To the Graduate Council:

I am submitting herewith a dissertation written by James Charles Fuscoe III entitled "Quantitative Analyses of Forward and Reverse Mutations at the hgprt Locus in CHO Cells." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

A. W. Hsie, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Vice Chancellor
Graduate Studies and Research

QUANTITATIVE ANALYSES OF FORWARD AND REVERSE MUTATIONS AT THE hgprt LOCUS IN CHO CELLS

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

James Charles Fuscoe III

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DEDICATION

This dissertation is dedicated to my wife Chris and my son Peter.

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ABSTRACT

This thesis describes (i) the relationship between the structures of 19 heterocyclic mustards (ICR compounds) and mutagenicity and cytotoxicity and (ii) the development of a reverse mutation assay to further characterized the types of mutations induced by ICR 191 and 170. Forward mutations at the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) locus in Chinese hamster ovary (CHO) cells were selected utilizing the purine analog 6-thioguanine. It was found that ICR 191 and 170 exhibit a concentration-independent phenotypic expression time of 7-9 days and that with pairs of compounds with the same heterocyclic nucleus, all secondary amine compounds (ICR 191, 371, 372, and 449) are less mutagenic than the tertiary amine compounds (ICR 170, 217, 292, 340, 355, and 368). All secondary amine compounds, but none of the tertiary amine compounds, showed a "plateau" in their concentration-dependent mutagenesis curves. When the 2-chloroethyl group of the side chain is replaced by a 2-hydroxyethyl group (ICR 170-OH, 191-OH, 292-OH, 340-OH, and 372-OH) or the amine linkage of the side chain is replaced by an ether linkage (ICR 283), mutagenicity is lost. Shortening of the side chain by 1 carbon (ICR 171) reduces mutagenicity. Substitution of a sulfur atom for a nitrogen atom in the side (ICR 342) or the addition of a second 2-chloroethyl group (ICR 220) results in increased mutagenicity and cytotoxicity. The distinct differences in mutagenicity between the secondary amine compounds and their tertiary amine analogs suggested that these compounds may be inducing different types of mutations. Reversion

analysis was used to investigate this possibility. An assay to quantify reverse mutations at the hgprt locus in CHO cells was defined in terms of optimal selective agent (azaserine) concentration, cell density, phenotypic expression time, and selective stringency. After treatment, replicate cultures of 10^6 cells are allowed a 48 hr phenotypic expression time in 100-mm plates. Azaserine (10μM) is then added directly to the growing culture and azaserine-resistant cells form visible colonies. It was found that all azaserine-resistant clones had re-expressed HGPRT enzyme and that two revertants contain altered HGPRT-indicating suppression. The results of reversion analysis with the mutagens ICR 191, ICR 170, and ethylnitrosourea suggest that these three agents may cause similar types of mutations in CHO cells.

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AP	Aminopterin
AS	L-azaserine
AS-F12FCM5	F12FCM5 containing 10 μM AS
AS^r	AS resistant
Bt_2cAMP	$N^6,O^{2'}$-dibutyryl adenosine 3':5'-cyclic monophosphate
CHO cells	Chinese hamster ovary cells
CRM	Cross reacting material
DTT	Dithiothreitol
EMS	Ethyl methanesulfonate
ENU	N-ethyl-N-nitrosourea
F12FCM5	Ham's F12 medium containing 5% extensively dialyzed fetal bovine serum
HGPRT	Hx-guanine phosphoribosyltransferase (EC 2.4.2.8.)
hgprt	Locus for HGPRT
Hx	Hypoxanthine
MF	Mutation frequency
MMS	Methyl methanesulfonate
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
PRPP	5-phosphoribosyl-1-pyrophosphate
TG	6-thioguanine
TG^r	TG resistant

CHAPTER I

INTRODUCTION

A cornerstone for the study of genetics is the availability of mutants. Mutations, until the recent developments initiated by recombinant DNA technology, represented the only means of inferring a particular wild type gene. Early geneticists depended upon naturally occurring alleles or the rare spontaneous mutant for their analyses of genetic markers.

In 1927, H. J. Muller (66) demonstrated for the first time that the low spontaneous frequency of recessive lethal, semilethal, and visible mutations in Drosophila melanogaster could be increased by an agent from the external environment. This demonstration of the mutagenicity of x-rays allowed the geneticist to enrich populations with mutants and also opened the field of mutagenesis—the study of mutations.

Despite much effort in searching for chemical agents which would induce mutations, it was not until 1944 that the first chemical of unquestionable mutagenicity was reported by Auerback and Robson (6). They showed that mustard gas could induce X-linked lethals in Drosophila. Because these data were classified, the identity of this chemical was not released until 1946 (7) and the details of the experiments were not published until 1947 (8).

The list of chemical, as well as physical, mutagens has increased dramatically since the 1940's as has the number of organisms used to detect mutations. Reviews of various detection systems can be found in

the "Chemical Mutagen" series edited by Hollaender (49) or Hollaender and de Serres (50).

Since the 1940's bacteria and bacteriophage have been used extensively in mutagenesis because of their ability to grow in defined media (thus permitting the selection of nutritional mutants) and because very large numbers of these organisms could be manipulated with ease. Through studies of the effects of chemical and physical agents on mutation induction in microorganisms, the modes of action of various mutagens have been deduced. Many reviews have been written on this subject (5, 27, 28, 29, 33, 86).

Basically, mutations can be divided into three types—point mutations (those that can be localized to a section of the chromosome through recombination studies), chromosome aberrations (those that are produced by breaking and rejoining of chromosomes), and meiotic or mitotic errors (those that result from unequal distribution of the chromosomes at meiosis or mitosis). Only point mutations will be discussed here.

Analysis of the types of DNA alterations resulting from point mutation were dependent upon four results from the fields of genetics, biochemistry, and molecular biology. They were (i) the finding that DNA is the genetic material, (ii) the one gene-one enzyme hypothesis of Beadle and Tatum, (iii) the ability to isolate, purify and establish the amino acid sequence of proteins, and (iv) the breaking of the genetic code. Wild type and mutant proteins could then be isolated and the amino acid sequence correlated with the known codons. In this way, base changes at the DNA level could be inferred.

These types of studies showed that point mutations can be divided into base substitution mutations (94) and frameshift mutations (91). There are two types of base substitution mutations that can occur—transitions and transversions (32). Transitions involve the substitution of one purine for another purine or one pyrimidine for another pyrimidine while transversions involve the substitution of a purine for a pyrimidine or vise versa. Frameshift mutations comprise either the insertion or deletion of one or more nucleotides in a gene.

The practice of analyzing base changes in DNA through sequencing of the protein gene product, however, has not been widely used to analyze mutations at the nucleotide sequence level since (i) it is often difficult to isolate and sequence proteins, (ii) mutant protein may not be detectable, and (iii) some well characterized genetic markers have no known protein product (e.g., rII locus in phage T4). Therefore, other methods have been used to determine the modes of action of mutagens.

One of the most successful alternatives is a method which involves the measurement of reverse mutation—reversion analysis. An operational definition of a reverse mutation is a mutation which fully or partially restores the activity of a mutant gene. Auerbach (4) offers a good discussion of reversion analysis as well as its strengths and weaknesses. The basic assumption in reversion analysis is that whereas the loss of a functional gene may be caused by a mutation in any one of many sites, the correction of that mutation (the reverse mutation) would require a much more specific alteration. For example, if a mutation in a gene was the result of a G-C to A-T transition, only a mutagen which causes A-T to

G-C transitions should revert it. A mutagen which induces G-C to A-T transitions, transversions or frameshifts would not be expected to revert this mutant. Similarly, a +1 frameshift mutation should be revertible by a mutagen which induces -1 frameshifts, but not by mutagens which cause +1 frameshifts, transitions, or transversions.

Therefore, using a battery of mutagens with different modes of action, mutagens could theoretically be divided into groups based upon their ability to revert a given mutant. Within each group, mutagens would be expected to have a similar mode of action. What this type of analysis does not show, however, is what specific mutagenic event any particular type of mutagen causes. It may show us which mutagens cause similar types of mutations and which cause different types of mutations, but in order to know what the mutagenic lesion is, additional, independent evidence is needed. In the case of Oeschger and Hartman (68), who used reversion analysis to study the types of mutations induced by acridine half-mustards (ICR compounds) in the histidine operon of Salmonella typhimurium, the additional evidence for concluding that ICR compounds cause almost exclusively frameshifts was (i) all mutants showed polarity, (ii) absence of extracistronic suppressors, (iii) demonstration of intracistronic reversion, (iv) absence of "leaky" mutants, (v) absence of intragenic complementation, and (vi) elimination of polarity in revertants. These types of independent observations, when coupled with reversion analysis, provide a powerful method for determining the types of mutations induced by a given agent.

In addition to the above study, several other groups have used reversion analysis to study ICR compound-induced mutants. Malling (60)

utilizing known Neurospora crassa base-pair substitution and frameshift ad-3B mutants concluded that ICR 170 produces mainly frameshifts but also base-pair substitutions at low frequencies. Munz and Leupold (67) analyzed 117 ICR 170-induced ad-6 and ad-7 mutants in Schizosaccharomyces pombe and also found evidence for frameshift mutations. Culbertson et al. (24) found that ICR 170 induced a set of his 4 mutations in Saccharomyces cerevisiae which exhibited characteristics similar to the bacterial frameshift mutations of Oeschger and Hartman (68). They also found what appeared to be a low frequency (3/30) of base-pair substitution mutations. Further studies by Yourno and Heath (96) and Yourno (95) on ICR 191-induced mutants at the his D gene in S. typhimurium showed that ICR 191 causes base additions or deletions in monotonous runs of G-C base-pairs.

Concurrent with these studies, cell biologists were developing techniques for the cultivation and manipulation of the cells of higher organisms in culture as reviewed by Pollack (79) and Puck (80). With the ability to grow animal cells in culture came the opportunity to study mutagenesis in animal cells in a manner similar to that used in microorganisms. Several studies, mostly by Harris (43, 44, 45) and Mezger-Freed (62, 63), however, suggested that mutation was not the basis for the observed variation in cultured cells. Their arguments were based mainly upon the ploidy independence of both spontaneous and mutagen-induced variation. DeMars (26) has refuted these charges and several recent studies have shown that there is a ploidy dependence on variant frequency (19, 51, 65, 83) and that genetic change is responsible for some specific markers in cultured animal cells (88). Banbury Report 2 (53) gives an up-to-date review of mutagenesis in cultured mammalian cells.

One locus in particular, hgprt, has been studied extensively in mammalian cell mutagenesis. The hgprt locus codes for the purine salvage enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT). The purine analogs 8-azaguanine or 6-thioguanine can be used to select for variants that have lost or altered HGPRT. Revertants can also be selected at this locus with aminopterin, amethopterin or azaserine so that reversion analysis should be possible. Chu et al. (20) utilized this selection system to do reversion analysis in Chinese hamster cells as did Peterson et al. (77). Recently, Hodgkins et al. (47) also analyzed agent-specific reversion at this locus in Chinese hamster cells. The main problem with these studies is that of quantification. When 8-azaguanine was used to select for forward mutations in these studies, factors such as serum components, cell density and phenotypic expression time, which effect recovery of the mutants, were not adequately defined. In addition, when aminopterin or amethopterin was used to select for the reverse mutations, many colonies developed which had not re-expressed HGPRT. It is, therefore, important to adequately define both forward and reverse mutation assays so that the results are reproducible and that colonies developing under the selective conditions have a known alteration.

The CHO/HGPRT assay (52, 69, 71, 72) has been designed to allow quantification of forward mutations at the hgprt locus in Chinese hamster ovary (CHO) cells. The second chapter of this thesis has been published (36) and presents the utilization of this quantitative assay in correlating the chemical structure of a series of heterocyclic

mustards (ICR compounds) with mutation induction and cytotoxicity. The ICR compounds were first synthesized in the early 1960's at the Institute for Cancer Research (73) in an effort to develop antitumor drugs. With the finding that an acridine nucleus linked to a reactive side chain had antitumor activity, a large number of structurally similar analogs containing various substitutions and modifications of the nucleus and side chain were synthesized (23). ICR 191 and 170 have been shown to be mutagenic to microorganisms (as pointed out earlier). ICR 170 has also been found to be mutagenic to Drosophila (15), to induce chromosome breakage in Vicia faba (55), to cause long patch, UV-type repair in human cells (84) but not to induce dominant lethal mutations in mice (30). ICR 191 has been found to cause mutations in human lymphoid cells (78), diploid human lymphoblasts (25), Chinese hamster cells (41, 92), and mouse lymphoma cells (34, 64). A comparison of the mutagenicities of the secondary amine ICR 191 and its tertiary amine analog 170 shows that ICR 191 is more mutagenic than ICR 170 in the S. typhimurium histidine reversion assay (13), while the reverse is true in a Saccharomyces methionine reversion assay (13), a Neurospora adenine reversion assay (12), and the CHO/HGPRT assay (70). This differential mutagenicity of ICR compounds may reflect actual differences in the modes of action of these mutagens in prokaryotes and eukaryotes, or it may result from intrinsic limitations inherent in reverse mutation assays. Also, ICR 170 is a potent antitumor agent against ascites tumor while ICR 191 is only slightly active (23). The reason for this is not clear.

The crystal structure of several of the ICR compounds has been determined by x-ray diffraction (10, 16, 38, 39, 90) in attempts to

discover those features responsible for the biological differences between ICR 191 and 170. The hydroxyl substituted, nonmutagenic derivatives were employed because they could be easily crystalized. Although information has been obtained on the three-dimensional conformation of these molecules, it is not yet possible to explain the biological properties through the structures.

The third chapter of this thesis describes the development of a quantitative reversion assay at the hgprt locus in CHO cells and its application in determining any differences in the types of mutations induced by ICR 191 and 170. Azaserine is used as the selective agent and the term azaserine-resistant (AS^r) is used to describe the cells of any colony which develops under the selective conditions of the reverse mutation assay. In addition, revertants were analyzed biochemically for evidence of suppression, a gene change which occurs at a different site from the initial mutation and can therefore be either intragenic or extragenic.

The final chapter contains some thoughts on possible future applications of the reversion assay to the study of mamalian cell mutagenesis.

CHAPTER II

MUTAGENICITY AND CYTOTOXICITY OF 19 HETEROCYCLIC MUSTARDS (ICR COMPOUNDS) IN CULTURED MAMMALIAN CELLS

Abstract

The mutagenicity and cytotoxicity of 19 ICR compounds, including 6 reported previously, have been determined in the CHO/HGPRT system. As with other physical and chemical agents, ICR 170 and 191 exhibit a phenotypic expression time of 7-9 days independent of concentrations tested. Thirteen of these compounds are mutagenic. At equimolar concentrations, the compounds with the tertiary amine-type side chain (ICR 217, 340, 355, 368, 170, and 292) are more mutagenic than the compounds with the secondary amine-type side chain (ICR 449, 371, 191, and 372). All secondary amine types show a "plateau" in their concentration-dependent mutagenesis curves at 3 to 4 μM. Shortening of the side chain by one carbon (ICR 171) results in a reduced mutagenicity. Substitution of a sulfur atom for a nitrogen in the side chain (ICR 342) increases both mutagenicity and cytotoxicity. The presence of 2 2-chloroethyl groups on the side chain (ICR 220) also results in greatly increased cytotoxicity and mutagenicity. When the 2-chloroethyl group of ICR 340, 372, 292, 191, or 170 is replaced by a 2-hydroxyethyl group (ICR 340-OH, 372-OH, 292-OH, 191-OH, 170-OH), a mutagenically inactive compound results which remains toxic. Replacement of the amine linkage with an ether linkage (ICR 283) also yields a mutagenically inactive compound.

Introduction

Recently, we have developed a mammalian cell gene mutation assay, CHO/HGPRT, which selects for TG^r variants in CHO cells (51, 52, 69). More than 98% of these variants show altered HGPRT activity in a set of well-defined conditions. This sytem has been used in studies of mutation induced by various chemical and physical agents (21, 22, 70, 85). The sensitive and quantitative nature of the assay has been utilized in investigations of the effects of alkyl-group modification on the cytotoxic and mutagenic activity of 10 direct-acting alkylating chemicals (21, 22) and 6 heterocyclic nitrogen mustards (ICR compounds) (70).

