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ABSTRACT

THE ISOLATION AND PARTIAL CHARACTERIZATION
OF CHINESE HAMSTER FIBROBLASTS WITH
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The isolation and characterization of bacterial mutants with abnormal UV sensitivity or defects in DNA repair have contributed extensively to our present understanding of prokaryotic DNA repair mechanisms and their role in mutagenesis. A similar approach is currently proving productive in the analysis of yeast and Drosophila DNA repair processes. Studies in mammalian somatic cells, however, have primarily been restricted to normal cells and cells from patients with one of a few known syndromes which exhibit abnormal responses to mutagens and/or altered DNA repair functions. Techniques have been reported whereby the induction, selection, and partial characterization of presumed mammalian DNA repair mutants have been described. The success of these efforts has been limited by the relative tedious nature of these selection techniques, instability of mutants isolated, or the use of cell lines not easily adaptable to mutation assays. Reported here is the

isolation and partial characterization of several Chinese hamster fibroblast cell lines with alterations in DNA repair and/or UV sensitivity.

The isolation procedure consisted of (1) mutation induction by 5-bromodeoxyuridine-black light and ultraviolet light (UV) treatments; (2) incorporation of tritiated thymidine in repair proficient cells at high temperature (38°C) following UV damage; (3) cold holding (4°C) of these cells to permit tritium-induced killing; and (4) recovery and testing of repair-deficient and UV sensitive cells which have survived and formed colonies at low temperature (34°C). Seventy-two surviving colonies were isolated from 2×10^7 cells plated for selection. Of the 72 colonies, 20 demonstrated potential interest and 4 were selected for extensive study. One mutant, identified as UV^S-7, was found to be slightly more sensitive to UV, not sensitive to x-rays or N-acetoxy-2-acetylaminofluorene (NAc-AAF) and exhibited a highly reduced level of unscheduled DNA synthesis (UDS), as compared to the parental line. Two additional lines, UV^S-40 and UV^S-44, were sensitive to UV, x-ray, N-methyl-N-nitro-N-nitrosoguanidine, and NAc-AAF but exhibited only slightly reduced UDS. A fourth line, UV^r-23, had enhanced UDS, was resistant to UV, but exhibited no abnormal sensitivity to x-ray or NAc-AAF. Mutation studies revealed UV^2-7 to exhibit a UV-induced hypermutability as compared to parental control cells at the three loci measured by

resistance to ouabain, 6-thioguanine, and diphtheria toxin. Analysis of the spontaneous mutation rate to 6-thioguanine resistance demonstrated that the rate for UV^S-7 was not at variance with that of the parental control cells. UV-induced mutation experiments also suggested hypermutability for UV^S-40, hypomutability for UV^r-23, and no significant deviation in the UV-induced mutability of UV^S-44, as compared to parental control cells.

The data confirm the contribution of deficient excision-repair to mutagenesis and support the existence of an alternative repair mechanism. Further characterization of the mutants discussed here or employment of the isolation technique to select new mutants should prove useful in the analysis of mammalian DNA repair processes and the details of mutation fixation.

To

Barbara

for sharing so very much

and having so much to share

To

my father and mother

for love and support

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LIST OF ABBREVIATIONS

AP	apurinic/apyrimidinic
AT	ataxia telangiectasia
BrdU	5-bromodeoxyuridine
cAMP	adenosine 3',5'- cyclic phosphoric acid
CHO	Chinese hamster ovary
dATP	deoxyadenosine 5'-triphosphate
dCTP	deoxycytidine 5'-triphosphate
D_o	statistic representing the increase in dose necessary to reduce survival by 1/e (.37) on the straight-line portion of a survival curve
DT	Diphtheria toxin
DT^r	Diphtheria toxin resistant
ER	Excision repair
FA	Fanconi's anemia
HAT	5 μg/ml hypoxanthine, 5 μg/ml thymidine, and 3.2 μM amethopterin
HCR	host cell reactivation
HGPRT	hypoxanthineguanine phosphoribosyltransferase
J	joules
Lf	floculating unit
MNNG	N-methyl N'-nitro-N-nitrosoguanidine
N	statistic representing the multiple of 100% to which the straight-line portion would extrapolate at 0 UV exposure

NAc-AAF	N-acetoxy-2-acetylaminofluorene
OUR^r	ouabain resistant
PBS	phosphate buffered saline
PR	photoreactivation
SCE	sister chromatid exchanges
6-TG	6-thioguanine
$6\text{-}TG^r$	6-thioguanine resistant
$^3H\text{-}TdR$	tritiated thymidine
UDS	unscheduled DNA synthesis
UV	ultraviolet-light
XP	xeroderma pigmentosum

INTRODUCTION

It is now recognized that repair processes play an essential role in the maintenance of genetic continuity, compensating for the instabilities of the DNA molecule. The DNA is constantly experiencing alterations of a spontaneous or induced nature, which, if uncorrected or miscorrected, may result in a mutation or a lethal event. The consequences of a lethal event are self-evident, however, those related to mutations, particularly as they occur in the somatic cell, are only beginning to be understood. Somatic mutations in mammals have now been implicated in a number of disease states including the carcinogenic (1, 2), atherosclerotic (3), and aging (2, 4) processes.

In an effort to help delineate the molecular mechanisms of mutagenesis, the past twenty years have seen a formidable effort put forth in the study of DNA repair. In prokaryotes, much has been learned and several mechanisms of repair have been identified. In addition to the repair of damaged DNA, prokaryotes have mechanisms of recombination that permit the "tolerance" of damage persistant through replication. In addition to these constituative mechanisms, inducible, "error-prone" functions have also been demonstrated. A significant portion of this knowledge has come

from the isolation and characterization of specific mutants with alterations related to repair functions. Despite the plethora of information that has been acquired from bacterial studies, the exact details concerning mutation fixation remain to be ascertained.

Studies in eukaryotes have been based primarily on the early observations in prokaryotes. Eukaryotic cells, particularly mammalian cells, have proven technically more difficult to handle. Researchers interested in the study of mammalian repair functions have been encouraged to focus on wild type cell lines and lines established from a few selected cancer-prone syndromes. These approaches have contributed to our understanding of mammalian repair and mutagenesis, suggesting the existence of some prokaryotic DNA repair and tolerance mechanisms in mammalian cells.

Considerable differences exist between prokaryotic and eukaryotic organisms. Eukaryotic DNA is complexed with protein, arranged in nucleosome structures, and confined by a nuclear envelope. Furthermore, eukaryotic gene regulation is complex, and multicellular organisms experience a magnitude of differentiated states at any given time. Such factors are likely to influence the damage of eukaryotic DNA, as well as the mechanisms and efficiency of the repair of that damage.

The identification of differences between prokaryotic and eukaryotic repair has been limited primarily to the

inability to identify, convincingly, prokaryotic functions in eukaryotic organisms. Apurinic endonuclease activity has not been identified in Drosophila and questions remain regarding the existence of photoreactivation or a recombinational postreplication repair in mammalian cells.

While such negative data suggest differences between the repair functions of various organisms, they do not confirm such differences, nor do they elude to what alternative functions may exist in higher organisms. An obvious approach to this problem is the isolation and characterization of eukaryotic mutants. Such an approach has proven successful with Drosophila, as a number of repair mutants have been isolated and are now being characterized in detail. Differences, however, are also likely to exist between Drosophila repair functions and those in mammalian cells.

Recently, techniques have been reported whereby the induction, selection, and partial characterization of presumed mammalian DNA repair mutants have been described. Due to the relatively tedious nature of these selection techniques, the instability of the mutants isolated, or use of cell lines not easily adaptable to mutation studies, these attempts have had limited success.

This dissertation reports an attempt at a new selective procedure for isolating mammalian cell mutants with potential alterations in DNA repair functions. Several

relevant Chinese hamster fibroblast cell lines have been isolated and partially characterized. This characterization includes selected studies of spontaneous and UV-induced mutagenesis.

LITERATURE REVIEW

The Use of Genetic Mutants to Examine DNA Repair and Mutagenesis

Bacteria

A great deal has been learned in the last two decades regarding prokaryotic DNA repair and mutagenesis. Much of this knowledge, including the initial suggestion that repair functions existed, was a consequence of the study of mutants with defects relevant to these functions. Mutants have contributed both to the discovery of new repair functions and to the understanding of the biological consequences of functions previously defined. Before discussing some specific contributions made by the study of such mutants, clarity is served by reviewing the mechanisms of prokaryotic DNA repair that are currently understood. Review and discussion will be kept brief.

