460.

267. Todd, P.W. and A.B. Hellewell. (1969) Phenotype and karyotype of a cultured Chinese hamster cell line defective for ultraviolet light resistance. Mut. Res., 7:129-132.

268. Topal, M.D. and J.R. Fresco. (1976) Complementary base pairing and the origin of substitution mutations. Nature, 263:285-289.

269. Trosko, J.E. and C-c. Chang. (1976) Role of DNA repair in mutation and cancer production. In: Ageing, Carcinogenesis and Radiation Biology, K.C. Smith (ed.), pp. 399-442, Pleum Press, New York.

270. Trosko, J.E. and C-c. Chang. (1981) The role of radiation and chemicals in the induction of mutations and epigenetic changes during carcinogenesis. In: Advances in Radiation Biology, J.T. Lett, H. Adler (eds.), Vol. 9, pp. 1-36, Academic Press, New York.

271. Trosko, J.E. and E.H.Y. Chu. (1975) The role of DNA repair and somatic mutation in carcinogenesis. In: Advances in Cancer Research, Kelin, Weinhouse, and Haddow (eds.), Vol. 21, pp. 391-425, Academic Press, New York.

272. Trosko, J.E., P. Frank, E.H.Y. Chu and J.E. Becker. (1973) Caffeine inhibition of post-replication repair of N-acetoxy-2-acetyl-aminofluorene-damaged DNA in Chinese hamster cells. Can. Res., 33:2444-2449.

273. Trosko, J.E. and J.D. Yager, Jr. (1974) A sensitive method to measure physical and chemical carcinogen-induced unscheduled DNA synthesis in rapidly dividing eukaryotic cells. Exptl. Cell Res., 88:47-55.

274. Tsai, Y-j., F. Hanaoka, M.M. Nakano and M-a. Yamada. (1979) A mammalian DNA$^-$ mutant decreasing DNA polymerase α activity at nonpermissive temperature. Biochem. Biophys. Res. Commun., 91:1190-1195.

275. Van Potter, R. (1980) Initiation and promotion in cancer formation: The importance of studies on intercellular communication. The Yale J. of Biol. Med., 53:367-384.

276. Van Zeeland, A.A. and J.W.I.M. Simons. (1975) The effect of calf serum on the toxicity of 8-azaguanine. Mut. Res., 27:135-138.

277. Van Zeeland, A.A. and J.W.I.M. Simons. (1976) The use of correction factors in the determination of mutant frequencies in populations of human diploid skin fibroblasts. Mut. Res., 34:149-158.

278. Vincent, B.R. de S. and G. Buttin. (1979) Studies on 1-β-D-arabinofuranosyl cytosine-resistant mutants of Chinese hamster fibroblasts: III. Joint Resistance to Arabinofuranosyl Cytosine and to Excess Thymidine - A Semidominant Manifestation of Deoxycytidine Triphosphate Pool Expansion. Somat. Cell Genet., 5:67-82.

279. Wade, M.H. and E.H.Y. Chu. (1979) Effects of DNA damaging agents on cultured fibroblasts derived from patients with Cockayne syndrome. Mut. Res., 59:46-60.

280. Waldren, C.A. and D. Patterson. (1979) Effects of caffeine on purine metabolism and ultraviolet light-induced lethality in cultured mammalian cells. Can. Res., 39:4975-4982.

281. Walters, R.A., R.A. Tobey and R.L. Ratliff. (1973) Cell-cycle-dependent variations of deoxyribonucleoside triphosphate pools in Chinese hamster cells. Biochim. et Biophys. Acta, 319:336-347.

282. Warren, S.T., R.A. Schultz, C-c. Chang, M.H. Wade and J.E. Trosko. (1981) Elevated spontaneous mutation rate in Bloom syndrome fibroblasts. Proc. Natl. Acad. Sci., USA, 78:3133-3137.

283. Warren, S.T. (1981) Bloom syndrome as a Human Mutator Mutation. Dissertation submitted to Michigan State University, East Lansing, Michigan, USA.

284. Warren, S.T., L.P. Yotti, J.R. Moskal, C-c. Chang and J.E. Trosko. (1981) Metabolic cooperation in CHO and V79 cells following treatment with a tumor promoter. Exptl. Cell Res., 131:427-430.

285. Watson, J.D. and F.H.C. Crick. (1953) Genetical implications of the structure of deoxyribonucleic acid. Nature, 171:964-967.