ICR compounds were synthesized by the Institute for Cancer Research as antitumor drugs (23), and several have been shown to be mutagenic (23, 25, 61) and carcinogenic (74, 75). The structural differences and similarities in their side chains and nuclei offer an opportunity for study of the dependence of mutagenicity and cytotoxicity on specific structural features. Our previous study (70) indicated that the tertiary amine types are 3 to 5 times more mutagenic than the secondary amine types at equimolar concentrations, that the 2-chloroethyl group is necessary for mutagenesis, that the secondary amine types show "plateaus" in their dose-dependent mutagenicity curves, and that the side chain seems to be more important than the nucleus in determining mutagenicity. We report here a continuation of our structure-activity (mutagenicity and cytotoxicity) study utilizing 13 additional ICR compounds.

Materials and Methods

Cell Culture. A subclone of the CHO-K_1 cell line, CHO-K_1-BH_4 (52), was used in this study. Stock cultures were routinely maintained in a logarithmic growth phase at low cell density in Ham's F12 medium supplemented with 10% fetal bovine serum under conditions of 5% CO_2 in air at 37° in an incubator humidified to 100%. For subculture, cells were removed with a minimal volume (e.g., 1 ml in a standard milk-dilution glass bottle) of 0.05% trypsin.

Chemicals. Fresh solutions (1 mg/ml) of ICR compounds were prepared in 0.01 N HCl (ICR 191, 191-OH, 170, 170-OH, 292, 292-OH, 372, 340-OH), H_2O (ICR 449, 372-OH, 220, 283, 340, 355, 368, 171, 371, 217), or 95% ethanol (ICR 342). Solutions stored at -10° were stable in terms of mutagenicity and cytotoxicity for more than a year. Dilutions were made in Hank's balanced salt solution.

Mutagen Treatment and Selection for TG Resistance. Mutagen treatment and the determination of mutation induction to TG resistance have been described in detail previously (52, 69, 70) and are summarized here: 5×10^5 cells were plated in 25 sq cm Falcon flasks containing 5 ml of medium F12 supplemented with 5% fetal bovine serum (K. C. Biological) extensively dialyzed by us (F12FCM5), and were incubated for 24 hr. The ICR compounds, with appropriate solvent controls as specified above, were added (time = 0), and the cultures were incubated for an additional 16 hr. The cells were then washed 3 times with saline G (82) and removed with trypsin for cell number determination with a Coulter Counter, Model B.

For determination of the effect of treatment of ICR compounds on the cellular cloning efficiency, 200 to 1000 cells (depending on the expected cell survival) were plated in triplicate on 60-mm dishes in F12FCM5. The untreated control cultures exhibit cloning efficiencies of 60 to 80%.

For phenotypic expression, approximately 10^6 treated cells were subcultured in F12FCM5 every 2 days and then selected for TG resistance on the 9th day after treatment. For selection, 2×10^5 cells were plated in 100-mm dishes (5 plates = 10^6 cells) in Hx-free F12FCM5 containing 10 μM TG. Cloning efficiency of the culture at the time of mutant selection was determined from 200 cells plated on 60-mm dishes in triplicate in Hx-free F12FCM5. After incubation in the selective medium for 7 days, mutant colonies with a minimum of 50 to 100 cells per colony were recognizable by the unaided eye. They were then fixed, stained, and enumerated. Mutation frequency (MF) was calculated as the total number of mutant colonies divided by the number of cells plaged (10^6), corrected for the cloning efficiency of the culture at the time of mutant selection; it is expressed as mutants/10^6 clonable cells. The MF is presented as a function of agent concentration in the figures; the spontaneous MF is not subtracted, but is given in the legend to each figure. We designate mutagenic activity as MF per μM compound tested based on the slope of the linear portion of the concentration-response curve under our experimental conditions.

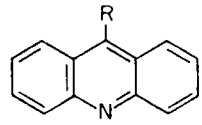
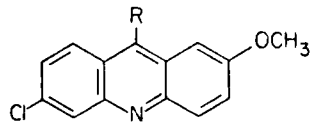
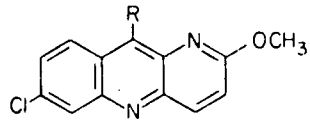
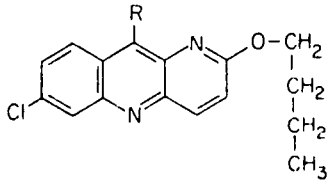
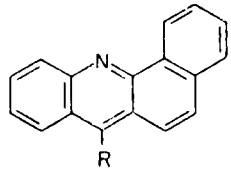
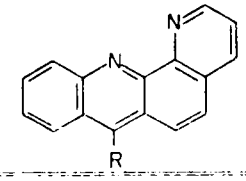
Results

Listed in Table 1 are the structures of the 13 ICR compounds plus 6 others (ICR 191, 191-OH, 170, 170-OH, 292, and 372) from our previous study (70). All code designations follow those of Creech et al. (23). ICR 449, 217, and 220 contain the same acridine nucleus (Nucleus C; Ref. 23) but different side chains: 449 contains a secondary amine side chain with a single 2-chloroethyl group; 217 contains a tertiary amine side chain with a single 2-chloroethyl group; 220 contains a tertiary amine side chain with 2 2-chloroethyl groups. ICR 283 and 171 contain the same methoxyacridine nucleus (Nucleus G) as 191 and 170 but have slightly modified side chains. The side chain of 283 and 171 is one carbon shorter, and 283 has an ether linkage in place of an amino linkage. ICR 372-OH, 340, 340-OH, and 342 contain the same methoxyazaacridine nucleus (Nucleus H) as does 372. ICR 340 has a tertiary amine side chain, while 342 has a sulfur mustard side chain (replacement of an amino linkage with a sulfur linkage). A hydroxyl group has been substituted for the chloride on the side chain in 372-OH and 340-OH. Both ICR 355 and 371 contain an n-butoxyazaacridine nucleus (Nucleus I) with either a tertiary amine side chain (ICR 355) or a secondary amine side chain (ICR 371). ICR 292-OH contains a hydroxyl substitution on the side chain of 292. ICR 368 contains a benzo(b)(1,10)phenanthroline nucleus (Nucleus M) with a tertiary amine side chain. With these diversified structural similarities and differences, we examined the relationship between structure and mutagenic and cytotoxic activities.

It has been shown for a variety of compounds that a phenotypic expression time of 7 to 9 days is needed for maximum stable mutation

TABLE 1

STRUCTURE OF 19 HETEROCYCLIC MUSTARDS (ICR COMPOUNDS)

NUCLEUS CODE	NUCLEUS STRUCTURE	ICR CODE	SIDE CHAIN (R)
C		449	$NH(CH_2)_3NHCH_2CH_2Cl$
		217	$NH(CH_2)_3N(C_2H_5)CH_2CH_2Cl$
		220	$NH(CH_2)_3N(CH_2CH_2Cl)_2$
G		191	$NH(CH_2)_3NHCH_2CH_2Cl$
		191-OH	$NH(CH_2)_3NHCH_2CH_2OH$
		170	$NH(CH_2)_3N(C_2H_5)CH_2CH_2Cl$
		170-OH	$NH(CH_2)_3N(C_2H_5)CH_2CH_2OH$
		283	$NHCH_2CH_2OCH_2CH_2Cl$
		171	$NHCH_2CH_2NHCH_2CH_2Cl$
H		372	$NH(CH_2)_3NHCH_2CH_2Cl$
		372-OH	$NH(CH_2)_3NHCH_2CH_2OH$
		340	$NH(CH_2)_3N(C_2H_5)CH_2CH_2Cl$
		340-OH	$NH(CH_2)_3N(C_2H_5)CH_2CH_2OH$
		342	$NH(CH_2)_3SCH_2CH_2Cl$
I		371	$NH(CH_2)_3NHCH_2CH_2Cl$
		355	$NH(CH_2)_3N(C_2H_5)CH_2CH_2Cl$
K		292	$NH(CH_2)_3N(C_2H_5)CH_2CH_2Cl$
		292-OH	$NH(CH_2)_3N(C_2H_5)CH_2CH_2OH$
M		368	$NH(CH_2)_3N(C_2H_5)CH_2CH_2Cl$

induction in the CHO/HGPRT system (69, 70, 72, 85). This is presumably the time necessary for the degradation of wild-type HGPRT enzyme and its mRNA and for replacement by the mutant HGPRT forms. This 7- to 9-day expression time was found for a secondary amine-type compound, ICR 191 (70), and also for a tertiary amine-type chemical, ICR 170. As shown in Figure 1, when treated cells were plated in selection on various days after treatment, a maximum and constant MF was obtained by days 7 to 9. There was neither a concentration dependence on expression time nor a loss of mutants up to day 13. An expression time of 9 days was used in all experiments.

The concentration-response curves for ICR 449 and 217 (Nucleus C; 449 with a secondary amine side chain and 217 with a tertiary amine side chain) are shown in Figure 2. At concentrations below 1 μM, there is a shoulder in the survival curve of 449, in which no appreciable loss of cellular cloning efficiency occurs, followed by an exponential increase in cellular lethality. MF increases linearly with increasing concentration and plateaus at about 4.2 μM. The survival curve of ICR 217 has a shoulder of approximately 0.5 μM followed by an exponential decrease in survival. MF increases linearly to 1.2 μM.

The concentration-response curves of ICR 340 and 340-OH are shown in Figure 3. At concentrations less than 1.0 μM, there is a shoulder in the survival curve of 340, followed by an exponential decline in survival. MF increases linearly with concentration to 2.2 μM. The hydroxyl substitution on the side chain (ICR 340-OH) yields a mutagenically inactive compound which also displays little cellular lethality over

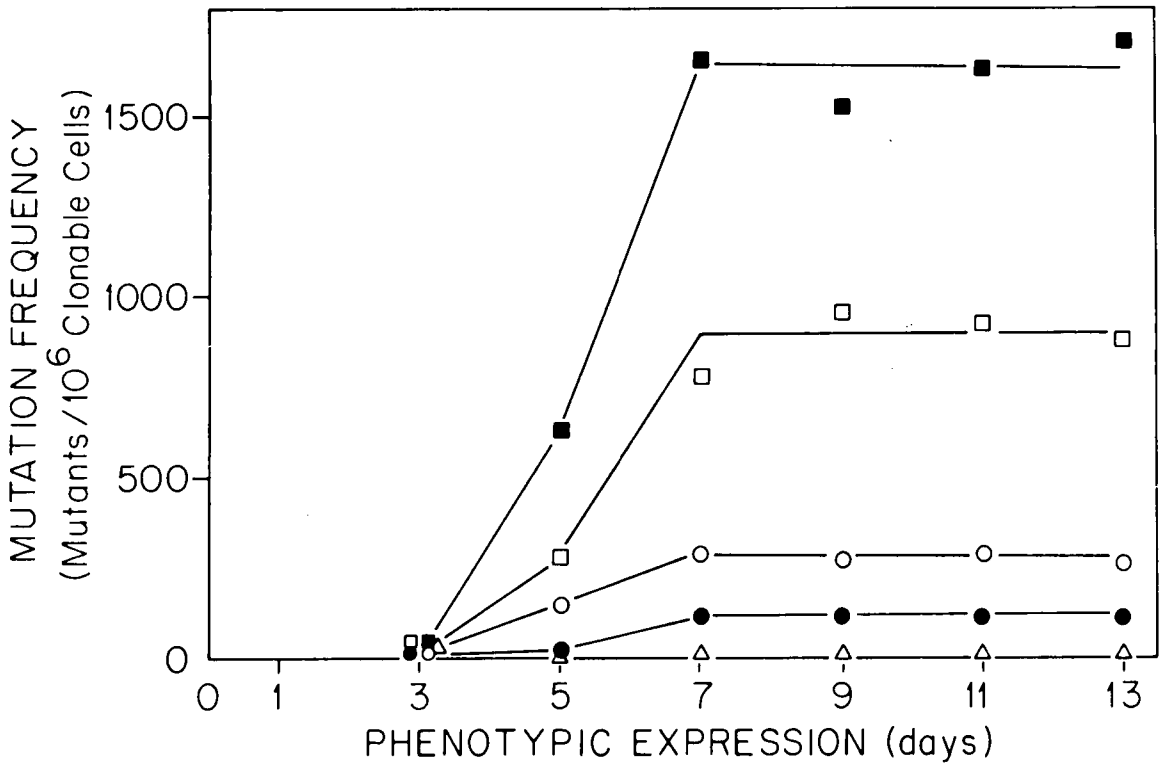


Figure 1. Phenotypic expression of ICR 170-induced TG^r phenotype as a function of time. Cultures were treated (initiation of treatment = day 0) as described with ICR 170 at 0(△), 0.1 (●), 0.2 (○), 0.6 (□), or 1.0 (■) μM for 16 hr. The cells were subcultured and plated for mutant selection every 2 days. The average spontaneous MF was < 1 (i.e., no mutant was found in 10^6 untreated cells).

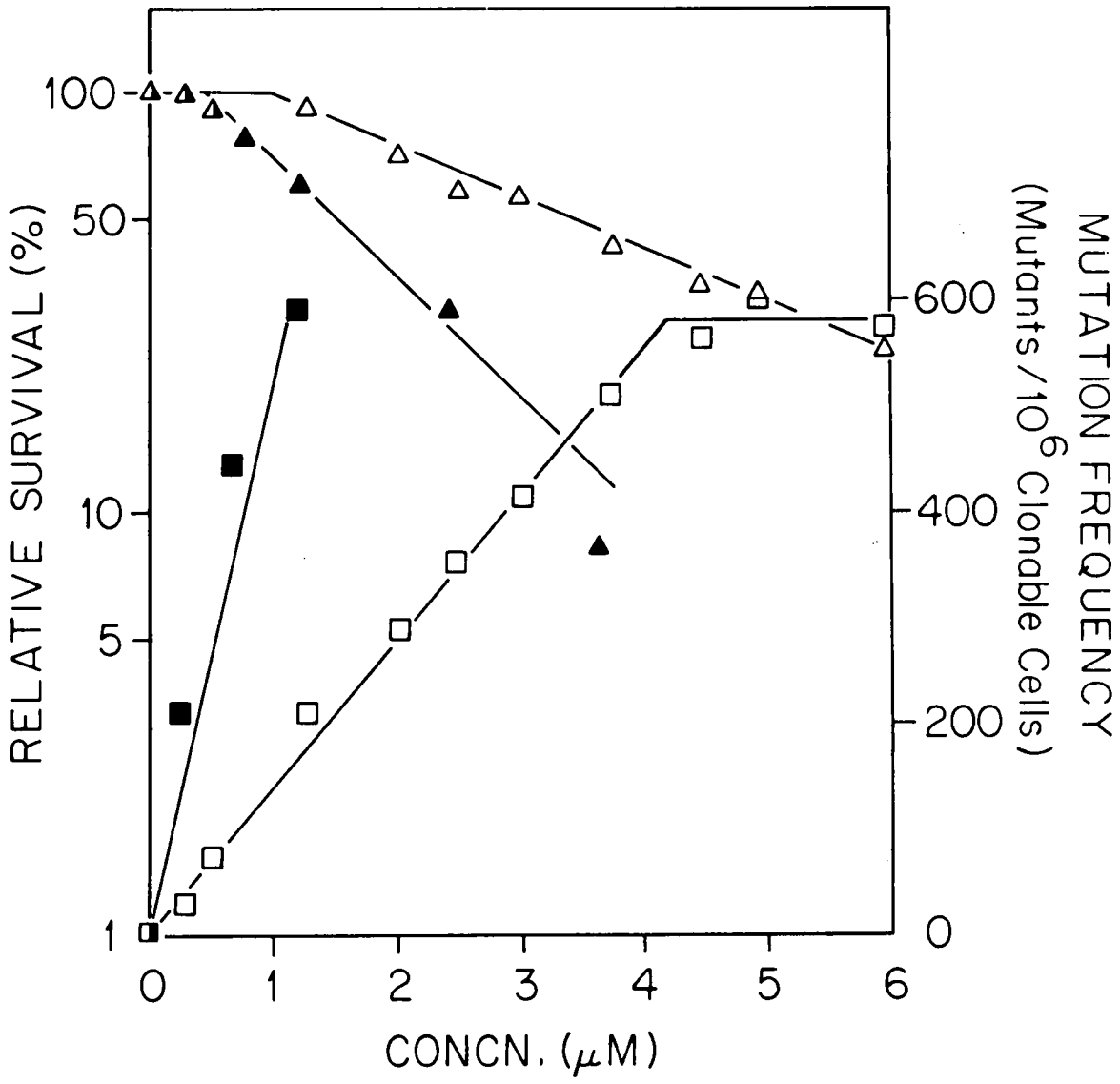


Figure 2. Cell survival (relative cloning efficiency) and mutation induction as affected by ICR 449 (□,△) and ICR 217 (■,▲). Cultures were incubated with various concentrations (CONCU.) as indicated for 16 hr; single-cell survival (▲,△) and induced MF (■,□) were determined as described. The average values from 3 separate experiments are presented. The spontaneous MF was 4.5.