Photoreactivation (PR) is one of the simplest of all repair functions. It involves the enzymatic repair of cyclobutane dimers through a light-dependent reaction. The reaction results in the restoration of DNA to an undamaged state without breakage of the sugar-phosphate backbone (5). The reaction is limited to action on pyrimidine dimers.

Excision-repair (ER) does not require light and is not restricted to action on pyrimidine dimers. Excision-repair may be classified in two forms, nucleotide ER and base ER. Nucleotide excision-repair involves (1) the incision of DNA near a recognized site of damage; (2) excision of the damaged base or bases plus a number (>20) of additional nucleotides or small oligonucleotides (long patch); (3) polymerization of new DNA using the undamaged strand as a template; and (4) ligation. The incision step appears to function differently in different organisms, and is not yet completely understood. Some organisms which exhibit radiation-resistance, such as Micrococcus luteus, posses a small protein with incision specificity for pyrimidine dimers (6). Most organisms, however, appear to rely on a series of gene products, all of which are necessary for incision to occur. This is exemplified by the uvrA, uvrB, and uvrC gene products of E. coli, which will be discussed later.

Base excision-repair (7) involves the generation of an apurinic or apyrimidinic (AP) site by the enzymatic or spontaneous removal of a damaged or mispaired base, incision by an apurinic endonuclease, excision of the AP site and a few additional bases (short patch), polymerization, and ligation.

Post-replication repair is a process whereby bacteria avoid the lethal effects of persistant damage by

leaving gaps in daughter strands opposite to such damage, and later filling these gaps by recombinational exchanges with a sister strand. The enzymatic details of this repair are not entirely clear, but will be eluded to later. The process results in the repair of the gaps left by replication past the damage, but as it does not result in the repair of the damage itself, the process is often referred to as a "tolerance" function, rather than repair.

Bacteria also possess an inducible, highly error-prone form of repair, designated the "SOS function." This function appears to work by filling the forementioned daughter strand gaps through the random insertion of bases. The induction of this function appears to be related to the presence of DNA degradation products occurring in response to inhibited DNA replication (8).

The literature on bacterial repair mutants is far too extensive to be reviewed in detail here. A few key examples and details will be cited to illustrate the tremendous contribution that mutant isolation and analysis has made to this field. The review of Hanawalt et al. (9), may be considered the most extensive to date on dark-repair in both bacteria and mammalian cells. Table 1 of that review illustrates both the vast quantity and variability of factors that may influence repair and mutagenesis. More than fifty different genes have been implicated in E. coli

K-12 repair processes, and the precise function of many of these remain unclear.

In 1978, Paul Howard Flanders presented a historical perspective on DNA repair. In his presentation (10), he pointed out that it was the study of a UV-sensitive mutant cell line of Escherichia coli, E. coli B_s, that first brought to light the existence of DNA repair functions for him and other researchers. Ruth Hill had observed that this mutant was incapable of reactivating ultraviolet light (UV)-irradiated bacteriophage. As the mutant was itself UV-sensitive, the likely explanation was that the mutant was incapable of repairing UV-induced damage. Howard-Flanders proceeded to isolate the E. coli K-12 mutants which map to the genes uvrA, uvrB, and uvrC (11). Shortly thereafter, Howard-Flanders and Clark began working with recA$^-$ mutants, which were found to be highly sensitive to both UV and ionizing radiation. They recognized this sensitivity to be a consequence of deficient recombination (12, 13). Double mutants possessing both uvr$^-$ and rec$^-$ phenotypes were determined to be highly sensitive to UV. It is now recognized that a single pyrimidine dimer in the DNA of these double mutants can prove lethal (14).

The initial isolation of these two classes of mutants, uvr$^-$ and rec$^-$, has stimulated an enormous amount of research examining DNA repair and its relation to mutagenesis. It has been determined that neither uvrA$^-$ nor

uvrB$^-$ mutants are capable of incision (15, 16) and although uvrC$^-$ strains do perform incision, the rate appears slower than in wild type cells (17). There is additional evidence that the defect is related to incision, as dimer-specific T4 endonuclease V can be used to restore viral reactivation ability to these mutants (18). Complementation studies have indicated that the uvrA gene product is separable from a product related to uvrB and uvrC (19). The latter may represent the formation of a complex.

The use of uvr$^-$ mutants was responsible for the first identification of postreplication-repair. Rupp and Howard-Flanders (20) noted that when mutants were exposed to low doses of UV, and then permitted to incorporate ^{3}H-thymidine, the labeled DNA sedimented more slowly than the DNA from similarly treated, but unirradiated controls. Allowing the irradiated cells a 70 minute chase time in unlabeled medium, resulted in a return to control sedimentation characteristics. The observation came to be recognized as "postreplication-repair."

The history of rec$^-$ mutants has been elusive and the details of postreplication-repair have remained unclear. The recA gene product has been suggested to play an important function in the "cutting" of the undamaged homologous DNA for recombination (10). There is preliminary evidence that recA$^-$ mutants may be defective in a step that permits the generation of recombinational intermediates, and thus

prevents the completion of exchange (21). Advances in current technology will be necessary to resolve recA function. In addition to the need for recombination in postreplication-repair, as illustrated by the need for the recA gene product, additional gene products are required which are not involved in normal recombination. These include the product of the recF gene.

In addition to the forementioned repair, the rec A and lex A genes appear to be coordinated in an inducible postreplication-repair function. The inducible responses have been summarized by Hanawalt et al. (9) and include inhibition of cell division, inhibition of post-irradiation DNA degradation, induced bacterial mutagenesis, and the induction of viral reactivation and mutagenesis by pre-irradiation of the host cell (Weigle reactivation and mutagenesis (22). For review, also see Witkin (23)). A model suggesting coordination between rec A and lex A for induction proposes that the lex A gene product is a repressor, controlling the rec A gene product (a protease activity) at a low, constitutive level (24, 25). At this low level, rec A product is capable of performing functions necessary for constitutive postreplication-repair. Upon induction, rec A product inactivates the lex A repressor and other repressors controlling SOS functions. One proposed consequence of these changes is the modification of a polymerase to permit error-prone "transdimer synthesis" (26).

There also appears to be an inducible nature to long patch excision-repair. This mechanism has been demonstrated to be dependent on rec A (27), requires protein synthesis as demonstrated by chloramphenicol inhibition, and is induced by UV (28). This form of excision-repair is presumed to be mutagenic. The observation that long patch repair increases with dose in a nonlinear fashion would support the induction hypothesis. However, the observation that mutation fixation can occur during the inhibition of protein synthesis (29) raises some doubt.

An attempt to unravel the enzymology of the excision and resynthesis steps of excision-repair have proven difficult, due to the apparent multiplicity of enzymes available for each task (30). Studies employing mutants defective in any of a variety of exonuclease activities have demonstrated the role of at least three exonuclease activities in excision (9). To complicate matters, mdf and dna B mutants exhibit normal UV-sensitivity, yet a reduced rate of excision, and a significant hypermutability (31, 32). The examination of E. coli mutants deficient in polymerase I, II, or III, have suggested that all three polymerases are involved in repair processes (9). Initial observations of pol A mutants (polymerase I-deficient) appeared to indicate enhanced repair (33). This observation is currently thought to reflect the involvement of polymerase I in short patch repair. The pol A mutants are therefore thought to

conduct an abnormal amount of long patch repair, in order to compensate for reduced short patch repair. This led researchers to observe a greater extent of repair, even though an equal or smaller number of damaged sites were being removed. A requirement for polymerase I and/or polymerase III has been demonstrated for postreplication-repair, as the single mutants pol A and pol C are capable of repairing daughter gaps, whereas the double mutants are not.

In addition to the examination of bacterial mutants that contribute directly to repair functions, there is a class of mutants that exhibit indirect effects. These mutants alter the physiological state of the cell in such a manner, that the repair functions and mutagenesis are affected. An example of such a mutant is dut$^-$, isolated by Hochhauser and Weiss (34). These cells are deficient in dUTPase and consequently have elevated dUTP pools. The cell misincorporates large levels of uracil into the DNA. The mutants generally exhibit an inordinately large number of Okazaki fragments, representing the incorporation of uracil followed by the base excision of that base. Through the generation of double mutants dut$^-$ mutants have been useful in identifying the function of uracil DNA glycosylase (ung gene), polymerase I and ligase in the base excision-repair of misincorporated uracil (7). In addition, the hypermutability of a ung$^-$ mutant is not enhanced by the

generation of a *ung*$^-$ *dut*$^-$ double mutant (35). The *dut*$^-$ mutants were therefore useful in demonstrating that the misincorporation of uracil does not substantially contribute to mutagenesis. It appears, rather, that cytosine deamination is the major cause of the mutator phenotype of *ung*$^-$ mutants.