286. Weichselbaum, R.R., J. Nove and J.B. Little. (1978) Deficient recovery from potentially lethal radiation damage in ataxia telangiectasia and xeroderma pigmentosum. Nature, 271:261-262.

287. Weichselbaum, R.R., J. Nove and J.B. Little. (1978) X-ray sensitivity of diploid fibroblasts from patients with hereditary or sporadic retinoblastoma. Proc. Natl. Acad. Sci., USA, 75:3962-3964.

288. Weinberg, G. B. Ullman and D.W. Martin, Jr. (1981) Mutator phenotypes in mammalian cell mutants with distinct biochemical defects and abnormal deoxyribonucleoside triphosphate pools. Proc. Natl. Acad. Sci., USA, 78:2447-2451.

289. Weissbach, A. (1975) Vetebrate DNA polymerase. Cell, 5:101-108.

290. Weissbach, A. (1977) Eukaryotic DNA polymerase. Annu. Rev. Biochem., 46:25-47.

291. Weissbach, A. (1979) The functional roles of mammalian DNA polymerase. Arch. Biochem. Biophys., 198:386-396.

292. Weymouth, L.A. and L.A. Loeb. (1978) Mutagenesis during in vitro DNA synthesis. Proc. Natl. Acad. Sci., USA, 75:1924-1928.

293. Whisk, W.J.D. and S. Shall. (1975) Stimulation of poly(ADP-ribose) polymerase activity by the anti-tumor antibiotic, streptozotocin. Biochem. Biophys. Res. Commun., 65:722-730.

294. Wist, E. and H. Prydz.. (1979) The effect of aphidicolin on DNA synthesis in isolated HeLa cell nuclei. Nucleic Acids Res., 6:1583-1590.

295. Witkin, E.M. (1967) Mutation-proof and mutation-prone modes of survival in derivatives of Escherichia coli B differing in sensitivity to ultraviolet light. Brookhaven Symp. Biol., 20:17-55.

296. Witkin, E.M. (1976) Ultraviolet mutagenesis and inducible DNA repair in Escherichia coli. Bacterial Rev., 40:869-907.

297. Wong-Staal, F., R. Dalla-Favera, G. Franchini, E.P. Gelmann and R.C. Gallo. (1981) Three distinct genes in human DNA related to the transforming genes of mammalian sarcoma retroviruses. Science, 213:226-228.

298. Yagura, T. T. Kozu and T. Seno. (1980) Partial purification and characterization of poly(dc)-dependent DNA polymerase and its stimulating factor. Biochem. Biophys. Res. Commun., 92:1289-1296.

299. Yang, L.L., V.M. Maher and J.J. McCormick. (1980) Error-free excision of the cytotoxic, mutagenic N^2-deoxyguanosine DNA adduct formed in human fibroblasts by (±)-7β, 8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo-[a] pyrene. Proc. Natl. Acad. USA, 77:5933-5937.

300. Yerganian, G., M.A. Nell, S.S. Cho, A.H.Hayford and T. Ho. (1968) Virus-associated gain and loss of proliferative and neoplastic properities of normal and virus-transformed diploid cell lines. Natl. Can. Inst. Monograph., 20:241-268.

301. Yoshida, S., S. Masaki, H. Nakamura and T. Morita. (1981) Cooperation of terminal deoxynucleotidyl transferase with DNA polymerase α in the replication of ultraviolet-irradiated DNA. Biochim. et Biophys. Acta, 652:324-333.

302. Yotti, L.P., C-c. Chang and J.E. Trosko. (1979) Elimination of metabolic cooperation in Chinese hamster cells by a tumor promoter. Science, 206:1089-1091.

303. Zamenhof, P.J. (1970) Studies on the possible product of a bacterial mutator gene. Mut. Res., 9:549-552.

304. Zeeland, A. A. Van and J.W.I.M. Simons. (1976) Linear dose-response relationships after prolonged expression times in V79 Chinese hamster cells. Mut. Res., 35:129-138.

305. Zelle, B., R.J. Reynolds, M.J. Kottenhagen, A. Schutt and P.H.M. Lohman. (1980) The influence of the wavelength of ultraviolet radiation on survival, mutation induction and DNA repair in irradiated Chinese hamster cells. Mut. Res., 72:491-509.