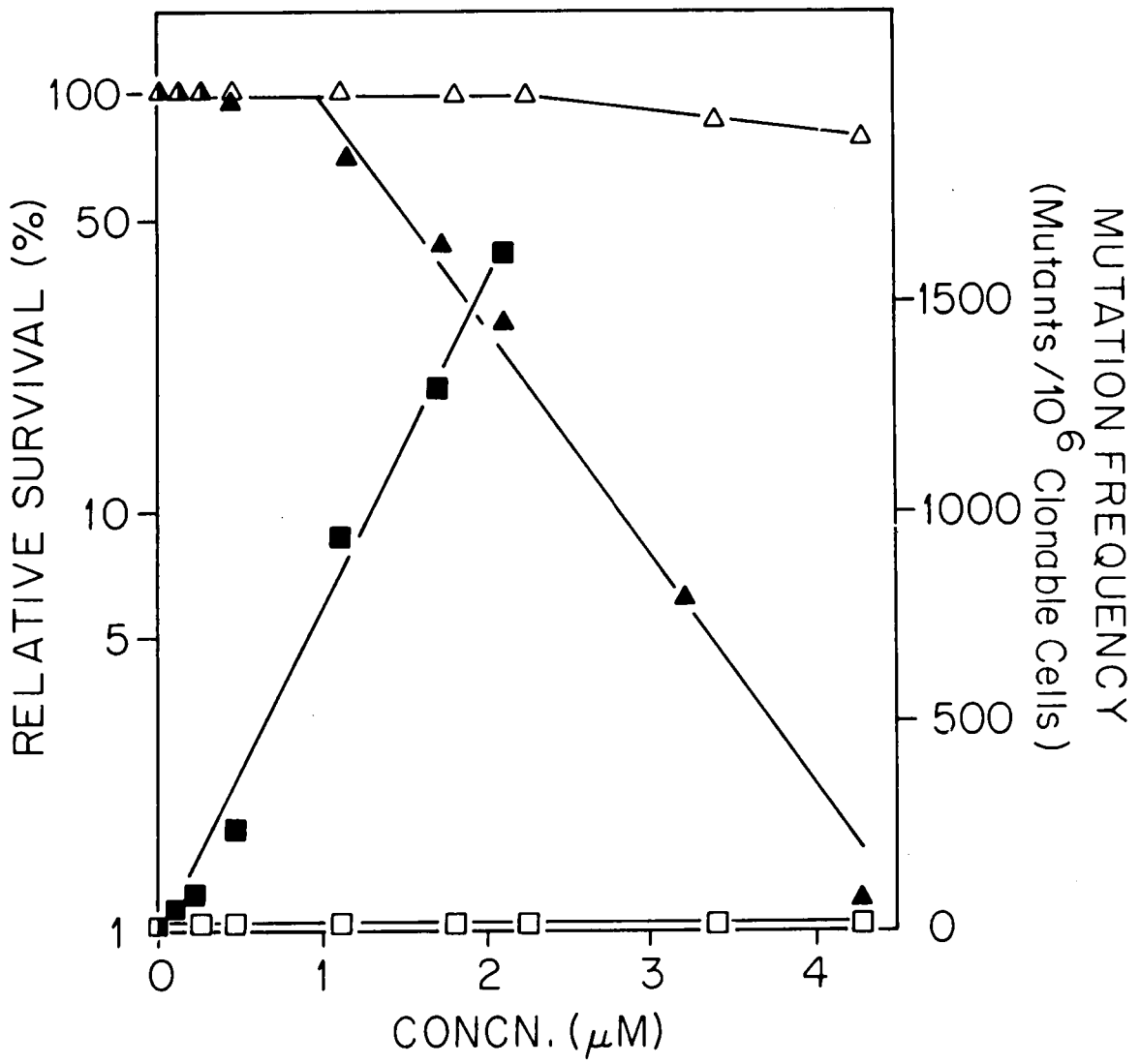


Figure 3. Cell survival and mutation induction as affected by ICR 340 (■,▲) and ICR 340-OH (□,△). Cultures were incubated with various concentrations (CONCN.) as indicated for 16 hr; single-cell survival (▲,△) and induced MF (■,□) were determined as described. The average values from 2 separate experiments are presented. The spontaneous MF was 2.0.

this range. Concentrations as high as 11.2 μM, which reduced cloning efficiency to ~2%, did not cause an appreciable increase of MF over the spontaneous frequency.

The concentration-dependent mutagenicity and toxicity of ICR 371 and 355 (Nucleus I; 371 with a secondary amine side chain and 355 with a tertiary amine side chain) are shown in Figure 4. Both survival curves have shoulders. MF increases linearly for both compounds, with 371 reaching a plateau at about 4 μM.

The survival curve of ICR 368 (Figure 5) has a shoulder at concentrations up to 0.1 μM, followed by an exponential decline in survival. MF increases linearly to 0.55 μM.

The concentration-response curve of ICR 342 is shown in Figure 6. The survival curve shows a biphasic exponential decline in cellular cloning efficiency. MF increases linearly and appears to approach a plateau at about 0.35 μM.

Figure 7 shows the mutagenicity of 5 hydroxyl-substituted analogues (ICR 340-OH, 191-OH, 170-OH, 292-OH, and 372-OH) (Figure 7A-E) and ICR-171 (Figure 7F). None of the hydroxyl-substituted analogues shows a concentration-dependent increase of mutagenicity. ICR 171 is weakly mutagenic compared with other mutagenic ICR compounds.

The mutagenicity of the compounds in Figures 2-5 (except 340-OH) and of 4 compounds from our previous study (ICR 191, 170, 372, and 292) is summarized in Table 2. The mutagenicity of ICR 191-OH, 170-OH, 340-OH, 372-OH, 292-OH, 283, 171, 342, and 220 is summarized in Table 3.

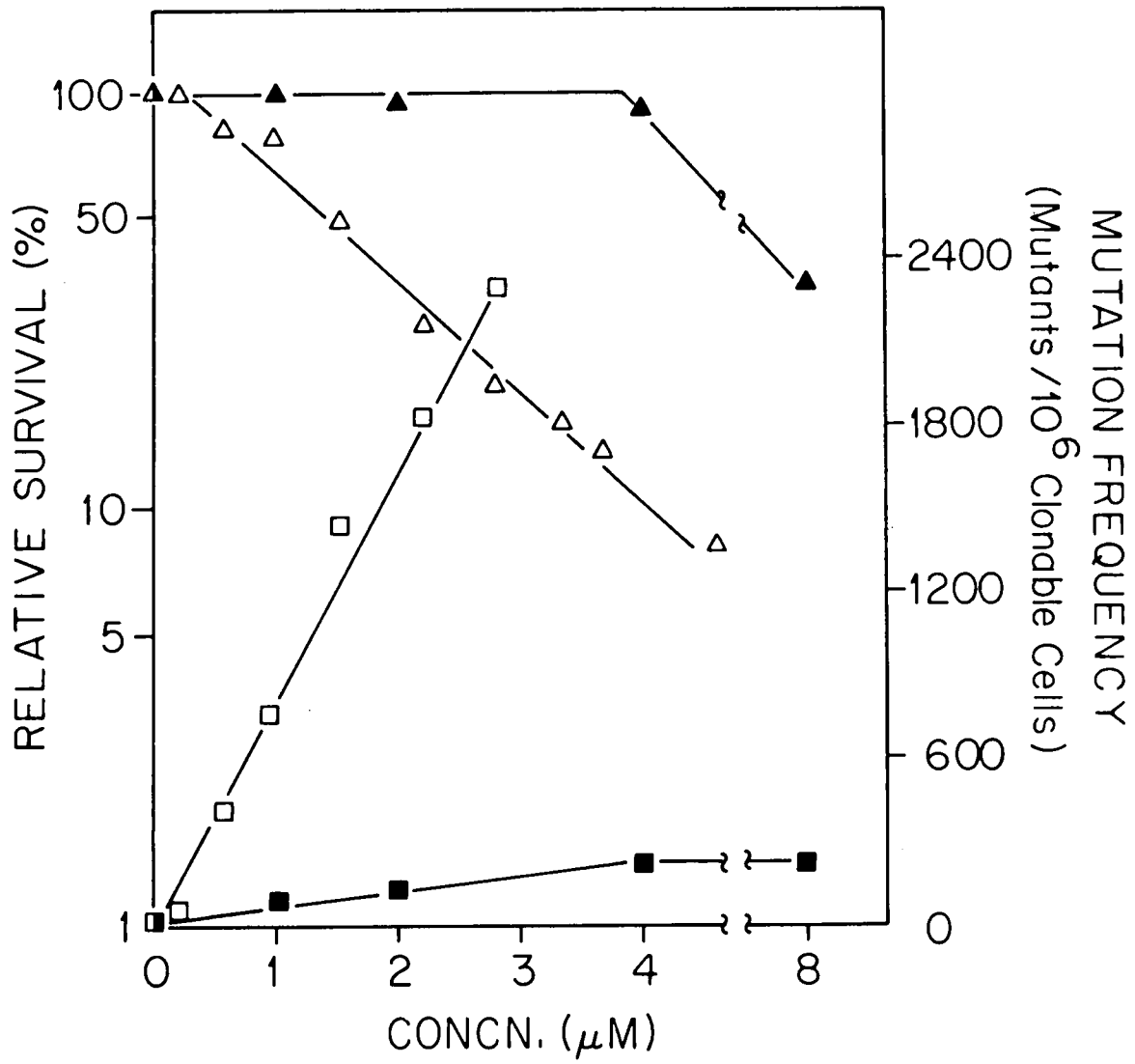


Figure 4. Cell survival and mutation induction as affected by ICR 355 (□,△) and ICR 371 (■,▲). Cultures were incubated with various concentrations (CONCN.) as indicated for 16 hr; single-cell survival (▲,△) and induced MF (■,□) were determined as described. The average values from 3 separate experiments are presented. The spontaneous MF was 1.3.

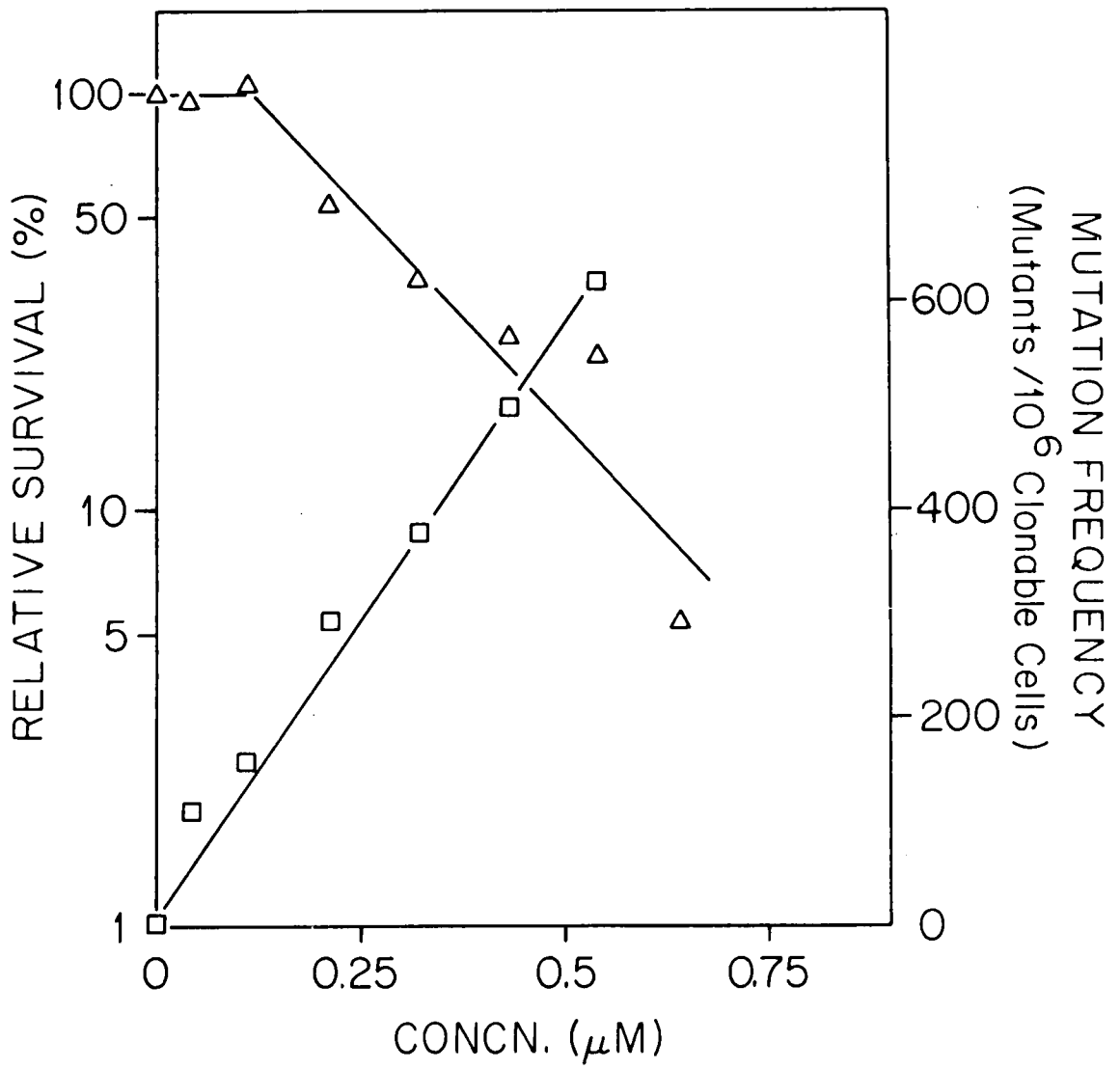


Figure 5. Cell survival and mutation inducation as affected by ICR 368. Cultures were incubated with various concentrations (CONCN.) as indicated for 16 hr; single-cell survival (△) and induced MF (□) were determined as described. The average values from 3 experiments are presented. The spontaneous MF was 3.5.