One additional aspect of the utilization of genetic mutants to study DNA repair and mutagenesis in prokaryotes has come through the employment of plasmids called R factors which carry antibiotic resistance genes. Ames and coworkers have developed a bacterial assay to detect mutagens (36). The assay examines the conversion from histidine requirement to prototrophy in strains of *Salmonella typhimurium* in which conversion is specifically dependent on base-pair substitution or a frameshift event. Sensitivity to mutagens in these lines can be enhanced by the addition of a mutation which eliminates excision-repair. In addition, McCann *et al*. (37) have demonstrated that the inclusion of R factor in these strains enhances the mutagenicity of certain mutagens. While the precise details of R factor action remain uncertain, the mutagen specificity suggests the involvement of a *rec*-dependent, error-prone process.

Lower Eukaryotes

The use of mutants has also been helpful in the study of repair and mutagenesis in lower eukaryotes. UV-sensitive mutants have been isolated in *Saccharomyces cerevisiae* (38),

and mutants sensitive to both x-rays and UV have been isolated in Saccharomyces (39), Neurospora crassa (40), Schizosaccharomyces pombe (41), and others (42). Many of these mutants have "rec-like" characteristics suggesting the existence of recombination functions in lower eukaryotes (42). Additional Neurospora crassa mutants have been isolated with defects in endo-exonuclease activity, which are mutagen-sensitive (43). Mutant studies in Dictyostelium discoideum have resulted in the identification of nine complementation groups (44). Through the analysis of double mutants, at least two repair pathways have been identified, one of which is similar to bacterial excision-repair.

Recently attention has focused on the identification of Drosophila mutants with relevance to DNA repair. It was soon learned that some mutants which had been selected on the basis of mutagen sensitivity (mus) shared characteristics with others selected for analysis as meiotic mutants (mei) (45). This initial observation suggested the existence of functions common to both DNA repair and meiotic recombination. Mutants have now been isolated that are specific to repair functions, meiotic recombination, and both repair and meiotic recombination. A few representative mutants illustrating this fact are presented in Table 1.

In addition to the excision and postreplication-repair functions identified in these mutants,

Table 1.--*Drosophila* Mutants of Meiotic and/or Mutagen-Sensitive Functions.

Mutant	Defective Repair Function	Meiotic Recombination	Sensitivity			
			UV	X-Ray	MMS	AAF
mei-9	Excision	Reduced	+	+	+	+
mei-41	Postreplication	Reduced	+	+	+	+
mei-218	?	Reduced	-	-	-	-
mus (1) 104	Postreplication	Normal	+	+	+	+

Refer to References 45-47.

photoreactivation has been demonstrated in Drosophila tissue culture cells (48) and larvae (49). The generally ubiquitous activity of uracil DNA glycosylase has remained undetectable in Drosophila cell lines, embryos, larvae, pupae, or adult flies (50).

Mammalian Cells

The major contributions to the present understanding of mammalian repair functions and mutagenesis have come through the study of human syndromes which exhibit mutagen sensitivity or a high incidence of cancer associated with altered repair function. Several such syndromes are listed in Table 2. The disease that has proved most beneficial in the study of mammalian repair is xeroderma pigmentosum (XP). Many reviews on XP have been published, including those by Cleaver (51) and Friedberg et al. (52).

XP patients exhibit a severe photosensitivity of the skin and a high incidence of skin cancer, restricted to exposed areas of the body. The first reported evidence that XP cells were hypersensitive to UV dates back to the work of Gastler in 1964 based on studies of total protein (53). Since then, many laboratories have demonstrated reduced XP colony forming ability following exposure to UV (54, 55, 56). This UV sensitivity has been correlated with an inability to reactivate UV damaged viral DNA (57), comparable to the observations made with E. Coli uvr A mutants (18).

Table 2.--Human Hereditary Disorders Which May Be Related to DNA Repair Deficiency.

Disorder	Inheritance
Ataxia telangiectasia	Autosomal recessive
Bloom's syndrome	Autosomal recessive
Cockayne's syndrome	Autosomal recessive
Dyskeratosis congenita	X-linked recessive
Fanconi's anemia	Autosomal recessive
Progeria	Autosomal recessive (?)
Retinoblastoma	Autosomal dominant (chromosome 13)
Xeroderma pigmentosum (Classical XP)	Autosomal recessive
Xeroderma pigmentosum (XP variant)	Autosomal recessive (?)

Sensitivity to additional mutagens has been examined. XP cells have been found sensitive to a wide variety of mutagens. These studies are summarized elsewhere (52). Slight XP sensitivity to x-rays and N-methyl-N-nitro-N-nitrosoguanidine (MNNG) have been observed by Lytle et al. (58) and Stich et al. (59), respectively. Maher and McCormick (60) have suggested that these sensitivities are not significant, and proposed that XP cells be used to identify those mutagens which cause major distortion in the DNA, possibly requiring a long patch repair which is not present in XP. Other mutagens (e.g., x-ray, MNNG) which cause strand breaks and minor base changes, they propose, are repairable in XP and may represent damage repaired by a short-patch mechanism.

Biochemical studies have clearly demonstrated reduced excision-repair in some XP cells. Cleaver demonstrated a reduction in repair synthesis in response to UV in XP cells (61). Setlow et al. (62) and Cleaver and Trosko (63) subsequently demonstrated that XP cells were not capable of excising pyrimidine dimers. A wide variety of techniques have now been used to examine many XP cell lines for excision-repair capacity. The data, in conjunction with complementation studies, indicate that XP is genetically heterogenous with at least eight complementation groups. Groups A-G are all excision-deficient to some degree, whereas the group classified as the XP variant exhibits

normal levels of excision-repair. Groups A-G are defective in ER to varying degrees, with only slight variation within any given group. Observations suggest that the degree of ER reduction does not correlate with UV-sensitivity (52). Sensitivity does appear to correlate with the degree of neurological involvement seen in the individual patients.

Despite normal ER, variant XP cells do exhibit a sensitivity to UV and some chemical mutagens (64) and a reduction in host cell reactivation (65, 66). In addition, XP variant cells exhibit a slower return to high molecular weight DNA following UV-irradiation than that observed in normal cells (67). This observation presumably demonstrates defective postreplication-repair in variant XP cells.

Studies of the UV and chemical-induced mutagenesis of XP cells have demonstrated all groups examined (including variant) to be hypermutable when mutation frequencies are examined per unit dose (56, 68). For classical XP cells, this observation corresponds to that in uvr$^-$ bacteria, where it has been assumed that reduced ER results in a greater dependence on error-prone postreplication-repair. For the variant XP cells, this observation is not easily explained by any currently understood mechanism of repair, but may reflect dependence on a yet undefined error-prone postreplication-repair, or the existence in variant cells of an error-prone physiological state. Classical XP cells exhibit normal mutability when mutations are considered per

unit survival (68), whereas XP variant cells may demonstrate a slight hypermutability based either on unit dose or unit survival (60).

Ataxia telangiectasia (AT) is another genetic disorder that has contributed to the study of mammalian repair. Although heterogeneity has been found in this disorder, all lines tested were sensitive to ionizing radiation (52). Using extracts from Micrococcus luteus known to catalyze strand breakage of γ-irradiated DNA, some AT cell lines have been shown to exhibit a reduced ability to remove enzyme-sensitive sites following γ-irradiation (68). It has not yet been possible to conclude that this apparent repair deficiency is the basic defect of AT. AT cells also exhibit chromosome instability.

Fanconi's anemia (FA) is another human syndrome which is associated with chromosomal instability and predisposition to cancer (69). Data examining the cytotoxicity, removal of damage, and mutability (70) in response to crosslinking agents suggest that FA is deficient in a short-patch repair function (69). Crosslink damage is repaired by a sequence of events involving the first half-excision from one strand, strand displacement and recombination, and final excision of the link to the second strand. FA cells appear to be defective in the first step of crosslink removal and not defective in the removal of thymine dimers. Classical XP cells are capable of first half-excision, so the

mechanism appears not to be related to the long-patch defect of XP. The data indicate that the first half-excision and excision of thymine dimers require different mechanisms.