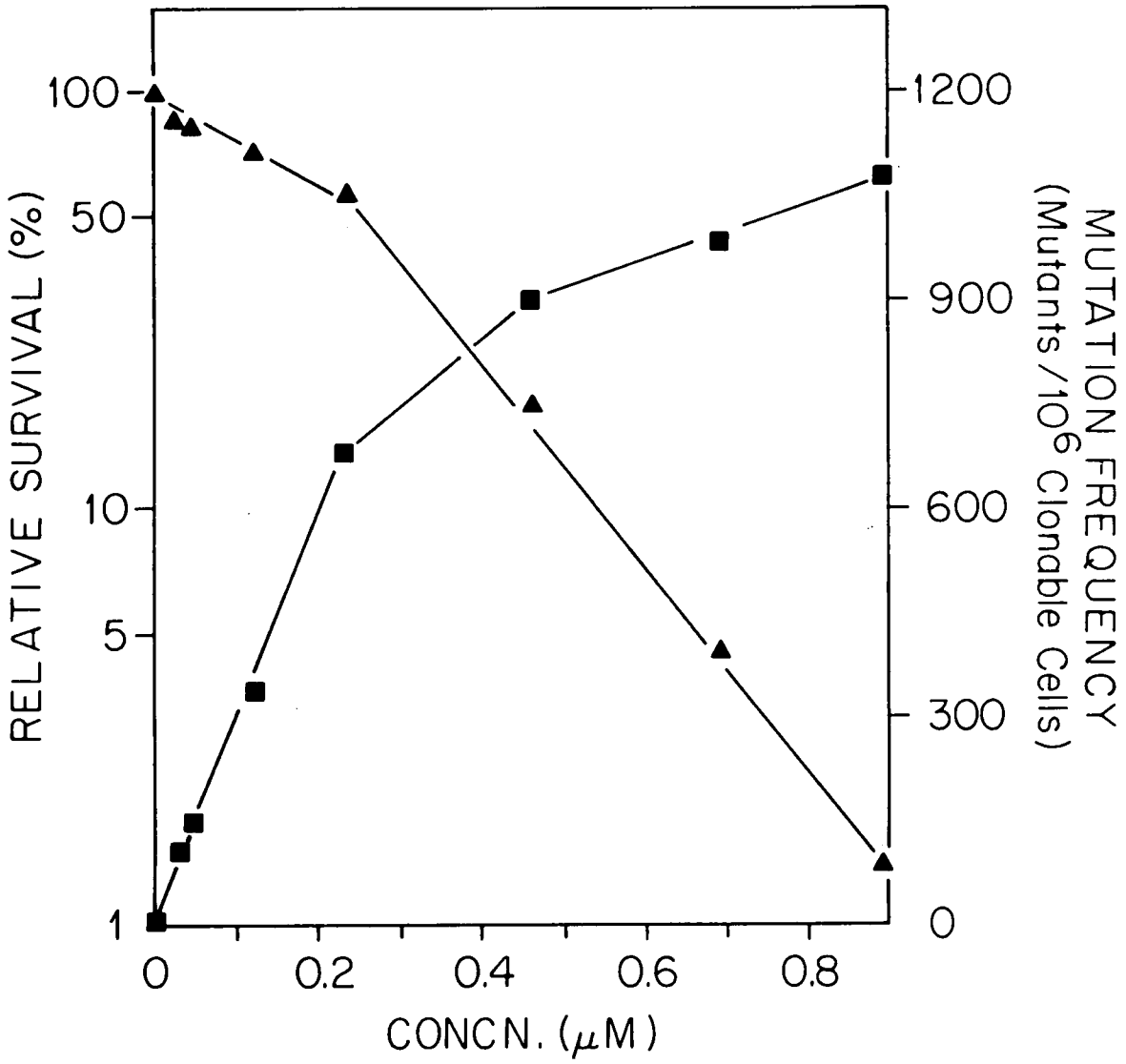


Figure 6. Cell survival and mutation induction as affected by ICR 342. Cultures were incubated with various concentrations (CONCN.) as indicated for 16 hr; single-cell survival (▲) and induced MF (■) were determined as described. The average values from 3 experiments are presented. The spontaneous MF was 12.0.

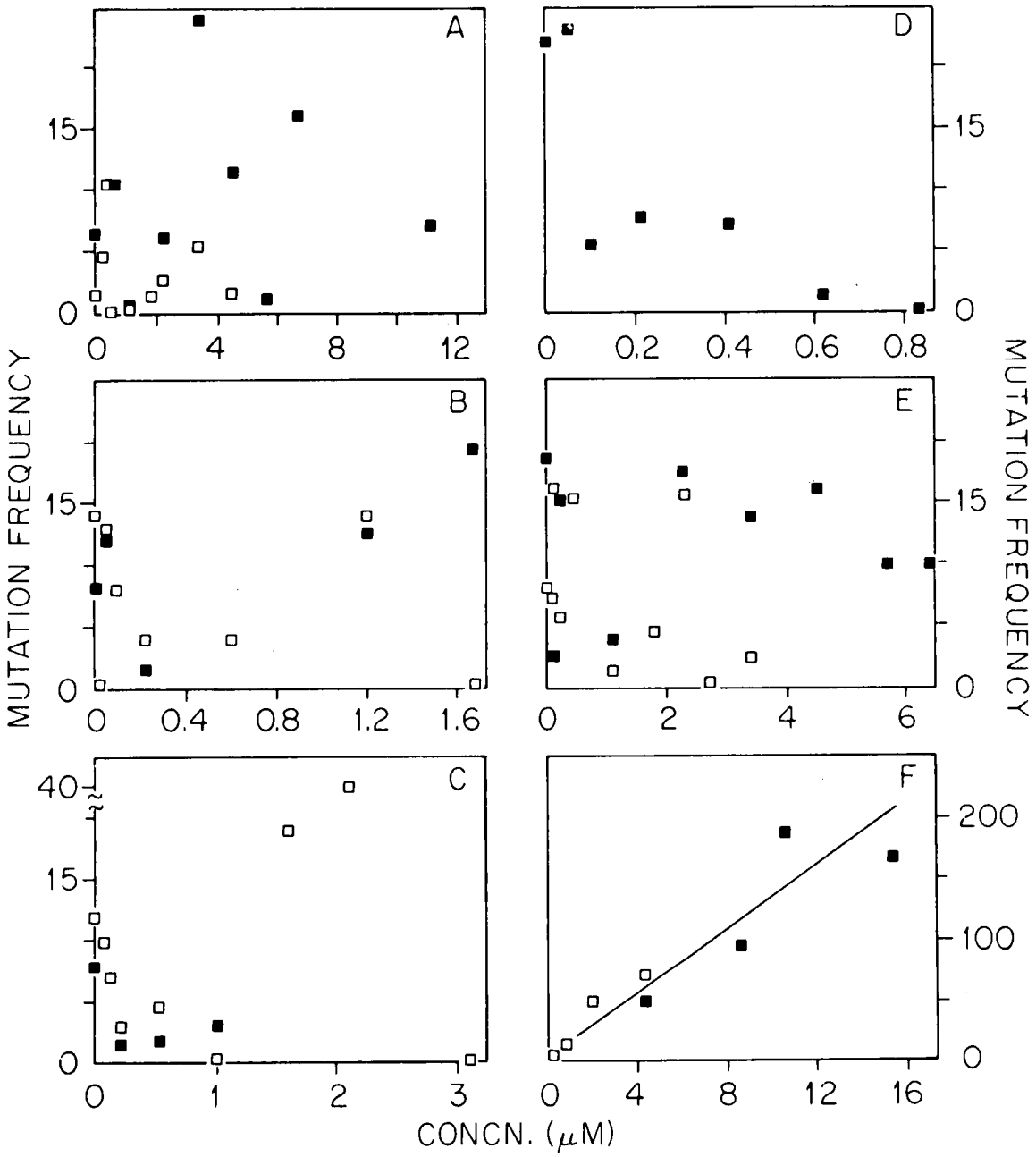


Figure 7. Mutation induction as affected by ICR 340-OH (A), 191-OH (B), 170-OH (C), 292-OH (D), 372-OH (E), and 171 (F). With the exception of D, the values from 2 independent experiments for each compound are presented. In (F) the spontaneous MF was < 1. CONCN., concentration.

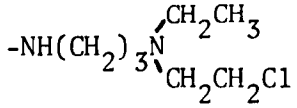
TABLE 2

MUTATION INDUCTION BY ICR COMPOUNDS:
EFFECT OF SIDE CHAIN AND NUCLEUS

	Side Chain					
	Secondary Amine Type[a]				Tertiary Amine Type[b]	
Nucleus	ICR Code	Mutagenic Activity (MF/μM)	Plateau Values[c] MF	μM	ICR Code	Mutagenic Activity (MF/μM)
C	449	140	580	4.2	217	500
G	191	250	750	3.2	170	1550
H	372	270	760	2.8	340	800
I	371	60	230	4.0	355	800
K					292	1700
M					368	1300

[a]The side chain for the secondary amine-type ICR compound is $-NH(CH_2)_3NHCH_2CH_2Cl$.

[b]The side chain for the tertiary amine-type ICR compound is



[c]MF's at the ICR concentration at which plateau of mutation induction begins.

TABLE 3

MUTATION INDUCTION BY OTHER ICR COMPOUNDS

Compound (ICR Code)	Concentration Range (μM)	Relative Survival Range (%)	Mutagenic Activity (MF/μM)
191-OH	0.02- 3.5	100-1	0[a]
170-OH	0.02- 1.5	100-1	0[a]
340-OH	0.02-15.7	100-1	0[a]
372-OH	0.02- 6.8	100-20	0[a]
292-OH	0.05- 1.0	100-1	0[a]
283	0.25-12.5	100-40	0[a]
171	1.1 -15.1	100-50	15
342	0.02- 0.9	100-1	3000[b]
220	0.01- 0.2	100-1	2600[c]

[a]At no concentration did values differ significantly from controls to which no ICR compound had been added.

[b]An extrapolated number since more than 90% of the cells were killed at 0.5 μM.

[c]An extrapolated number since more than 90% of the cells were killed at 0.1 μM.

Discussion

Of the 13 ICR compounds reported here and the 6 others described previously (70), 13 have been shown to be mutagenic in the CHO/HGPRT system. All but 1 of these (ICR 171) were found to possess moderate to high antitumor activity (23). However, 5 of these (ICR 220, 342, 371, 355, and 368) were reported to be nonmutagenic in the Salmonella typhimurium histidine reversion assay (23). ICR 372-OH is not mutagenic in the CHO/HGPRT assay but was slightly mutagenic in the Salmonella test (23). It should be noted that the mutagenicity data in the Salmonella test reported in 1972 (23) employed strains of bacteria different from the ones currently in use (2). The mutagenicity of several of these compounds has been reinvestigated in Salmonella typhimurium strains TA1535, TA1536, TA1537, TA1538, TA98, and TA100; in general, it has been found that the secondary amine-type compounds are more mutagenic than their corresponding tertiary amine analogues based on maximum revertants per nmole of chemicals (personal communications from D. M. De Marini and H. E. Brockman, Illinois State University). This differential activity may reflect actual differences in the mode of action of these ICR compounds in Salmonella and CHO cells, or may result from the fact that the Salmonella assay measures reverse mutation while the CHO/HGPRT assay measures forward mutation. Recently, it has been reported that ICR 191 failed to induce DNA repair in a haploid Rana pipiens cell line and a diploid human fibroblast cell line (93), although San and Stich (87) found that ICR 191 induced DNA repair in another human fibroblast line.

The side chain appears to be more important in determining mutagenic activity than the heterocyclic nucleus (Table 2), although Creech et al.

(23) found that a heterocyclic nucleus of appropriate size and conformation in a monofunctional mustard was necessary for antitumor and mutagenic activity. Tertiary amine-type compounds were at least 3 times as mutagenic as the corresponding secondary amine analogs bearing the same nucleus, and a plateau of mutation induction was observed for secondary amine-type compounds at similar concentrations (Table 2). With 1 exception (ICR 371), MF at the plateau was also similar among these compounds. It is possible that the lower plateau frequency of ICR 371 is due to interaction between the n-butoxy group of the nucleus and the side chain, since the only difference between ICR 372 (which exhibits a plateau MF similar to that of 191 and 449) and 371 is substitution of an n-butoxy group for a methoxy group on the nucleus. ICR 342, a sulfur mustard, also appears to exhibit a plateau (Figure 6, page 22).

When the 2-chloroethyl group is linked by an oxygen atom instead of a nitrogen to the rest of the molecule (ICR 283), mutagenicity is lost and toxicity is low. Because ICR 283 also has 1 less carbon in its side chain, a comparison between it and ICR 191 or 170 (which have the same nucleus as 283) may not be appropriate since the shortening of the chain alone may cause the compound to become inactive. To deal with this possibility, we determined the mutagenicity of ICR 171, which has the same nucleus as ICR 283 and a shortened side chain but has a nitrogen substituted for the oxygen. As illustrated in Figure 7F, ICR 171 was found to have low mutagenic activity. It appears that both the side-chain shortening and the substitution of an oxygen for the nitrogen reduce mutagenic potency. On the other hand, the presence of

2 2-chloroethyl groups on the side chain (ICR 220 versus 217 or 449) or substitution of a sulfur for a nitrogen on the side chain (ICR 342 versus 372) results in greatly increased mutagenicity and cytotoxicity (Tables 2 and 3). On a molar basis, MF is increased more than 5-fold by the addition of a 2nd 2-chloroethyl group and more than 3-fold by the sulfur substitution.

Replacement of the 2-chloroethyl group by a 2-hydroxyethyl group (ICR 191-OH, 170-OH, 340-OH, 372-OH, and 292-OH) results in mutagenically inactive compounds (Figure 7 and Table 3). There also appears to be no direct correlation between mutagenicity and cytotoxicity, since all hydroxyl-substituted analogs are nonmutagenic but exhibit less cytotoxicity (ICR 340-OH), equal cytotoxicity (ICR 372-OH, 292-OH, and 170-OH), or greater cytotoxicity (ICR 191-OH) compared with the parental mutagenic compounds.

The relationships between a concentration-dependent cytotoxicity and mutagenicity for these compounds is of interest in view of the recent proposal that cytotoxicity provides an estimate of "mutagenic potency" in mammalian cells (17). The observation that the hydroxyl derivatives of the ICR compounds are cytotoxic but not mutagenic, and that the mutagenic compounds show activity at concentrations which result in little or no detectable cytotoxicity raises questions about the proposed relationship between these two biological effects. To investigate this further we have applied two methods of data transformation. In the first, shown in Figure 8, the MF is graphed as a function of $\underline{D}/\underline{D}_o$, where $\underline{D}_o$ is the concentration of agent which is needed to reduce cell survival by 63%, as measured in the linear portion of the cytotoxicity curve. The

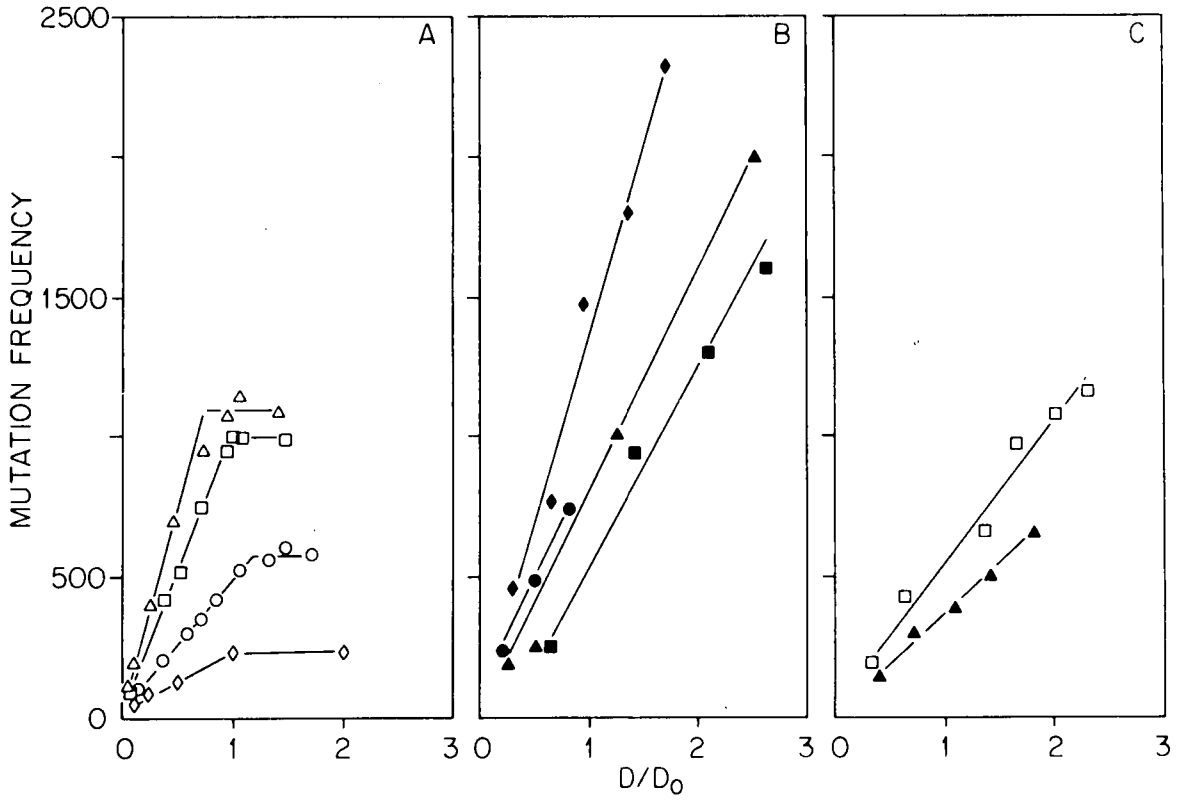


Figure 8. Comparison of the mutagenicity of different ICR compounds. The concentration of each agent is expressed in terms of its D_0 value. A, agents with a secondary amine side chain: ICR 191 (△), 372 (□), 449 (○), and 371 (◇); B, agents with a tertiary amine side chain and the same 4 nuclei as in A: ICR 170 (▲), 340 (■), 217 (●), and 355 (◆). C, 2 other agents with the tertiary amine side chain: 292 (□) and 368 (▲).

average number of lethal events, if the random-hit model is assumed (81). As shown in Figure 8, each of the 10 ICR compounds exhibits an apparent linear increase in mutagenic activity with increasing values of $\underline{D}/\underline{D}_o$. The secondary amine types (Figure 8A) show the plateau in mutagenic activity, while the tertiary amine types (Figure 8B and C) do not. At a $\underline{D}/\underline{D}_o$ value of 1, an MF range of 230 to 1400 is observed for these 10 compounds. The second method, shown in Figure 9, presents the MF as a function of cell survival from 90 to 10%. Due to the variations in the magnitude of the shoulder in the cytotoxicity curve for the 10 compounds, the MF at 100% survival varies from 100 to 600, and an equally wide range of values is found at any given survival, e.g., 230 to 1200 at 50% survival. A similar range of MF at equal cytotoxicity was previously reported with alkylating agents (21, 22). The data of Figures 8 and 9 show that the relationship between mutation induction and cytotoxicity is not the same for these 10 compounds of similar structure. These differences may reflect the fact that mutagenic lesions are not the same as cytotoxic lesions, and show that a comparison of these 2 biological effects of chemicals should be made with caution.

Although mutagenic activity is greatly dependent upon the side chain, the heterocyclic nucleus does play a role in modifying this activity. The addition of a chloro group and a methoxy group (ICR 449 and 217 versus 191 and 170) to the acridine structure increases the mutagenic activity 2- to 3-fold when the side chains are held constant (Table 2, lines 1 and 2, page 24). Introduction of a nitrogen into the nucleus, to form an azaacridine, results in a decrease in mutagenicity when the

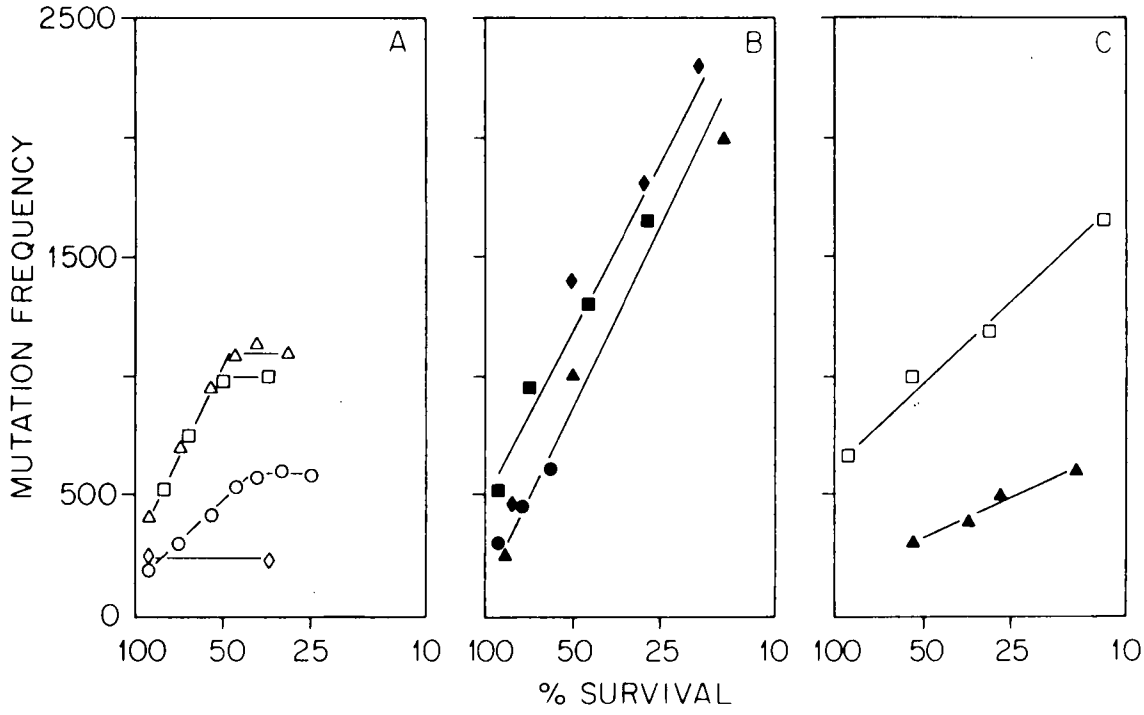


Figure 9. Mutagenicity as a function of cytotoxicity. The MF for the same 10 ICR compounds as in Figure 8 is presented to show the relationship with cytotoxicity. Symbols are the same as in Figure 8.

ratio of a given concentration D, to the D_o value corresponds to the side chain is a tertiary amine (ICR 170 versus 340) and no change in mutagenicity when the side chain is a secondary amine (ICR 191 versus 372) (Table 2, lines 2 and 3). Replacement of the methoxy group on the azaacridine nucleus with an n-butoxy group does not change the mutagenicity when the side chain is a tertiary amine (ICR 340 versus 355) but reduces mutagenicity when the side chain is a secondary amine (ICR 372 versus 371) (Table 2, lines 3 and 4). The finding that modifications of the heterocyclic nucleus do not always affect the mutagenicity of the secondary amine-type and tertiary amine-type compounds in the same way suggests that the side chains may interact differently with the nuclei to induce the mutagenic event.

Tumorigenicity data are available for 6 of these compounds. Four, found to be highly mutagenic in our studies (ICR 170, 340, 292, and 342) are carcinogenic, and the other 2 (ICR 191 and 191-OH) are reported to be noncarcinogenic in a mouse lung tumor system (74, 75). ICR 191-OH is nonmutagenic in the CHO/HGPRT system, but ICR 191 is a potent mutagen not only in our system but also in several others (25, 61). Apparently, ICR 191 is a mutagenic noncarcinogen. This may be due to the fact that these mutagenic studies (25, 61, 70) were conducted without the addition of a metabolic activation system. Our preliminary studies show that the addition of our standard Aroclor 1254-induced rat liver S-9 metabolic activation system to the mutagenesis protocol of ICR 191 greatly reduced the mutagenicity and cytotoxicity of this compound (unpublished observations). Perhaps the lack of carcinogenicity of ICR 191 in the mouse lung tumor system can be explained by its being inactivated in the mouse

at the target site (lung) or before it reaches the lung; thus application of the chemical by another route might induce tumors. The finding that an S-9 system can reduce the mutagenicity and cytotoxicity of a compound such as the direct-acting mutagen ICR 191 indicates a potential problem in quanitification of mutagenic activity and in correlation of chemical mutagenicity in a short-term assay with carcinogenicity in laboratory animals.

ICR compounds are a unique class of mutagens in the CHO/HGPRT system. They induce MF's of more than 10^3, with relative survivals greater than 10% and often greater than 50% (ICR 340 and 355). The finding of distinct differences in the concentration-dependent mutagenicity curves of the secondary amine-type compounds, which exhibit a plateau of mutation induction, and the tertiary amine types, without such a plateau, suggests that different mechanisms of mutation induction are operating in these 2 types of compounds. These might include differences in the types of lesions induced or in the repair or fixation of the lesions. Incomplete phenotypic expression cannot account for the plateau, since in expression time experiments (data not shown) MF reaches a maximum stable value by days 7 to 9 at plateau concentrations. The mechanisms by which these extremely mutagenic chemicals cause mutations while inducing little cellular lethality is currently under investigation.

CHAPTER III

QUANTIFICATION AND CHARACTERIZATION OF REVERSE MUTATIONS AT THE *hgprt* LOCUS IN CHINESE HAMSTER OVARY CELLS

Abstract

We describe an assay for the quantification of reverse mutations at the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) locus in Chinese hamster ovary cells utilizing the selective agent L-azaserine (AS). Conditions are defined in terms of optimal AS concentration, cell density, and phenotypic expression time. After treatment, replicate cultures of 10^6 cells are allowed a 48-hr phenotypic expression time in 100-mm plates. AS (10 μM) is then added directly to the growing culture and AS-resistant (AS^r) cells form visible colonies. This assay is used to quantify ICR 191-, ICR 170-, and N-ethyl-N-nitrosourea-induced reversion of independently isolated $HGPRT^-$ clones. The AS^r phenotype is characterized both physiologically and biochemically. All AS^r clones isolated are stably resistant to AS and aminopterin but sensitive to 6-thioguanine. They also have re-expressed HGPRT enzyme. In addition, several revertants are shown to contain altered HGPRT. The data provide further evidence that ICR 191 and ICR 170 cause structural gene mutations in mammalian cells and also suggest that ICR 191, ICR 170, and N-ethyl-N-nitrosourea induce similar types of mutations in Chinese hamster ovary cells.

Introduction

We (36, 70) have reported the mutagenicity of a series of heterocyclic mustards, the ICR compounds, in Chinese hamster ovary (CHO) cells. Employing a forward mutation assay, CHO/hypoxanthine-guanine phosphoribosyltransferase (HGPRT) (69), which utilizes the purine analog 6-thioguanine (TG) to select for mutants at the hgprt locus in CHO cells, various relationships between structure and mutagenicity were established. One of the most intriguing findings was a distinct difference in mutagenicity between those compounds with a secondary amine side chain and those with a tertiary amine side chain. The tertiary amine-type compounds, such as ICR 170, were always more mutagenic than their secondary amine-type analogs, such as ICR 191, at equimolar concentrations. Furthermore, all of the secondary amine-type, but none of the tertiary amine-type, compounds exhibit a plateau of mutation induction in their concentration-dependent mutagenicity curves. This suggested different mechanisms of mutation induction may be operating in these two types of compounds.

While a mutation in any of a number of locations in a gene may cause loss of a functional gene product, compensation for the original lesion would be expected to require a much more specific mutation. Measuring reverse mutations, therefore, may give insights into the types of mutations induced by physical and chemical agents (4). In order to investigate possible differences in the types of mutations induced by these compounds, we studied the reversion of independently isolated ICR 191- and 170-induced TG-resistant (TG^r) clones by ICR 191, ICR 170, and

the alkylating agent N-ethyl-N-nitrosourea (ENU). The types of mutations induced in bacteria by these three chemicals have been inferred from reversion studies in which it was found that ICR 191 and 170 cause mostly frameshifts (1, 3, 68) and ENU causes mostly base-pair substitutions (89). Some studies in microorganisms have extended the analysis of ICR 191-induced mutants to the amino acid sequence level and found evidence consistent with a frameshift mutation (11, 42, 54).

In mammalian cells, medium containing aminopterin or amethopterin (HAT medium) has been used extensively to select for revertants at the hgprt locus (18, 20, 31, 47, 48, 77). However, the ability to grow in HAT medium does not necessarily reflect the re-expression of HGPRT (48; Machanoff and O'Neill, unpublished observations).

For this reason, we have chosen to redefine conditions for the selection of revertants utilizing the glutamine analog L-azaserine (AS). AS acts as an irreversible competitor of glutamine in the de novo purine biosynthetic reaction catalyzed by phosphoribosyl-formylglycineamidine synthetase (46). The ability of $HGPRT^-$ clones to grow in AS, therefore, is dependent upon the re-expression of HGPRT. Parameters which affect quantification of reversion at the hgprt locus have been defined including AS concentration, cell density, phenotypic expression time, and selective stringency.

Reversion analysis of various TG^r clones, as well as physiological and biochemical characterization of TG^r mutants and their revertants, provides further evidence of the genetic origin of ICR 191- and 170-induced TG resistance. Preliminary results have been reported previously (35).

Materials and Methods

Cell lines and cell culture methods. A subclone of the CHO-K_1 cell line, CHO-K_1-BH_4 (52), was used as the parental cell line in this study. Stock cultures were routinely maintained in monolayer in Ham's F12 medium supplemented with 5% fetal bovine serum (F12FCM5) under conditions of 5% CO_2 in air at 37° in an incubator humidified to 100%. All sera (K. C. Biological) were heat inactivated and extensively dialyzed in this laboratory. Cells were removed with 0.05% trypsin for subculture.

Selection for ICR 191- and ICR 170-induced TG resistance has been described by Fuscoe et al. (36). TG^r clones were obtained by picking individual colonies from TG-selection plates with a Pasteur pipette and replating them in hypoxanthine (Hx)-free F12FCM5 containing 10 μM TG. Five to seven days later the cells were placed in nonselective medium F12FCM5. All TG^r clones used in this study were independently isolated and found to maintain their TG^r phenotype after growth under nonselective conditions for at least 100 population doublings (2 months).

Chemicals. ICR 191 and ICR 170 were a gift from R. M. Peck of the Institute for Cancer Research, Fox Chase, Pennsylvania, and were prepared at 1 mg/ml in 0.01 N HCl. ENU (NCI Repository and IIT Research Institute) was prepared in dimethyl sulfoxide at 100 μM immediately prior to use. Stock solutions of 10^{-2}M AS (Calbiochem) were prepared in H_2O and stored frozen for a maximum of 3 weeks. The sodium salt of 5-phosphoribosyl-1-pyrophosphate (PRPP) and the ^{3}[H]Hx were purchased from Sigma and Schwarz/Mann, respectively.

Mutagen treatment and selection for resistance to AS. Cells (3.5×10^6 TG^r) were plated in 150 sq cm Corning flasks containing 20 ml F12FCM5 and incubated for 24 hr. The chemical agent, with appropriate solvent controls, was added, and the cultures incubated for an additional 24 hr. The cells were then washed three times with phenol red-free Saline G (82) and removed with trypsin for cell number determination.

For determination of the effect of mutagen treatment on survival (cellular cloning efficiency), 200 cells were plated in triplicate on 60-mm dishes in F12FCM5. Seven days later, the colonies were fixed, stained, and counted. Untreated controls exhibit 80-100% cloning efficiency. Revertants were determined by plating 10^6 cells in each of 6-10 100 mm dishes containing F12FCM5 (10 ml total). This represents at least 1/2 of the culture. Forty-eight hr later the medium was changed to 10 μM AS in F12FCM5 (AS-F12FCM5). After incubation for an additional 7 days, colonies were fixed, stained, and counted. In determining the phenotypic expression time 42×10^6 ICR 191-induced $HGPRT^-$ cells (clone J1) were treated with 4.4 μM ICR 191 for 24 hr. Aliquots of 10^6 cells were plated on 150-mm dishes in F12FCM5 and divided into seven groups of 10 plates each. Each group, therefore, contained 10^7 treated cells. At 12-hr intervals the media of one group was changed to AS-F12FCM5 and incubated for 8 days, at which time colonies were fixed, stained, and counted. Reversion frequency is expressed as revertants/10^7 clonable cells and was calculated as the number of revertant colonies divided by the number of cells plated and corrected for the survival (cloning efficiency) of the culture.

HGPRT enzyme assay. Cell-free extracts were prepared according to a modified method of Capecchi et al. (14). Briefly, 60-80% confluent cultures were washed three times with Saline G and drained for 5 min. At 4°, extract buffer (10 mM Tris, 10 mM $MgCl_2$, 30 mM KCl, 1 mM DTT, 1% Triton X-100, pH 7.0 for specific activity studies, or 50 mM KH_2PO_4, 1 mM DTT, 1% Triton X-100, pH 7.0 for heat inactivation studies) was spread over the plate. After 20 min, the plate was tilted, drained for 5 min and the extract collected. Following centrifugation at 20,000 × g for 20 min, the supernatant fraction was collected. Revertant extracts were prepared immediately prior to use. Wild-type extracts could be stored for 2 weeks at -100° with little loss of activity.

Protein concentrations were determined as described by Lowry et al. (59). HGPRT enzyme activity was measured in extracts as described previously (52) except that the assay was performed at 35°. The reaction mix contained 20 µl of 4 mg/ml PRPP dissolved in Tris buffered salt solution (250 mM Tris, 25 mM $MgCl_2$, 50 mM KCl, pH 7.5), 20 µl of ^{3}[H]Hx (approximately 0.2 mCi/ml; specific activity approximately 3.6 Ci/mmol) and the reaction was initiated by the addition of 40 µl cell extract. HGPRT activity was measured at four or five reaction times and the specific activity was calculated from the fitted linear regression line. The specific activity of wild-type cell extracts is 3.0-5.0 nmol/min/mg protein.

Heat inactivation. Preliminary experiments indicated that incubation of cell-free extracts prepared in the previously described extract buffer at 74° gave a satisfactory heat inactivation profile for wild-type enzyme.

This temperature was used throughout this study. Samples (0.1 ml) were placed in 250-µl polyethylene tubes and capped. An aluminum heating block with holes to accommodate these tubes was maintained at 74° ± 0.1°. Duplicate samples were placed in the block, removed at various times, and placed in a similar block at dry-ice temperatures. Within 2 hr, the samples were thawed, vortexed, centrifuged, and 40 µl samples assayed for HGPRT activity as described above.