Dyskeratosis congenita represents another example of a syndrome with altered crosslink repair, as measured by an excessive induction of sister chromatid exchanges (SCE) following psoralen-UV light treatment (70). Bloom's syndrome fibroblasts should also prove useful in the study of repair mechanisms, exhibiting enhanced spontaneous levels of SCEs (71). Retinoblastoma may also prove useful, demonstrating a relationship between x-ray sensitivity and a deletion in a specific region of chromosome 13 (72). Cockayne's syndrome represents an additional example of a condition that exhibits specific sensitivity (damage requiring long-patch repair) without the identification of a precise repair defect (52). Additional syndromes have been cited (52).

Collectively, the many mammalian syndromes studied with potential importance to the mechanisms of DNA repair and mutagenesis, suggest the existence of a wide variety of relevant genotypes. Technical limitations, including poor plating efficiency and limited lifespan, have complicated the study of many of these syndromes. Conflicting results have been published regarding characteristics of many of these lines (52), which likely reflect genetic heterogeneity, alterations in individual culture conditions, or

significant limitations of the tecnhiques employed. Therefore, although many unique strains have been examined, the delineation of repair and mutagenesis mechanisms in mammalian cells is far from complete.

Repair in Chinese Hamster Cell Strains

In Chinese hamster cells, Elkind (73) observed that x-ray exposure was less toxic if administered at lower dose over a longer period of time. This was one of the first reported observations that inferred the existence of mammalian DNA repair. Excision-repair has now been confirmed in mammalian cells. Chinese hamster cells, however, have much lower levels of excision-repair than normal human cells (74). Trosko et al. (75) demonstrated the difficulty in detecting dimer removal in hamster cells. Hamster cells have been shown to conduct levels of repair replication equivalent to those of human cells at times shortly after UV (76). Additional differences between normal human and hamster excision-repair are seen in studies examining the repair of NAc-AAF-induced damage. Data indicate that the repair of UV and NAc-AAF are additive in human cells (77) but not in hamster cells (78). The data suggest that in human cells, different repair pathways are necessary to repair the two types of damage. This disagrees with the previously reported observations for XP cells (60) and with the results of similar experiments reported by Brown et al. (79). The results in human cells are unclear and those in hamster cells suggest similar repair pathways for the two

types of damage. In addition to the observations related to excision repair, the postreplication-repair phenomenon has been observed in Chinese hamster cells (80).

Additional Factors Relevant to Mammalian DNA Repair and Mutagenesis

Although the study of the aforementioned syndromes has suggested the existence of base and nucleotide excision-repair and postreplication-repair in mammalian cells, the data are far from complete. Disagreement has arisen in regard to the interpretation of certain data. Lehman (81) has demonstrated the time-dependent closure of daughter strand gaps in mammalian cells, suggesting postreplication-repair. Setlow and D'Ambrosio (82) have demonstrated the enhancement of such a phenomenon by a split-dose protocol and suggested this represented evidence of an inducible mammalian repair function. Cleaver et al. (83) and Painter (84) have argued that their data may be reinterpreted to indicate that gaps formed are only the consequence of delays in normal synthesis, and not at all related to a repair function. To date this argument has been unresolved.

An additional discrepancy has arisen in the literature in regard to the identification of mammalian photoreactivation. For many years it was assumed that mammalian cells did not possess this function (85, 86). Recently, Sutherland and Oliver were able to demonstrate a culture condition dependent photoreactivation in human cells (87).

Mortelmans et al. have examined these specific conditions and suggest that the observations may represent the action of "nonspecific photosensitizers" rather than an actual PR enzymatic event. The specificity of the conditions under which PR can be observed in mammalian cells also raises questions regarding its function in vivo.

Confusion also exists in the literature regarding the demonstration of recombinational events in mammalian cells in conjunction with repair events. A number of laboratories have demonstrated such events (88, 89), but Cleaver et al. (75) remain skeptical, proposing that artifacts make data interpretation difficult. They suggest that small daughter strands may exist at the time of irradiation, permitting the induction, rather than the transfer, of dimers into those strands. Meneghini and Menck (90) used two techniques, (1) synchronized cells blocked with hydroxyurea; and (2) the incorporation of 5-bromodeoxyuridine (BrdU) until the time of irradiation, to eliminate artifacts in the measure of parental strand dimers transferred to daughter strands. The measurements revealed a 10-20 percent transfer. This would be considerably less transfer than that observed in bacterial cells. Additional mechanisms must be involved in the bypass of dimers.

One additional area of uncertainty in mammalian repair that has received considerable attention is the question of inducible functions. This has been reviewed

by Hanawalt et al. (9). Observed results with three experimental techniques support the existence of an inducible function in mammalian cells. The first is the observation of Weigle reactivation and Weigle mutagenesis, which involves the irradiation of a host cell to stimulate the reactivation and mutagenesis of an irradiated virus. Weigle reactivation has been observed in mammalian cells for Herpes virus (91), Kilham rat virus (92), adenovirus (93), and SV40 (94). Weigle mutagenesis was noted to occur with Herpes (95). Reactivation in mammalian cells has been demonstrated to be inhibited by cycloheximide treatment (92) suggesting a dependence on protein synthesis.

Split-dose enhancement of daughter strand gap closure has been interpreted as evidence for induction of a repair function. D'Ambrosio and Setlow (82, 96) have demonstrated that a small inducing-dose of UV several hours before a large damaging dose can stimulate the recovery of DNA to full size quicker than the recovery observed without stimulation. This effect can be inhibited by cycloheximide (82). The observations of Chang et al. (97), using a split-dose protocol, revealed reduced mutagenesis with Chinese hamster cells. With the addition of caffeine and cycloheximide, they observed enhanced mutagenesis, and suggested the existence of an error-free postreplication-repair in Chinese hamster cells to account for the results.

A third line of evidence supporting inducible mammalian repair is the ability to enhance the repair of alkylation damage. Pretreatment with low doses of an alkylating agent stimulates the repair of damage caused by a subsequent large dose. This procedure has been used in whole animals to enhance the removal of 0^6 methyl guanine from the DNA of rat liver (9).

Painter (84) has suggested that the split-dose work might be reinterpreted, and that it does not support the inducibility of a repair function. He proposes that the initial dose results in a reduction in DNA synthesis and potentiates the occurrence of a higher molecular weight species than would be seen in unirradiated controls. This species would lead to an earlier production of large molecular weight DNA in an enhanced postreplication-repair protocol.

Another hypothesis used to discount the data in support of inducible mammalian repair is derived from the work of Loeb and coworkers. Using an _in vitro_ assay to examine the fidelity of DNA replication of an amber mutant of ϕX174 (am3ϕX174) with _E. coli_ polymerase I, Weymouth and Loeb (98) demonstrated that the substitution of Mn^{+2} for Mg^{+2}, with specific nucleotides, could enhance the error frequency of the polymerase. An additional experiment (99) demonstrated that (1) Co^{+2} for Mg^{+2} could produce similar results, (2) the error rate was dependent on the nucleotide dCTP

and dATP levels, and (3) the relative increase in dATP levels was directly proportional to an enhanced error rate. The data are consistent with the *in vivo* work of Peterson *et al.* (100) which demonstrates that a 3- to 10-fold increase in mutagenesis is observed in the presence of specifically altered nucleotide pools. Recently, the isolation of a hypermutable cell strain with an inherently altered dCTP level (101) and a similar strain with normal polymerase activity (102) have been reported. These further support the *in vitro* observations.

The data suggest that the physiological state under which normal enzymes must function can drastically alter the fidelity with which they act. It seems likely that most repair enzymes will have restricted environments in which they can efficiently function. The UV exposure used for the induction of the split-dose phenomenon or the Weigle reactivation affects normal DNA synthesis and may affect additional cell functions leading to an altered physiological state. This altered state may result in Weigle effects and enhanced removal of 0^6 methyl guanine.

Isolated Mammalian Cell Mutants with Altered UV-Sensitivity and/or DNA Repair

A number of attempts to isolate mammalian cell mutants using nonselective procedures, with the aid of massive screening or replica plating techniques, have been reported (103, 104, 105, 106). These researchers have

chosen nonselective techniques either because of a lack of success with alternatives, or a concern over the harsh procedures necessary for mutant enrichment (103). Sato and Hieda (105) utilized massive screening to identify a mutant mouse lymphoma cell line sensitive to methyl methanesulfonate and x-ray, which was not altered in UV sensitivity. Kuroki and Miyashite (103) used Lederberg style replica plating which, although tedious, yielded a 100% plating efficiency. They used MNNG to provide a mutagenized population of mouse cells before selection and were able to isolate seven UV-sensitive clones. Many of these mutants proved not to be stable. Stamato and Waldren (104) employed a nylon cloth replica plating technique to isolate a stable, UV-sensitive Chinese hamster ovary clone. This clone has not been characterized in great detail. Nonselective mutant procedures have also been used to examine the effects of temperature (107), viral transformation (108), and ploidy (109) on DNA repair and/or mutagen sensitivity.

Procedures for general mammalian mutant selection or enrichment have been reviewed elsewhere (110, 111). These procedures have been used in an effort to isolate UV-sensitive and/or repair mutants. The most commonly employed technique involves the incorporation of BrdU and subsequent exposure to visible light as a means of lethal growth. The employment of this technique for the isolation of mutagen-sensitive or repair mutants requires

modifications of the techniques initially described by Puck and Kao (112) for the selection of nutritional mutants.

Randtke et al. (113) used such a technique to isolate thirteen clones of Chang liver cells that were presumed slow to progress into normal synthesis following a sublethal dose of UV-irradiation. Normal cells which do progress into synthesis incorporate BrdU and are killed when exposed to visible light. The hypothesis appeared correct as unirradiated groups produced no survivors. One of the isolated clones was UV-sensitive.

Isomura et al. (114) isolated two UV-sensitive clones using a similar approach with HeLa S3 cells. MNNG was used to mutagenize the initial population before selection. The clones were found to be more sensitive to 4-nitroquinoline 1-oxide, but not x-ray, than the parental HeLa cells.

Klimek et al. (115), using similar selection, isolated both UV-resistant and UV-sensitive clones of HeLa S3 cells, which appeared to exhibit differential repair as measured by BrdU photolysis.

A more recent enrichment technique, which should prove useful in future studies, is the use of irradiated viruses to select UV-sensitive and repair mutants. Repair-proficient cells will reactivate the virus (host cell reactivation (HCR)) and be killed, whereas repair-deficient cells should survive. This technique has been used with

human FL cells and Herpes virus (116). The results were dramatic, with nineteen of 238 isolated clones exhibiting UV-sensitivity. Of four clones studied, two were stable for extended periods and only one of these two appeared to be HCR deficient when reexamined. Chinese hamster cells do not appear to incorporate the viruses commonly used for such studies (Dave Lytle, personal communication), so alternative methods will have to be devised for selection with these cells.

Recent experimentation in repair related research has seen a renewed interest in the isolation of mammalian cell mutants. The isolation of hamster mutants with defects related to postreplication-repair were recently reported (117). The cell lines exhibited hypersensitivity to a variety of mutagens. The selection procedure for these lines did not employ any major improvements upon old isolation techniques. In addition to these mutants, another laboratory has reported the isolation of seven CHO clones apparently excision-repair deficient and UV-sensitive (118). Two of the lines exhibit hypermutability. The lines were isolated by a unique technique that utilized the photographic examination of colony growth following UV exposure to determine sensitivity.

The most important mammalian mutants discussed here are summarized in Table 3.

Table 3.--Selection of Mammalian Mutants.

Cell Line	Isolation Technique	Mutant Characteristics	Reference
Mouse lymphoma	Nonselective	X-ray-sensitive	105
CHO-K1	Replica plating	UV-sensitive	104
Mouse FM3A	Replica plating	UV-sensitive	103
Chang liver cells	BrdU + light	UV-sensitive	113
HeLa S3	BrdU + light	UV-sensitive ER deficient	114
HeLa S3	BrdU + light	UV-sensitive Altered ER	115
Human FL	Viral suicide	UV-sensitive	116
CHO	Replica plating + photographic identification	UV-sensitive ER deficient Hypermutable	118

MATERIALS AND METHODS

Cell Strain

The parental cells used for the isolation of potentially mutant cell lines and used throughout these experiments for control values were V79 Chinese hamster lung fibroblasts. This transformed, aneuploid cell line was originally derived from a male Chinese hamster, Cricetulus griseus, 2n=22 (119). The cell line can be grown in mass culture attached to the bottom surface of plastic or glass flasks and plastic tissue culture dishes and is capable of forming colonies from a single cell in such vessels with nearly 100% plating efficiency.

Culture Medium

Cells were grown and experiments conducted in Eagle's Minimal Essential Medium (MEM) (120) with Earle's salts (GIBCO, Grand Island, New York), supplemented with a 50% increase in all essential amino acids except glutamine, 100% increase in all nonessential amino acids, 50% increase in all vitamins, and 1 mM sodium pyruvate. The concentration of bicarbonate was adjusted to 1.5 g/l. Sterilization was achieved by positive pressure filtration through nucleopore filters (Nucleopore Corporation,

Pleasanton, California). Sterile medium was stored in the dark, in a 4°C cold room. Prior to use, the medium was supplemented with 5% fetal calf serum (GIBCO, Grand Island, New York, Flow Laboratories, Inc., Rockville, Maryland, or Pel Freeze, Rogers, Arkansas) which had been stored at -20°C, thawed, and heat inactivated at 56°C for 25 minutes if not previously inactivated by the manufacturers. The medium was further supplemented with penicillin G (100 units/ml) and streptomycin (100 µg/ml).

Culture Vessels and Incubation Conditions

Stock cultures were grown in sterile glass bottles or plastic flasks (Corning Glass Works, Corning, New York). When sufficiently dense, cells were subcultured with 0.01% crystalline trypsin (Sigma Chemical Company) in phosphate buffered saline (PBS) without calcium and magnesium ions. Cultures were routinely subcultured one or two days prior to an experiment at sufficient density to ensure log phase cells at the time of experimentation. For most experiments, cells were grown in 9 cm plastic culture dishes (Falcon Plastics, Oxnard, California or Corning Glass Works, Corning, New York). Both stock cultures and experimental plates were incubated in water jacketed incubators which provide a stable temperature of 37°C (unless otherwise indicated), with humidified air and 5% CO_2.

Cell and Colony Counts

Trypsinized cells in suspension were counted using a hemacytometer. Colonies developing from single cells were scored visually when sufficiently large, or after staining by rinsing with 0.85% saline, fixing with 95% ethanol, and staining with 2.5% Giemsa stain.

Plating Efficiency and Cell Survival Determinations

Plating efficiencies and replating efficiencies were determined by plating 200-300 cells in 10 ml of medium in a 9 cm plate, with 3 or 4 plates per treatment group. For replating efficiencies, plates were handled identical to treatment plates with the exclusion of selective medium.

Cell survival was determined for a variety of mutagens described below. In all cases, a sufficient number of cells was plated for each treatment group, to permit an estimated 50-300 colonies surviving per plate, with 3 to 4 plates per treatment group. Colonies were scored as previously described.

Mutant Induction

The procedure for mutant induction and selection is outlined in Figure 1. Cells were mutagenized by a two-step procedure consisting of (1) the incorporation of 5-bromodeoxyuridine (BrdU) (National Biochemicals Corporation, Cleveland, Ohio), and subsequent exposure to black light, and (2) UV irradiation. 12×10^6 cells were plated in 40

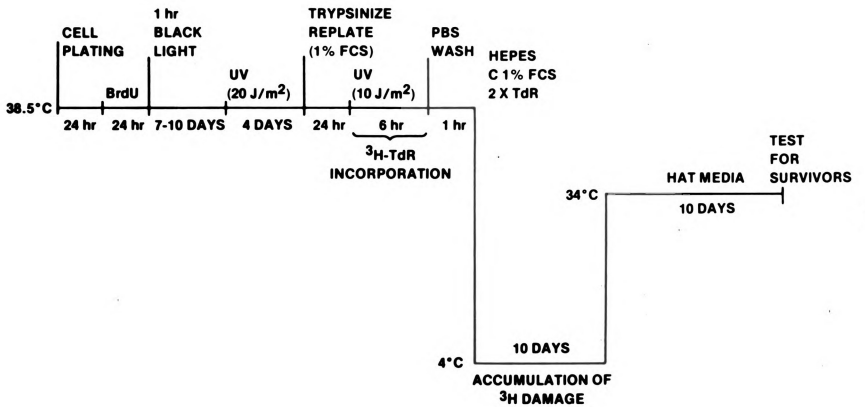


Figure 1. Mutant Selection Protocol.

plastic tissue culture dishes and allowed 24 hours to attach and achieve log phase growth at 38.5°C. Cells were grown for an additional 24 hours in the presence of 0.1 mM BrdU in the dark and were subsequently exposed to black light from a series of bulbs (General Electric F15T8-BL 15W), at a distance of 5.5 cm for one hour with medium remaining on the cells. Cells were washed with PBS and supplied with fresh medium. After approximately 10 days of growth, subculturing when necessary, 12 x 10^6 cells of the resulting population were plated and allowed 3.5 hours for attachment. Medium was removed and cells were exposed to 20 joules/m^2 from a germicidal lamp (General Electric 25T8-25W) which was positioned to deliver a fluence of 1 joule/m^2/second, as measured by a Blak-Ray Ultraviolet Meter Model J-225 (Ultraviolet Products, Inc., San Gabriel, California).