Results

Twenty-four ICR 170- and 39 ICR 191-induced TG^r clones are characterized in Table 4. Twenty-four/twenty-four ICR 170-induced TG^r clones and 38/39 ICR 191-induced TG^r clones are stably resistant to TG and sensitive to AS and aminopterin (AP). Eight/eight independently isolated ICR 170-induced TG^r clones and 9/10 independently isolated ICR 191-induced TG^r clones lack detectable HGPRT activity. These $HGPRT^-$ clones were used in subsequent experiments. One ICR 191-induced clone, designated I2, contains 5% wild type enzyme activity and is capable of growing in both TG and AP, but not AS.

Analysis of kinetic properties and thermal sensitivity of HGPRT in I2 showed that the K_m for PRPP is six times that of wild type and the V_{max} 15 times lower than wild type (data not shown). Heat inactivation profiles showed that wild type enzyme has a heating time in min required to reduce the initial specific activity by one-half (t1/2) of approximately 13.7 min while I2 enzyme has a t1/2 of approximately 0.7 min at 74° (Figure 10).

TABLE 4

CHARACTERISTICS OF TG^r CLONES

Mutagen	Cloning Ability*					HGPRT Enzyme	
	F12	TG	AP	AS	Proportion	Specific Activity	Proportion
ICR 170	+	+	-	-	24/24	<1%	8/8
ICR 191	+	+	-	-	38/39	<1%	9/9
	+	+	+	-	1/39	5%	1/1

*F12, TG, AP, and AS designate F12FCM5, Hx-free F12FCM5 containing 10 μM TG, F12FCM5 containing 1 μM AP, and F12FCM5 containing 10 μM AS, respectively. (+), 75-100% cloning efficiency. (-), <1% cloning efficiency. Specific activity is expressed as percentage of wild type in cell free extracts as described in materials and methods.

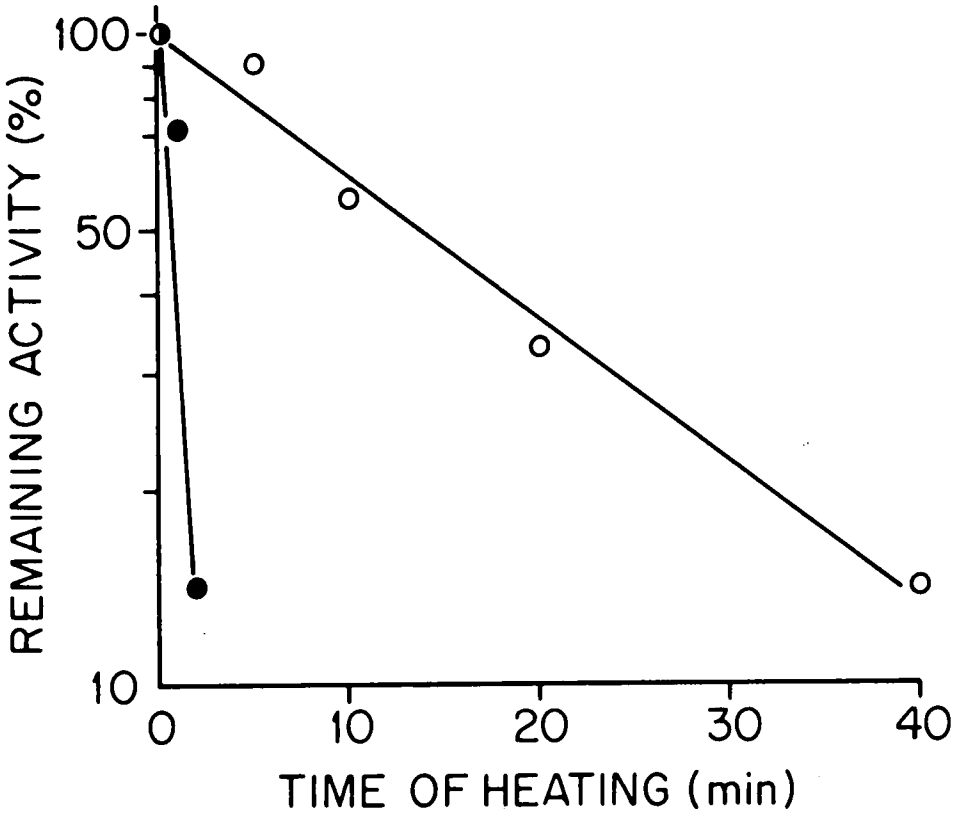


Figure 10. Heat inactivation profile of HGPRT activity in cell free extracts from wild type (○) and I2 (●) cells at 74°. Specific activities at 0 time were 4.17 nmol/min/mg protein for wild type and 0.18 nmol/min/mg protein for I2 cells. I2 is an ICR 191-induced TG^r clone.

In studying the effects of AS on cellular cloning efficiency, it was found that AS concentrations of 0.5-50 μM in F12FCM5 resulted in essentially 100% cloning efficiency of $HGPRT^+$ cells and $<10^{-6}$ cloning efficiency of $HGPRT^-$ cells (Table 5). AS (10 μM) in F12FCM5 (AS-F12FCM5) was used for all subsequent experiments.

The time-dependent killing of $HGPRT^-$ cells in AS-F12FCM5 (Figure 11A) showed that after 1 day in AS-F12FCM5 the cloning efficiency of the cultures was reduced to approximately 3%. This high initial rate of killing is then replaced by a more gradual loss of cellular cloning efficiency, about 50% per day. There is a loss of approximately 50% of the cells during the first 2 days in AS-F12FCM5 followed by no change in cell number to day 7 (Figure 11B). The initial loss of cells from the plate could reflect either a failure of some cells to attach in AS-F12FCM5 or a fraction of the dead cells detaching from the plate. A comparison of the data on the colony forming ability of $HGPRT^-$ cells in Table 5 (10 μM AS) and Figure 11 shows that while $HGPRT^-$ cells cannot form colonies in AS-F12FCM5 (Table 5), $HGPRT^-$ cells remain viable after 7 days in AS-F12FCM5 at a frequency of 10^{-4} - 10^{-3} and can be rescued by replating in nonselective F12FCM5 (Figure 11). Therefore, it is particularly important when picking AS^r colonies to replate the cells in AS-F12FCM5. Otherwise, it is possible a viable $HGPRT^-$ cell may contaminate the AS^r clone.

Based upon preliminary experiments, we hypothesized that the expression time would be short for the reverse mutation compared to the 7-9 day expression time for the forward mutation at the hgprt locus (35, 70, 72). Preliminary data also suggested that the reverse mutation

TABLE 5

EFFECT OF AS CONCENTRATION ON CELLULAR CLONING EFFICIENCY OF $HGPRT^+$ AND $HGPRT^-$ CELLS

AS Concentration (μM)	Absolute Cloning Efficiency	
	Wild Type*	$HGPRT^-$ Mutant†
0	1.00	0.95
0.5	0.88	$<1 \times 10^{-6}$
1.0	1.07	$<1 \times 10^{-6}$
5.0	1.10	1×10^{-6}
10	1.02	$<1 \times 10^{-6}$
50	0.96	$<1 \times 10^{-6}$

*One hundred wild-type cells were plated in F12FCM5 containing the indicated concentrations of AS. After 7 days incubation, colonies were fixed, stained, and counted.

†One hundred $HGPRT^-$ cells were plated in F12FCM5 and 10^6 $HGPRT^-$ cells were plated in F12FCM5 containing the indicated concentrations of AS. After 7 days incubation, colonies were fixed, stained, and counted.

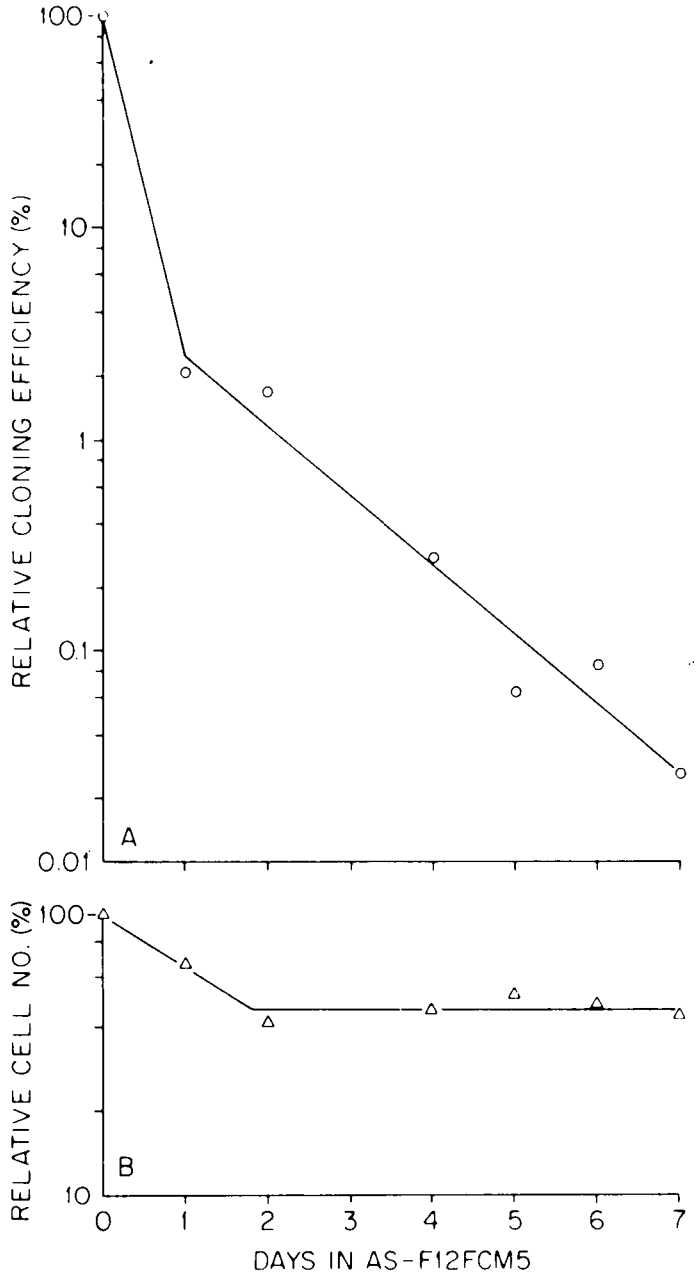


Figure 11. Time course of AS-induced killing of HGPRT$^-$ cells. (A) The effect on clonal growth: 10^6 cells were plated in replicate in AS-F12FCM5 on day 0. Absolute cloning efficiency was 1.30. At various times cells were washed, removed with trypsin, counted, and replated in F12FCM5. After 7 days incubation, colonies were fixed, stained, and counted. (B) The effect on population growth: Relative cell number of the cultures as a function of days in AS-F12FCM5.

frequency would be 1-2 orders of magnitude lower than the forward mutation frequency. Therefore, the cells were plated in nonselective F12FCM5 after treatment and allowed time to express the revertant phenotype; then selective medium was added directly to the growing culture (in situ expression). In this way we avoided sampling errors which subculture might introduce and also allowed the selection of large numbers of cells in relatively few plates.

To assess the effects of $HGPRT^-$ cells on the colony-forming ability of $HGPRT^+$ cells, a reconstruction experiment was conducted in which 10^6 $HGPRT^-$ cells and 100 $HGPRT^+$ cells were cocultured in F12FCM5 (Figure 12). At various times the media was replaced with AS-F12FCM5. There was full recovery of $HGPRT^+$ colonies after 0-72 hr of growth before the addition of AS. Thus, there appears to be no effect on recovery of $HGPRT^+$ cells under these conditions.

An experiment was then performed to determine the expression time of the AS-resistant (AS^r) phenotype utilizing in situ expression. As shown in Figure 13, even immediately after treatment (0 hr) there is expression of the AS^r phenotype. The frequency increases for about 36 hr, after which it remains constant to 72 hr. The expression time is short for the reverse mutation as compared to the forward mutation. A 48-hr expression was used in all subsequent experiments.

Thirty-two AS^r clones derived from independently isolated TG^r clones after treatment with ICR 191 and 170 have been isolated under these defined conditions of AS concentration, cell density, and expression time. All 32 clones are stably resistant to 1 μM AP and 10 μM AS but

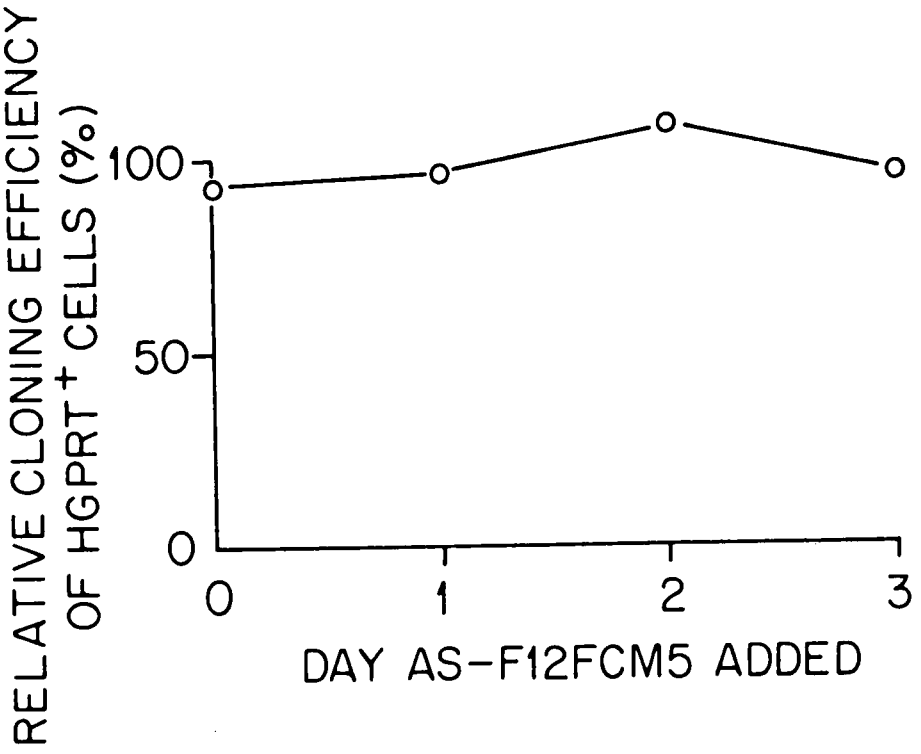


Figure 12. Effect of cocultivation of HGPRT$^-$ and HGPRT$^+$ cells on the recovery of HGPRT$^+$ cells. HGPRT$^-$ (10^6) cells and 100 HGPRT$^+$ cells were plated at time 0 in 10 ml F12FCM5 on 100-mm dishes. After 24, 48, and 72 hr, the medium was changed to AS-F12FCM5. Eight days later, colonies were fixed, stained, and counted. Absolute cloning efficiency of HGPRT$^+$ and HGPRT$^-$ cells was 1.05 and 0.81, respectively, under nonselective conditions.

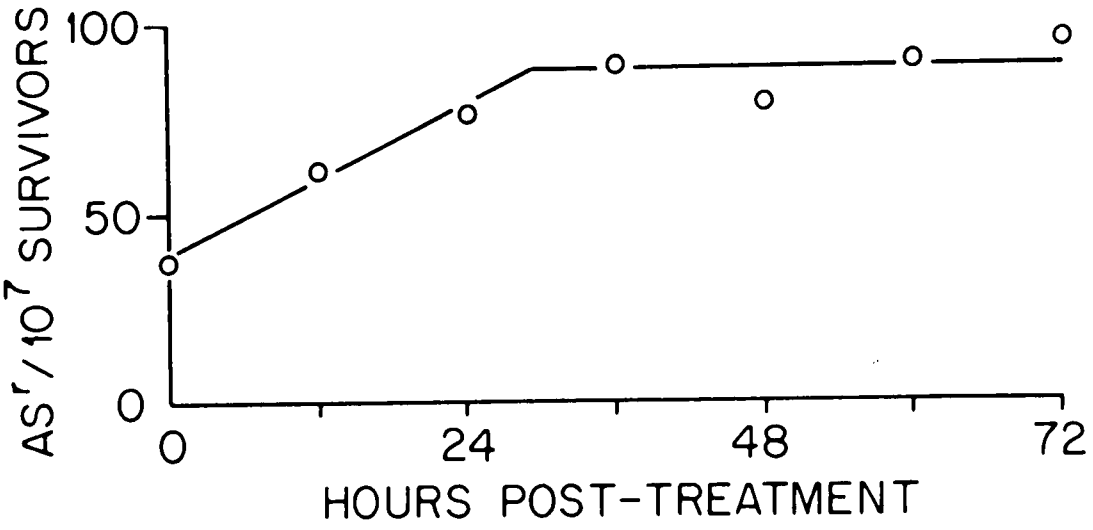


Figure 13. Phenotypic expression time of AS^r phenotype. ICR 191-induced $HGPRT^-$ cells (clone J1) were treated with 4.4 μM ICR 191 for 24 hr. The absolute cellular cloning efficiency of the treated culture was 0.70. Aliquots were plated in F12FCM5 as described and the media changed to AS-F12FCM5 at the times indicated. Spontaneous reversion frequency of an untreated control culture was 1×10^{-7}/clonable cell at 0 hr and 2×10^{-7}/clonable cell at 72 hr.

sensitive to 10 μM TG. They have also re-expressed the enzyme HGPRT. Table 6 summarizes the HGPRT specific activities of 27 revertant clones derived from 6 independently isolated $HGPRT^-$ clones.