Mutant Selection and Isolation

Mutagenized cells were trypsinized and replated at 2.5 x 10^6 cells/plate (8 plates) in medium containing 1% fetal calf serum. Twenty-four hours later the cells were exposed to 10 joules/m^2 UV and supplied with medium containing 1% fetal calf serum plus 20 μCi/ml ^{3}H-thymidine, 64.7 Ci/m mol (New England Nuclear, Boston, Massachusetts), for 6 hours. Cells were washed twice with PBS and then incubated in medium containing Hepes buffer, 1% FCS and 2x cold thymidine (4.12 x 10^{-5} M) for 1 hour. Plates were

then moved to a Sherer automatically controlled cold room (4°C) for 10 days.

Following radioactive thymidine killing the cells around the periphery of each plate were removed with a rubber policeman to eliminate survival due to a shadowing effect. HAT medium [5 µg/ml hypoxanthine (Sigma Chemical Company), 5 µg/ml thymidine (Sigma Chemical Company), and 3.2 µM amethopterin (National Biochemicals Corporation)] was added to each plate and cells were grown for 10 days at 34°C. Surviving clones were isolated and grown in duplicate in 24-well plates (Corning Glass Works, Corning, New York).

UV Sensitivity

One set of multiwell plates containing isolated colonies was reserved to provide cells for the establishment of individual cell lines for freezing, the unscheduled DNA synthesis assay, and additional work. The duplicate set of multiwell plates was used as a preliminary screen for UV sensitivity. With medium removed, cells in multiwell plates were exposed to 10 joules/m^2 UV. Fresh medium was added and several days later individual wells were examined for qualitative evidence of sensitivity or resistance. Wells exhibiting abnormal response as compared to the parental line were considered to be of special interest, and the cell lines established from their duplicate wells were examined by more quantitative means. For each cell line

demonstrating preliminary sensitivity or resistance, UV sensitivity was determined by means of colony forming ability after exposure to 5.0 and 20 joules/m^2 UV. Survival was determined by averaging the percentage of survivors in quadruplicate plates for each dose of UV. Detailed survival at many doses of UV was then determined for a few select lines in a similar manner.

Unscheduled DNA Synthesis (UDS)

Excision-repair capacities of the various cell lines were estimated by means of the unscheduled DNA synthesis assay as developed by Trosko and Yager (121). Preliminary measurements were made by establishing one monolayered 60 x 15 mm petri dish of cells for each cell line isolated through the employment of the mutant selection protocol. Monolayered cells were maintained on deficient medium lacking arginine and isoleucine (GIBCO, Grand Island, New York) and supplemented with 5% dialysed fetal calf serum (GIBCO, Grand Island, New York) for 24 hours. Medium was then changed to fresh deficient medium with 5% dialysed fetal calf serum for an additional 48 hours. Five mM hydroxyurea (Sigma Chemical Company) was added 1 hour prior to the collection of medium and exposure of the cells to 20 joules/m^2 of UV irradiation. ^{3}H-thymidine (40-60 Ci/m mol) was added to the collected medium to a concentration of 5 μCi/ml. Medium was returned to the cells for 3 hours of incorporation. The parental cell line was assayed as a control,

using duplicate plates and groups with and without UV, to allow for more accurate estimations of normal values.

At 3 hours all cells were collected in PBS, frozen, and later assayed via trichloroacetic acid (TCA) extraction of the DNA. Specific activities (DPM/μg DNA) were calculated for each cell line. A few selected lines were analyzed in greater detail using the same techniques with the arginine/isoleucine deficient medium. Various doses of UV were examined with duplicate plates per dose.

Sensitivity to Additional Mutagens

Selected lines were examined for sensitivity to several mutagens, in addition to the aforementioned ultraviolet light. For x-ray sensitivity cells were trypsinized, suspended in medium in 25 cm^2 plastic flasks, and irradiated with x-rays generated by a General Electric Maxitron 300 x-ray machine. The exposure rate was 184 R/min (250KV, 20mA, with 3 mm Aluminum filtration). Cells were immediately plated in 9 cm petri dishes and 7-10 days later, colonies were scored. Percent survival was again determined by averaging quadruplicate plates per point.

For examining sensitivity to N-methyl-N-nitro-N-nitrosoguanidine (MNNG) (Sigma Chemical Company), trypsinized cells were plated in 10 ml of medium and allowed 3.5 hours for attachment. MNNG was dissolved in acetone and immediately added to plates with acetone alone added to some plates as control. After 2.5 hours the plates were

washed with PBS and supplied with fresh medium. Seven to ten days later colonies were scored and percent survival determined as previously described. Similar experiments were conducted with N-acetoxy-2-acetylaminofluorene (NAc-AAF) using dimethylsulfoxide (DMSO) as a solvent and 24 hour exposure. NAc-AAF was a gift to Dr. James Trosko from Dr. James Miller.

Cell Growth Rate

For each cell line to be examined, approximately 1×10^5 cells were plated in a sufficient number of 9 cm tissue culture dishes and at various times thereafter, two plates were trypsinized for each cell line, and the cells were counted and the values averaged.

Recovery to High Molecular Weight DNA Following UV-Irradiation

Following UV-irradiation, daughter strand DNA is found to be of low molecular weight. This observation suggests either the occurrence of postreplication "gaps" adjacent to pyrimidine dimers (81), or the inhibition of normal DNA synthesis by such dimers (83, 84). With increasing time, the DNA returns to high molecular weight as a consequence of either the filling of gaps or resumed replication. Since the examination of this recovery to large molecular weight has been proposed as a measure of postreplication repair, it was of interest to examine the mutants reported here and evaluate their competence at

conducting this molecular function. UV^r-23, UV^s-40, and UV^s-44 were examined with a parental control in two separate experiments.

In the first experiment, cells were grown in 60 mm tissue culture dishes and labeled with ^{14}C-thymidine for 24 hours. All cells were rinsed with PBS, exposed to 7.5 joules/m^2 UV. Fifteen minutes after irradiation, cells were exposed to ^{3}H-thymidine (67 μCi/ml, 50 Ci/m mol) for 30 minutes, rinsed with PBS, and subsequently permitted 0, 45, or 90 minutes chase with cold thymidine. The experimental procedures were spaced so all groups were ready to collect at the same time. Cells were exposed to 1500 Rads of x-ray and collected. A 50 ml aliquot of each treatment group was lysed for 1 hour in 1.0 N NaOH and 0.01 M EDTA on top of a 5-20% alkaline sucrose (high salt) gradient in a 7/16 inch diameter x 2-3/8 inch polyallomer tube (Beckman Instruments, Inc., Palo Alto, Ca.). Sedimentation occurred at 30,000 revolutions/minute at 20°C for 130 minutes. Gradients were collected in 5 drop fractions from the bottom, and radioactive content of the fractions was determined for both ^{3}H and ^{14}C.

The second experiment was conducted in a similar manner with the following changes. Only UV^s-44 and control were examined, and all cells were permitted a 45 minute chase time. Cells were exposed to UV doses of 0 or 7.5 joules/m^2 or a split-dose procedure of 2.5 and 7.5 joules/

m^2, separated by 2 hours. The split-dose procedure has been demonstrated to enhance the recovery of DNA to high molecular weight, possibly by inducing a repair mechanism (82).