Figures 14 and 15 illustrate the concentration-dependent increase in reversion frequency of two independently isolated ICR 191-induced $HGPRT^-$ clones treated with ICR 191. Induced reversion of clone C1 (Figure 14) and clone J1 (Figure 15) can be expressed as approximately 1×10^{-7}/clonable cell/μM ICR 191 and 29×10^{-7}/clonable cell/μM ICR 191, respectively. While there is nearly a 30-fold difference in revertibility between these 2 clones, their sensitivity to ICR 191-induced cellular lethality is nearly identical (Figure 16).

In attempts to analyze any differences in the types of mutations induced by ICR 191 and ICR 170, reversion frequencies after treatment with ICR 191, ICR 170, and ENU of 12 independently isolated $HGPRT^-$ clones (6 induced by ICR 191 and 6 induced by ICR 170) were measured (Tables 7 and 8). While all ICR compound-induced TG^r clones are revertible by the two ICR compounds, most are also revertible by ENU.

We have further examined the HGPRT enzyme in revertants in attempts to discover revertants with second site mutations. Heat inactivation profiles of two revertants which contain HGPRT with altered thermal sensitivities are shown in Figure 17. Clones 511 and 604 have t1/2's of 10.7 and 7.6 min, respectively. Heat inactivation profiles of 19 revertants, including those in Figure 17, are summarized in Table 9, and it appears that only 2/19 revertants (clones 511 and 604) have significantly altered t1/2's.

TABLE 6

HGPRT ACTIVITY OF AS^r REVERTANTS

TG^r (HGPRT-) Mutant	Number of Revertant Clones	Specific Activity (% of Wild Type)							
		0-9	10-29	30-59	60-74	75-99	100-124	125-149	150-250
E2	5	0	0	0	1	2	2	0	0
H1	3	0	0	0	1	2	0	0	0
J1	8	0	0	0	0	0	5	3	0
201	4	0	1	0	2	1	0	0	0
204	5	1	0	1	0	1	0	0	2
207	2	0	0	0	0	0	1	1	0
Total	27	1	1	1	4	6	8	4	2

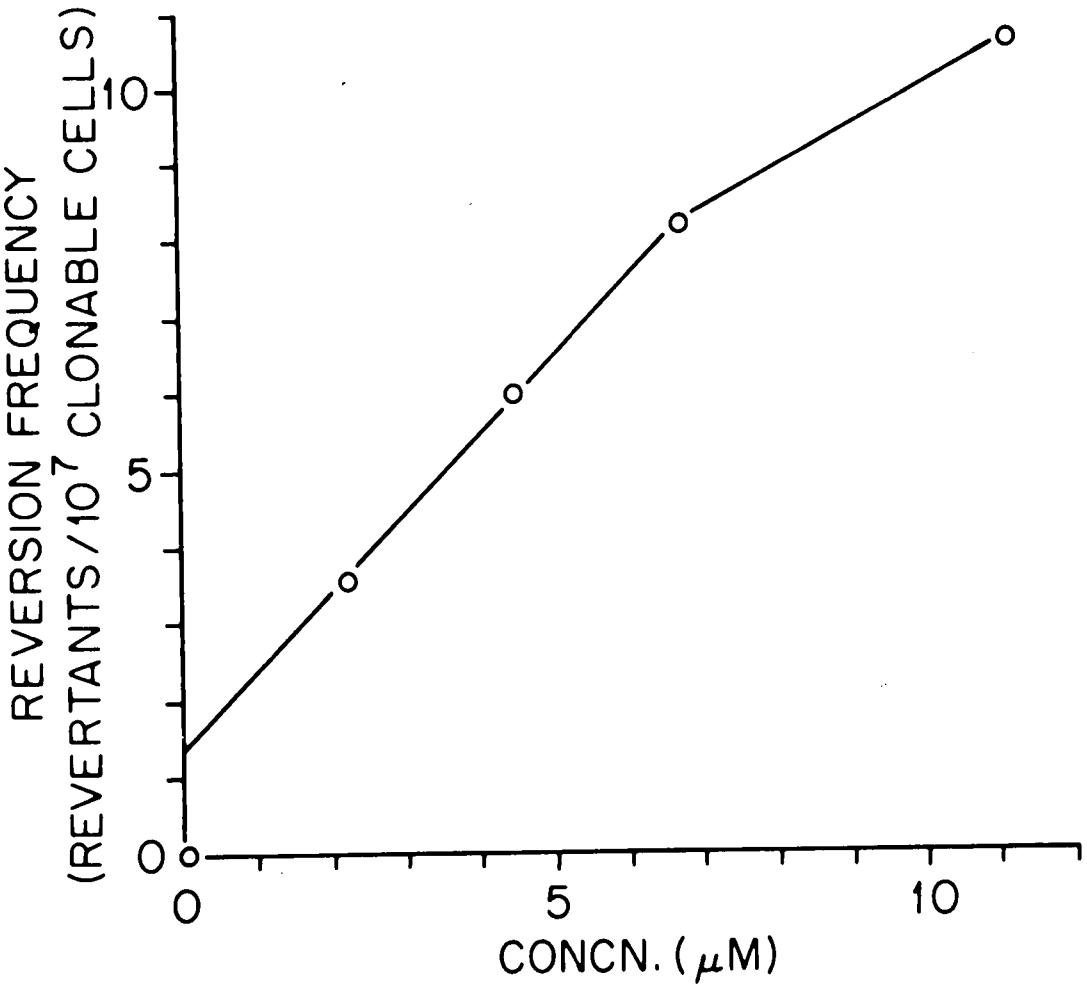


Figure 14. A concentration-dependent induction of reversion to AS^r phenotype of clone C1 by ICR 191. Clone C1 is an ICR 191-induced TG^r clone and has no detectable HGPRT activity. Cultures were incubated with the indicated concentrations (CONCN.) of ICR 191 for 24 hr. Spontaneous reversion frequency was 1×10^{-7}/clonable cell.

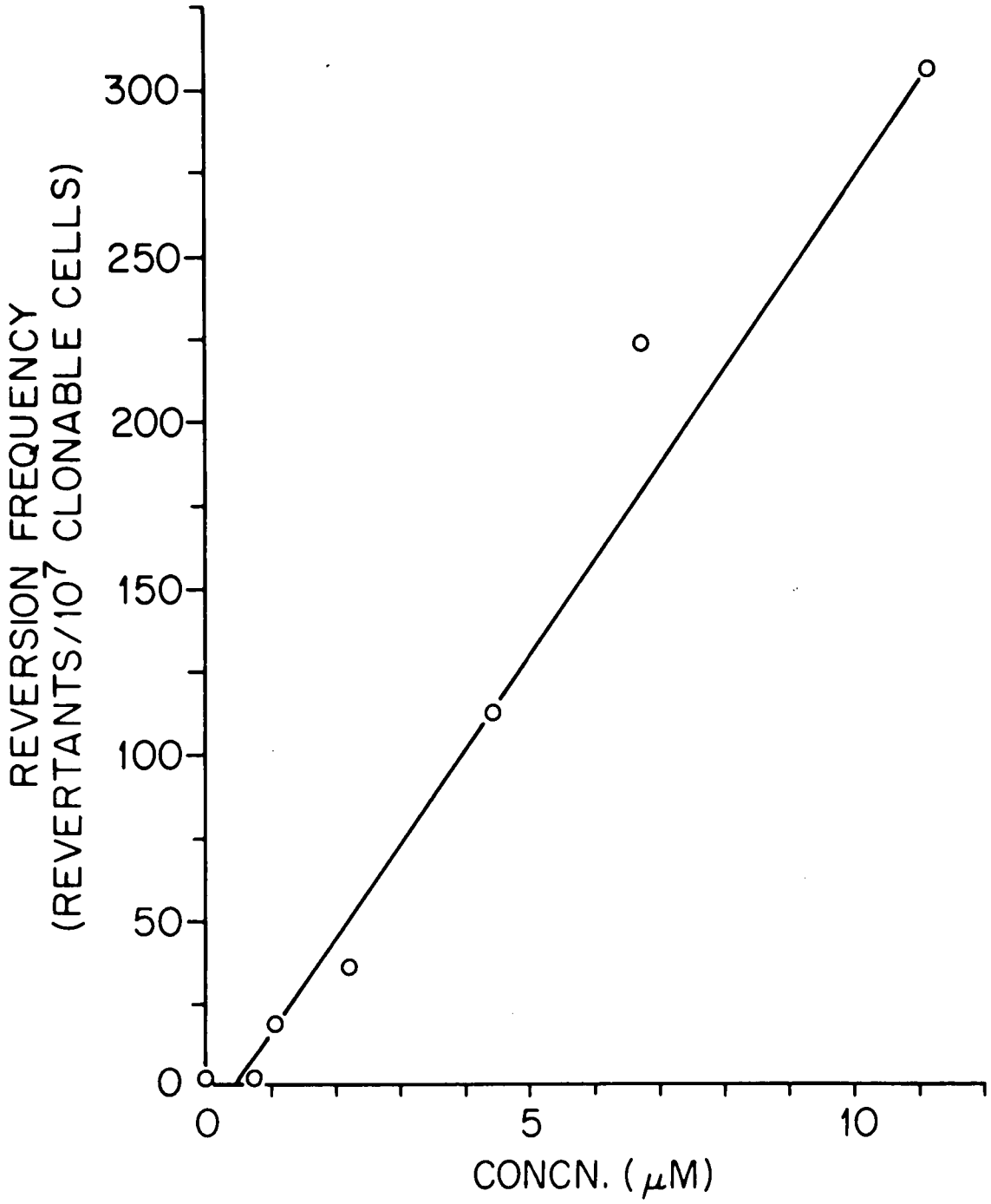


Figure 15. A concentration-dependent induction of reversion to AS^r phenotype of clone J1 by ICR 191. Clone J1 is an ICR 191-induced TG^r clone and has no detectable HGPRT activity. Cultures were incubated with the indicated concentrations (CONCN.) of ICR 191 for 24 hr. Spontaneous reversion frequency was 1×10^{-7}/clonable cell.

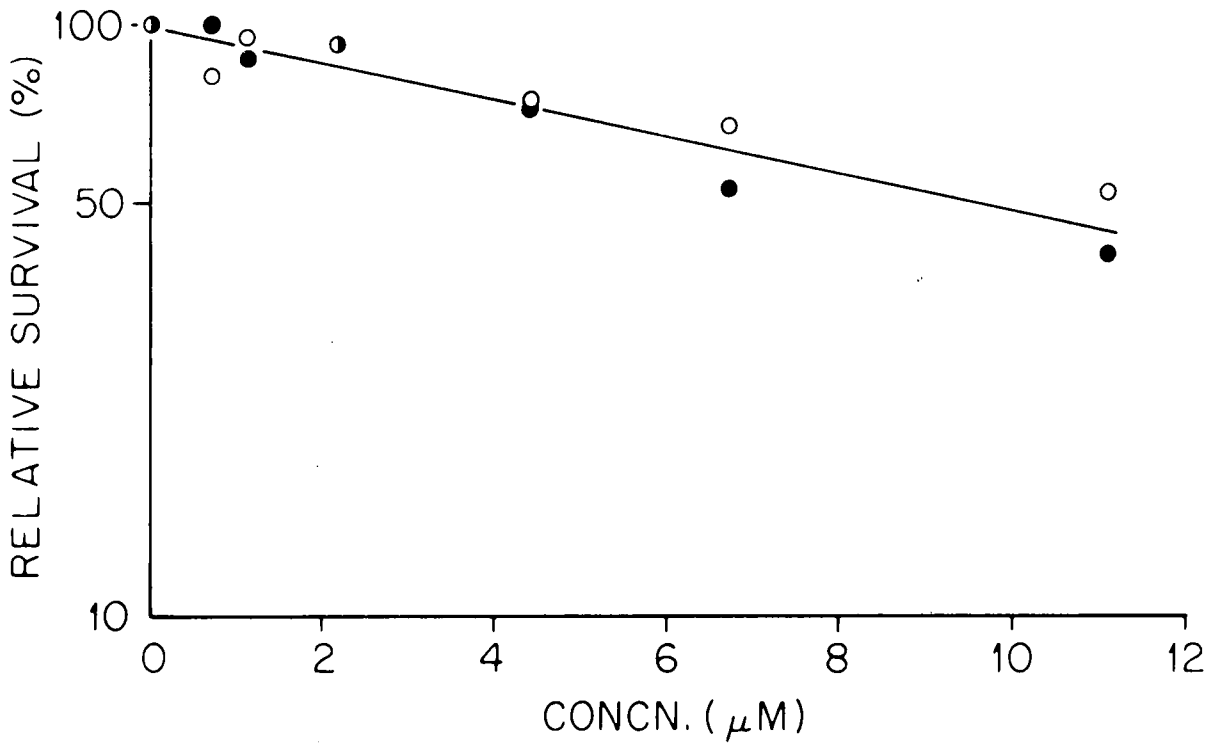


Figure 16. The effect of ICR 191 on the cloning efficiency of clone C1 (○) and clone J1 (●). Cultures were incubated with the indicated concentrations (CONCN.) of ICR 191 for 24 hr. Absolute cloning efficiency of an untreated control culture was 0.91 for clone C1 and 0.80 for clone J1.

TABLE 7

REVERTIBILITY OF ICR 191-INDUCED TG^r CLONES

	Revertants per 10^7 Clonable Cells*			
Clone	Untreated	ICR 191	ICR 170	ENU
J1	0.9	178	92	68
E2	<0.9	43	58	20
H1	<1	24	10	< 2
G1	<0.7	12	3	< 2
D6	<0.7	10	5	< 2
C1	<0.7	10	6	< 2

*Mutagen concentration and relative survival of treated cultures were as follows: 4.4 μM ICR 191, 65% (range = 57-72%); 0.6 μM ICR 170, 60% (range = 50-67%); 1.0 mM ENU, 50% (range = 38-61%).

TABLE 8

REVERTIBILITY OF ICR 170-INDUCED TG^r CLONES

Clone	Revertants per 10^7 Clonable Cells*			
	Untreated	ICR 170	ICR 191	ENU
218	0.7	140	140	121
207	<1	80	76	< 7
211	0.7	74	133	163
201	<1	56	76	56
204	<1	38	48	8
224	<0.8	37	87	91

*Mutagen concentration and relative survival of treated cultures were as follows: 4.4 μM ICR 191, 70% range = 61-82%); 0.6 μM ICR 170, 54% (range = 44-61%); 1.0 mM ENU, 49% (range = 43-57%).