Chromosome Analysis

Cells were inoculated into 60 mm tissue culture dishes (Falcon Plastics, Oxnard, Ca.) containing 5 ml of medium and a sterile glass cover slip. Twenty-four hours later, colcemid (GIBCO, Grand Island, New York) was added to a final concentration of 0.1 µg/ml for 3 hours. Cover slips were collected, treated with 0.7% sodium citrate for 20 minutes and fixed with methanol and acetic acid (3:1) for a few minutes. After air drying, cover slips were stained in Giemsa stain for 10 minutes. Giemsa stain consisted of 3 ml Giemsa stock selection (Bio/Medical Specialties, Santa Monica, Ca.) and 100 ml 0.025 M phosphate buffer (pH 6.8). Slips were then rinsed in water, air dried, soaked in xylene and mounted for examination.

Establishment of Mutation Frequencies

A variety of resistant markers and mutation protocols were used throughout these experiments. The specific procedures employed for any given experiment are indicated with the data in the RESULTS portion of this dissertation. The details of the procedures are described here.

Mutation induction was always achieved with UV from one of several germicidal lamps, positioned to deliver a

fluence of 1.0-1.4 joules/m^2/second. Trypsinized cells were plated in 9 cm tissue culture dishes in 10 ml of medium at the density indicated for each experiment, and allowed 3.5 hours for attachment. Exposure consisted of removing both medium and dish cover for a sufficient time to achieve the total dose desired. In addition to those cells plated for mutation selection, an appropriate number of cells for each treatment group were plated to determine both plating efficiency and UV lethality, as previously described.

The induction of ouabain resistance in Chinese hamster cells by UV has been quantitatively characterized (122). Ouabain resistance is rendered by mutations in the membrane-bound Na^+/K^+ ATPase (123). Mutation expression of ouabain resistance has been achieved by either *in situ* or "replating" techniques. Both techniques were utilized for the experiments presented here. For *in situ* expression, survivors of UV mutagenesis were permitted to develop into colonies before exposure to 1 mM ouabain (Sigma Chemical Company). Resistant clones were scored visually in 6-7 days. Expression time permitted before exposure to the selective agent was determined either in terms of cell division or time (e.g., days). Maximum expression of wild type Chinese hamster fibroblasts has previously been shown to occur at four cell divisions (122). Therefore, in some experiments, the visual identification of 16-cell colonies was used as evidence that maximal expression had occurred.

For the replating technique, mutagenized cells were permitted to grow continuously in log phase in the tissue culture dishes. When necessary, these cells were subcultured and transferred to plastic flasks to maintain sufficiently low density and encourage log phase growth. At 3, 6, 9 and 12 days following mutagenesis, cells were replated in tissue culture dishes, allowed 3.5 hours for attachment and exposed to ouabain. Colonies were scored visually when sufficiently large.

A second locus utilized in these experiments was that of hypoxanthineguanine phosphoribosyltransferase (HGPRT). Inactivity at this locus has been demonstrated to be responsible for 6-thioguanine resistance (124). The induction of 6-thioguanine-resistant (6-TG^r) mutants by both UV and x-rays has been reported (122), and 6-7 days has been determined to be sufficient time for maximum expression when utilizing a replating technique (122). Therefore, the UV-induced 6-TG^r mutagenesis experiments discussed here employed a replating technique identical to that described for ouabain, with the substitution of 10 μg/ml of 6-TG (Sigma Company) as a selective agent.

The forward mutation to diphtheria toxin resistance (DT^r) has also been characterized as a UV-inducible mutation assay in Chinese hamster fibroblasts (125). Maximum mutant yield is achieved by employing a combined replating-*in situ* protocol. The DT^r assay was used to examine UV-induced

mutagenesis in one experiment. The protocol is presented in Figure 2. Mutagenized cells were grown for various lengths of time and plated as per the replating technique. Before the addition of 0.1 Lf/ml (LF = floculating unit) of the diphtheria toxin (Connaught Laboratories, Toronto, Canada), however, the cells were permitted to undergo four rounds of replication. After two days, toxin was removed and fresh medium added. Following sufficient time for development, colonies were scored visually.

Determination of Spontaneous Mutation Rates

To determine the rate at which spontaneous mutations occur in the parental Chinese hamster cell line and one isolated mutant line, a modification of the technique initially developed by Newcombe (126) was used. As illustrated in Figure 3, the technique consists of the determination of mutation frequencies at various times and a concurrent monitoring of cell divisions. This permits the determination of the change in frequency per number of cell divisions.

Cells for both lines were grown in HAT medium (previously described) for 16 days, subculturing as necessary. After an additional 5 days growth in normal medium, cells were trypsinized and plated in 144 tissue culture dishes at 5×10^4 cells/dish. In addition to the dishes, a total of 20×10^6 cells were distributed to four 150 cm^2 flasks. After 3.5 hours, cells in dishes were exposed to

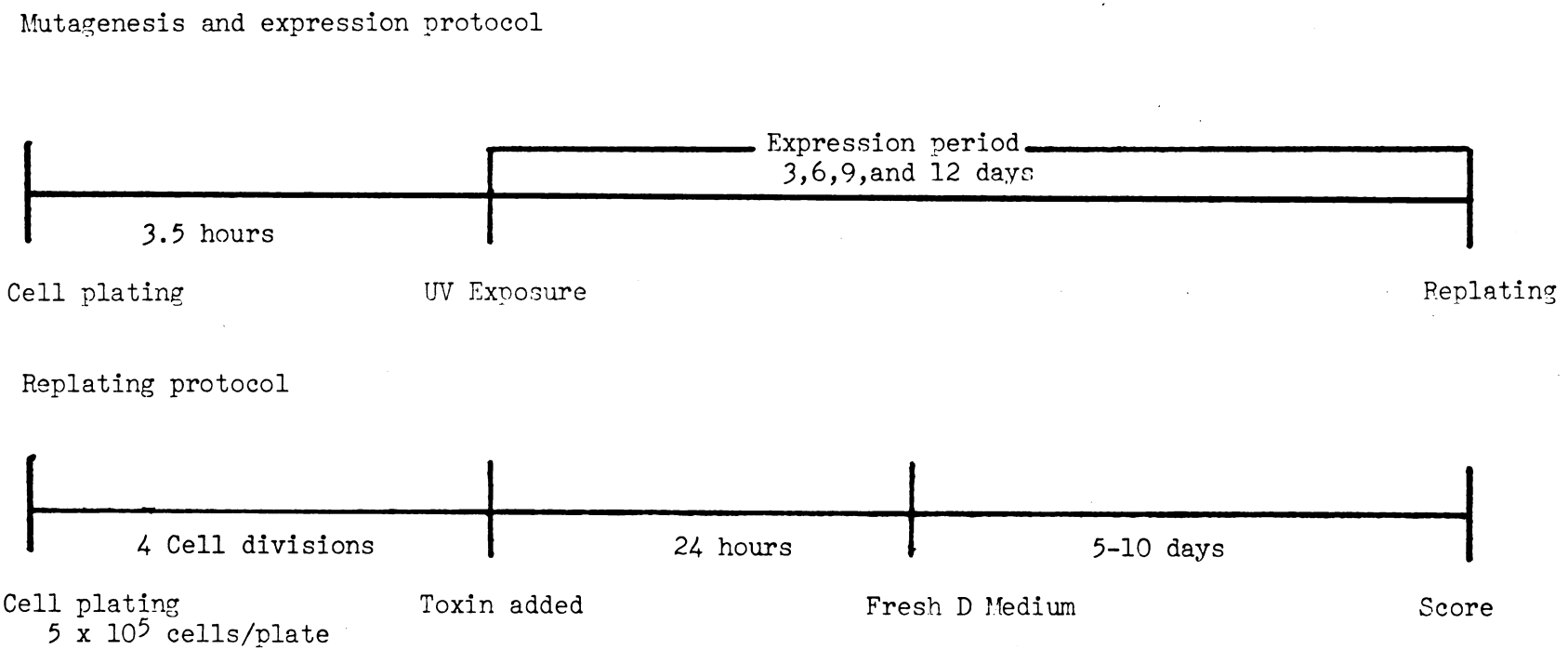
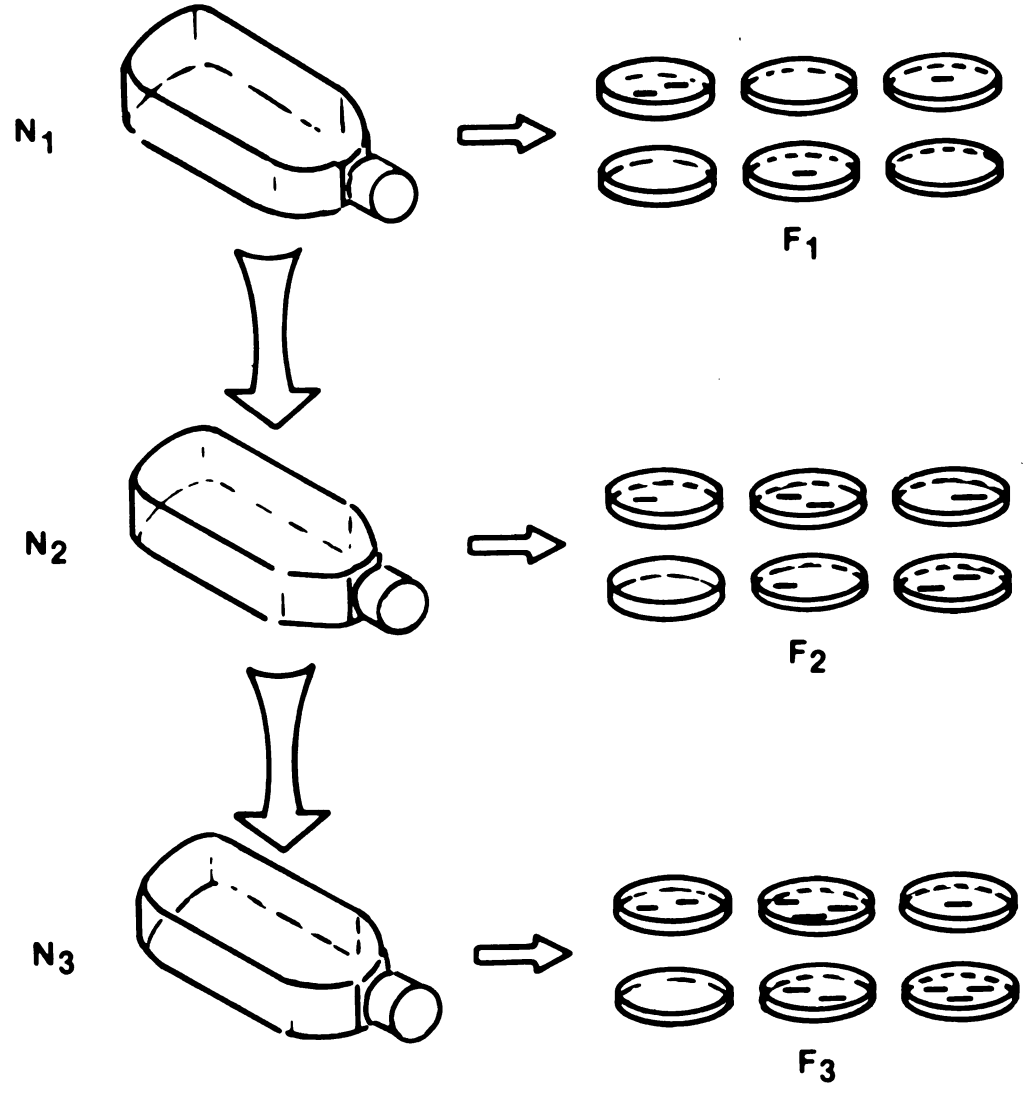