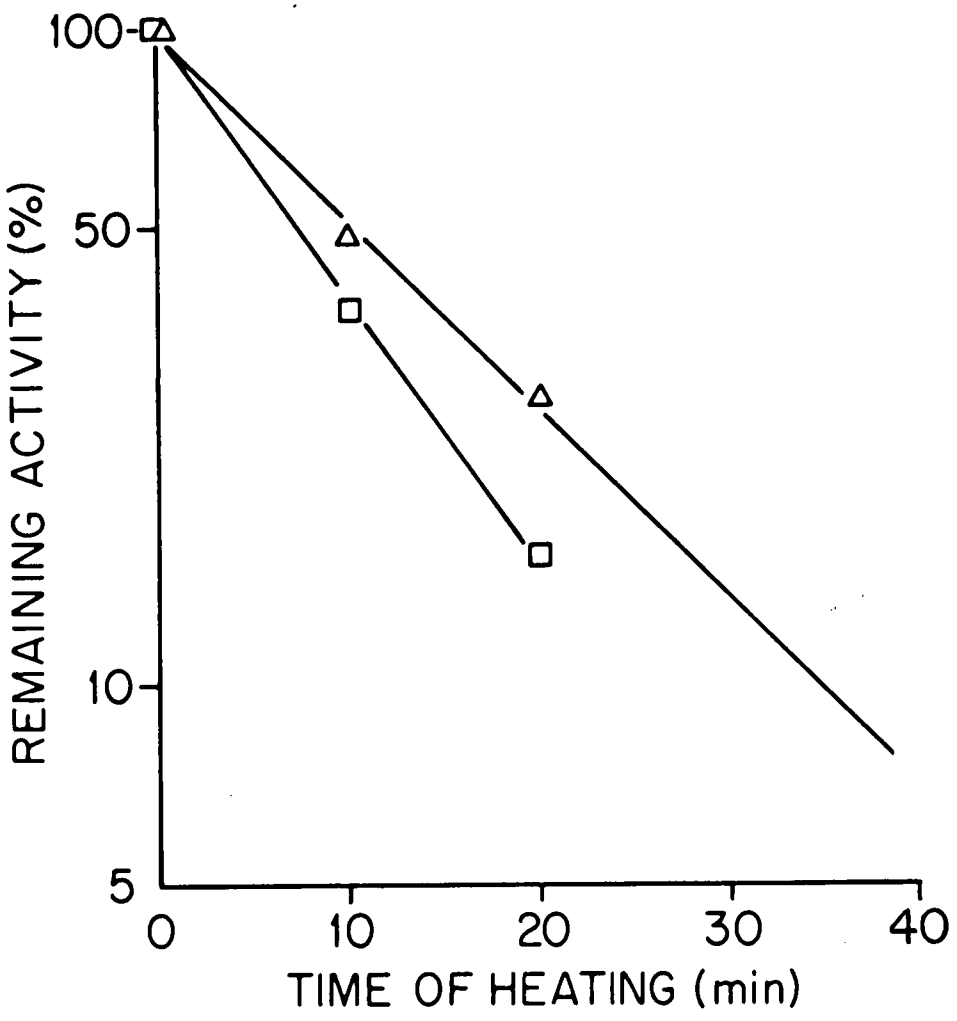


Figure 17. Heat inactivation profile of HGPRT activity in cell-free extracts from revertant clones 511 (△) and 604 (□) at 74°. Specific activities at 0 time were, 1.05 and 0.38 nmol/min/mg protein for 511 and 604, respectively.

TABLE 9

HGPRT HEAT STABILITY OF REVERTANTS

TG^r (HGPRT-) Mutant	AS^r ($HGPRT^+$) Revertant	Specific* Activity	t1/2†
E2	351	1.03	13.7
	352	0.73	14.9
	353	0.92	14.5
	403	0.99	12.9
H1	356	0.65	16.0
	404	0.97	13.8
J1	357	1.46	13.2
	359	1.49	15.0
	364	1.21	14.1
	367	1.23	15.5
	407	1.08	13.8
	408	1.14	13.2
	410	1.00	13.3
	411	1.29	11.7
201	511	0.28	10.7
	601	0.69	13.3
204	605	2.44	12.9
	604	0.09	7.6
207	607	1.43	11.5

*Specific activity is expressed as the ratio of revertant/wild type specific activity.

†t1/2 is the heating time in min required to reduce the initial specific activity by one-half. t1/2 for wild type ranged from 11.5-13.7 and averaged 12.9.

Discussion

In order to develop a quantitative assay measuring reverse mutations at the hgprt locus in CHO cells, we have defined (i) the concentration of the selective agent AS, (ii) optimal cell density conditions under which there is no effect on revertant recovery, and (iii) the phenotypic expression time, that is, the time needed for the change at the level of the gene to be reflected at the physiological level. We have also shown that the colonies developing under these defined conditions contain HGPRT activity and that in some cases altered enzyme can be demonstrated.

Since all 12 independently isolated $HGPRT^-$ clones were revertible, ICR 191 and ICR 170 do not appear to induce total gene loss under these conditions. Also, there is a 20- to 30-fold difference in reversion frequency induced by a given agent among the clones. Different $HGPRT^-$ clones also have qualitatively different reversion patterns. For example, clone 207 is readily revertible by ICR 170 and ICR 191 but not by ENU, while clone 218 is revertible by all three agents (Table 8). These observations are consistent with the notion that ICR 191 and ICR 170 act as point mutagens and that many different mutations are present in these $HGPRT^-$ clones. Furthermore, the spontaneous reversion frequency is less than 10^{-7} revertant/clonable cell. This is at least an order of magnitude lower than the spontaneous forward mutation frequency at this locus (69).

Not only are all of these clones revertible by both ICR 191 and ICR 170, but the ability of the two ICR compounds to induce reversion in these clones is similar; that is, those clones which are highly revertible by ICR 191 are also highly revertible by ICR 170 and those clones which

are less revertible by ICR 191 are also less revertible by ICR 170. This suggests that ICR 191 and 170 induce similar types of mutations in this system.

Based upon the mode of action of ICR compounds in prokaryotes (1, 3, 11, 42, 54, 68), it might be inferred that the mutations induced are frameshifts. Studies of ICR compounds in eukaryotes have not been as extensive, but there are several reports which provide indirect evidence for frameshift induction. These studies are based upon a compound's differential ability to induce mutations in essential or nonessential genes in a single cell line. Friedrich and Coffino (34) measured resistance to ouabain, TG, and $N^6,O^{2'}$-dibutyryl adenosine 3':5'-cyclic monophosphate (Bt_2cAMP) in S49 mouse lymphoma cells. Ouabain resistance and in some cases Bt_2cAMP resistance (types A and D) were assumed to require an altered, yet functional, protein and should, therefore, be induced mainly by missense mutation. TG resistance, on the other hand, could be induced by agents which cause missense, frameshift, or more extensive alterations in DNA. It was found that ICR 191 induced resistance to TG at high frequencies, to Bt_2cAMP (type A and D) at low frequencies, and to ouabain not at all. Ouabain resistance also could not be induced by ICR 170 in another mouse lymphoma line (L5178Y cells), yet trifluorothymidine resistance, which results from loss of a nonessential enzyme (thymidine kinase), could be induced at high frequencies (64). In contrast to these studies, Gupta and Siminovitch (40) found that ICR 191 induced both TG and ouabain resistance in CHO cells. They also found that ICR 191 induced resistance to emetine and

diphtheria toxin (each assumed to involve an essential gene function). Thus, there is evidence from cultured mammalian cell systems that ICR 191 may cause missense mutations in addition to frameshifts.

In the present study, ENU was chosen as a mutagen whose mode of action would be dissimilar to ICR 191 and ICR 170. This alkylating agent reacts mainly as an S_N1 reagent (Swain-Scott s value of 0.26) and causes a high proportion of O^6-ethylguanine (76). In bacteriophage, there is good correlation between the ability to alkylate guanine at the 0-6 position and mutagenesis (57, 58). O^6-Alkylguanine has also been shown to cause mispairing in vitro (37). This correlation suggests this mispairing may also operate in vivo to produce base-pair substitution mutations.

In general, however, ENU induced reversion of these clones in a pattern parallel to the ICR compounds (Tables 7 and 8, pages 54 and 55). Since it seems unlikely that extracistronic suppression could account for reversion of all the $HGPRT^-$ clones, we suggest that these three agents may induce similar types of mutations in CHO cells. Based upon the types of mutations induced by these three mutagens in bacteria, clone 207, which is revertible by the ICR compounds but not by ENU, appears to be the best candidate for containing a frameshift mutation. It is possible that our findings result from cellular processes (e.g., repair) that modify the initial reaction of these agents with DNA and that these compounds induce more than one type of mutation.

The physiological and biochemical characteristics of ICR 191- and 170 induced TG^r clones do not appear to be significantly different. The

finding that all but one clone (I2, which may be of spontaneous origin) have no detectable HGPRT activity is consistent with the notion that these clones contain an alteration at the hgprt locus such as a deletion or frameshift. The former explanation may be ruled out since 12/12 of these HGPRT$^-$ clones are revertible (Tables 7 and 8).

The fact that we were able to detect I2 demonstrates that cells with small amounts of HGPRT activity, as might be expected if a base-pair substitution had occurred, can grow under our TG selection conditions. In addition, Riddle and Hsie (85) found that 6/15 UV-induced TG^r clones, isolated from the CHO/HGPRT assay, contained detectable HGPRT activity. Further, about 20% of EMS-induced TG^r clones have physiological characteristics of reduced but detectable levels of HGPRT (O'Neill and Machanoff, unpublished observations). This indicates that we are not biasing our sampling of TG^r clones to include only those with no enzyme activity. The heat inactivation profile of I2 extract and its altered K_m indicate that this clone contains an altered HGPRT. Cappechi et al. (14) have shown that altered HGPRT in mouse L cells is degraded faster than wild-type HGPRT. This may explain why I2-HGPRT also has a reduced V_{max}.

The distribution of specific activities among the revertants (Table 6, page 50) suggests that many of these clones contain HGPRT which is different from wild type. In fact, only about one-half (14/27) contain wild-type levels of the enzyme (75-124%). The heterogeneity also indicates that there are different alterations in different revertants suggesting suppression. For example, one revertant of clone 204 has 9%

of wild-type HGPRT activity while another revertant has 250% the HGPRT activity of wild type. Furthermore, the HGPRT specific activity of the revertants is strongly influenced by the TG^r clone from which it was derived. Revertants of TG^r clones E2, H1, J1, and 207 all have HGPRT specific activities close to wild type, while many revertants from TG^r clones 201 and 204 have significantly altered HGPRT specific activities. Even though all the TG^r clones examined have no detectable HGPRT, these data and the revertibility of these TG^r clones (Tables 7 and 8, pages 54 and 55) are consistent with the notion that these different TG^r clones contain point mutations in the gene coding for HGPRT.

An alternative explanation is that a regulatory element which controls the amount of HGPRT expressed is being altered instead of the hgprt structural gene. Evidence for a regulatory gene for HGPRT is based upon (i) re-expression of HGPRT in cell hybrids (9) and (ii) the finding that the specific activity of HGPRT varies from tissue to tissue in an organism (56). While same-site reversion or alterations in a regulator would produce an unchanged HGPRT in the revertant, second-site reversion could lead to an altered HGPRT. In order to show that alterations at a regulatory locus are not responsible for our observations, we have looked for altered HGPRT protein in revertants by measuring heat stability of HGPRT specific activity in cell-free extracts.

We have examined the heat stability of 19 revertants and found that 17 have heat inactivation profiles indistinguishable from wild type. The half-lives of HGPRT activity (t1/2) in these clones are summarized in Table 9, page 57, along with the HGPRT specific activities and the

TG^r clone from which the revertants arose. Two revertants, 511 (derived from TG^r clone 201) and 604 (derived from TG^r clone 204), show an increase in heat lability (Figure 17, page 56) demonstrating an altered HGPRT protein. We suggest, therefore, that TG^r clones 201 and 204 contain mutations in the hgprt gene and that clones 511 and 604 arose through second-site reversion. It is interesting to note that clones 201 and 204 can also give rise to revertants with HGPRT heat stability of wild type, thus indicating more than one mechanism of reversion. Although both TG^r clones 201 and 204 were induced by ICR 170, as was revertant clone 511, revertant clone 604 was induced by ICR 191. These observations provide further evidence that both ICR 191 and ICR 170 induce similar gene mutations in mammalian cells.

The quantitative nature of this reversion assay coupled with further biochemical analyses of mutants and revertants will prove useful in studies of the mechanisms of mammalian cell mutagenesis.

CHAPTER IV

EPILOGUE

Reversion analysis has been valuable in the elucidation of the modes of action of mutagens on microorganisms. With the development of the quantitative reverse mutation assay described here, it is now possible to apply reversion analysis to mammalian cells in culture. This thesis describes the reversion by three agents (ICR 191, ICR 170, and ENU) of ICR 191- and 170-induced TG^r clones.

Future studies should involve attempts to revert these TG^r clones with mutagens with different properties such as base analogs (e.g., 6-hydroxylaminopurine, 5-bromodeoxyuridine), other monofunctional alkylating agents (e.g., ethyl methanesulfonate, N-ethyl-N'-nitro-N-nitrosoguanidine), polyfunctional alkylating agents (e.g., nitrogen mustard, triethylene-melamine) and other bacterial frameshift mutagens (e.g., 2-nitroso-fluorene, 4-nitrosobiphenyl). These types of studies may permit the division of these agents into groups of similar acting mammalian cell mutagens. In addition, other mutagens, such as the ones mentioned above, should be used to induce the TG^r clones for reversion studies.

Since so little is known about spontaneous mutations, reversion analysis would also be useful in characterizing spontaneously occurring lesions.

Of particular interest, is the finding that ICR 191, ICR 170, and ENU may cause similar types of mutations in cultured mammalian cells. This suggests that the sort of mutagen specificity found in bacteria and

bacteriophage may not apply to higher organisms. A theoretical explanation for this has been put forward by Drake (28). This theory is based upon the following observations of the dependence of mutation induction on mutagenic repair systems. First, inactivation of the mutagenic repair system of phage T4 does not affect mutation induction by ethyl methanesulfonate (EMS) or N-methyl-N-nitro-N-nitrosoguanidine (MNNG) but abolishes most mutation induction by methyl methanesulfonate (MMS). Second, in E. Coli, inactivation of mutagenic repair reduces slightly to moderately the mutagenicity of EMS and MNNG, but completely that of MMS. In yeast and Neurospora, essentially all mutagenicity of these compounds is abolished when mutagenic repair is inactivated. This suggested to Drake "a phylogenetic trend in which mutagenicity by direct base mispairing has been progressively reduced in higher organisms, leaving only mutagenic repair as the residual mechanism." This indicates that the types of mutations induced by particular agents in mammalian cells may depend upon the indirect action of a mutagenic repair system and not upon the direct chemical reaction of a mutagen with DNA. Isolation of mammalian cell mutants with deficient repair systems would be necessary to further test this theory.

An interesting observation from the analysis of revertants is that some revertants contain up to 250% of the enzyme activity of wild type cells. This could be due either to the revertant manufacturing an enzyme at a higher rate than wild type or to a gene dosage effect. Karyotypic analysis may answer this question.

Antibody to HGPRT has recently been made available to this laboratory through the courtesy of T. Caskey, Baylor College of Medicine.

Analysis of the TG^r clones for cross-reacting material (CRM) may further define the molecular lesion responsible for TG-resistance induced by ICR 191 and 170. If frameshifts were induced, then no CRM would be expected while if missense mutations were induced, CRM may be present. Suppression by extracistronic methanisms (e.g., altered tRNA anticodons) could also be looked for. This type of revertant would be expected to express low levels of HGPRT (through suppression by an altered tRNA) and also the mutant HGPRT since there are multiple loci for each tRNA and only one is likely to be altered. By reverting a TG^r clone with detectable CRM, this type of suppression may be discovered.

Heat inactivation studies of the HGPRT of revertants revealed two revertants with altered enzyme. This may indicate suppression. It is possible that other revertants also contain altered HGPRT which has the same thermal sensitivity as wild type HGPRT. If revertants do contain altered HGPRT, it may be detectable through altered antigenic sites. Other methods which may prove useful in screening revertants for altered HGPRT are kinetic studies for the substrates PRPP and Hx and isoelectric focusing of HGPRT activity. Isolation of revertants with altered HGPRT will be useful in determining the types of suppression that occur in mammalian cells.

AS^r clones with very low HGPRT specific activity may prove useful in measuring forward mutations at this locus. If the long (7-9 days) phenotypic expression time in the CHO/HGPRT assay is due to dilution of preexisting HGPRT, then a revertant with only 10% of wild type enzyme would be expected to have a much shorter phenotypic expression time. It may then be possible to adapt the forward muation assay to <u>in situ</u>

expression of the TG^r phenotype and thus not only save time, but also avoid the sampling errors which subculture introduces.

The continuation of this thesis project along the above mentioned lines should provide further information on the process of mutation induction in mammalian cells as well as the ways in which correction of these mutations can occur.

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