Figure 2. Mutation Assay Measuring the Frequency of Diptheria Toxin Resistance

Mutation Rate Methodology



$$\text{mutation rate} = \frac{2(F_\beta - F_\alpha)}{\ln N_\beta / N_\alpha} \ln 2$$

where: α = initial
β = after x divisions
F = mutation frequency
N = total cell number

Figure 3. Mutation Rate Methodology.

10 μg/ml 6-TG. Flasks were subcultured when necessary by pooling and counting the total cells at each subculturing and seeding 20 x 10^6 cells into four new flasks. On the basis of cell counts, it was possible to determine the total cell growth that occurred between two mutation frequency samplings. Cultures were maintained for a total of 80 days, with a number of platings for frequency determinations to permit the calculation of several different mutation rate values for each cell line. The calculated mutation rates for the two lines were compared by means of a standard Student t-test.

RESULTS

Selection Protocol

The selection protocol was designed for the isolation of mutants defective in excision repair or hypersensitive to UV for alternative reasons. Protocol design, therefore, focused on qualitative rather than quantitative characteristics. Past laboratory experience has demonstrated that 24 hour BrdU incorporation and 1 hour black light commonly yields less than 1% survival and 20 joules/m^2 UV yields less than 10% survival. Although measures were not employed to examine quantitatively, the lethality of these mutagenic treatments, visual examination confirmed that significant cell killing had occurred. The tritium suicide technique involving the incorporation of high specific activity tritiated thymidine after exposure to 10 joules/m^2 UV and subsequent accumulation of damage at low temperature was also noted to produce significant cell killing.

All culture dishes showed greater survival around the periphery than in the center of the dish. As culture dishes commonly produce a shadowing effect around the periphery during UV exposure, all the plates were scraped at the periphery with a sterile rubber policeman to eliminate

clones that may have survived as a result of this shadowing.

Surviving clones were grown in HAT medium for 10 days before isolation to permit the elimination of cells that survived due to an inability to utilize exogenous thymidine.

Of 2 x 10^7 mutagenized cells plated for selection, approximately 100 survived to produce colonies through the procedure. Seventy-two colonies were isolated and tested for preliminary evidence of UV-sensitivity or deficiency in unscheduled DNA synthesis.

Initial Estimates of UV Sensitivity

Exposure of a duplicate set of all isolated clones in multiwell plates permitted the qualitative identification of 9 UV-sensitive (No. 17, 27, 32, 33, 40, 44, 54, 58, 59) and 4 UV-resistant clones (23, 26, 61, 64).

Preliminary analysis of repair synthesis for 57 clones is presented in Table 4. The results were used to identify lines 13, 17, 19, 32, 33, 41, 42, and 59 as potentially deficient in excision repair. Ten additional clones were analyzed at a later date permitting the identification of clones 4, 7, and 20 as potentially deficient. Of the 11 potentially repair deficient mutants, only four (No. 17, 32, 33, 59) were among the 9 lines identified as UV-sensitive in the qualitative prescreening.

Table 4.--Initial Crude Repair.

Cell Line	Sp. Act.*	Cell Line	Sp. Act.
V79**	722.49	35	872.36
1	1132.16	36	1414.47
2	5555.84	37	918.65
5	879.37	39	790.39
6	975.39	40	674.82
9	737.94	41	289.04
10	930.55	42	299.34
11	646.66	43	765.48
12	702.75	44	605.72
13	415.64	45	714.76
14	600.65	46	626.44
15	562.58	47	488.39
17	424.51	48	522.45
18	995.84	49	937.34
19	362.34	52	953.15
21	1023.54	53	891.43
22	480.03	54	799.35
23	597.88	55	715.65
24	601.60	57	617.38
25	845.70	58	507.73
26	738.75	59	198.25
27	775.45	60	398.08
28	894.65	61	750.91
29	1036.39	62	866.56
30	819.52	63	501.40
31	839.59	65	638.58
32	240.78	66	774.26
33	325.61	67	527.08
34	674.26	68	897.23

*Sp. Act. = Specific Activity (DPM/μg DNA).

**V79 background Sp. Act. = 156.24.

To further evaluate the 20 strains of potential interest, more quantitative UV survival experiments were conducted. The experiments illustrate that the strains exhibit a wide range of UV sensitivity, as exemplified by the data in Figure 4.

Although all of the 20 variants appeared to be of further interest, it seemed most efficient to concentrate on a few cell lines for further characterization. Therefore, attention was focused on the two lines most sensitive to UV (UV^s-40, UV^s-44), the one line most resistant to UV (UV^r-23) and the one line demonstrating the greatest deficiency in UDS (UV^s-7).

Radiation Sensitivity

Detailed UV and x-ray survival curves for the four cell lines analyzed are presented in Figure 5. Because of its very slow growth characteristics, UV^s-7 was often analyzed after the other cell lines. As shown in Figure 5A and 5C, UV^s-40 and UV^s-44 are sensitive to both UV and x-ray. UV^r-23 is similar to wild type in x-ray sensitivity but more UV-resistant. UV^s-7, which was not detected as UV-sensitive in the preliminary screening, is slightly, but consistently UV-sensitive as presented in Figure 5B. The x-ray sensitivity of UV^s-7 (Figure 5D) is not at variance with the wild type.

The UV survival curves have been used to estimate D_o and N values for the four mutant lines and controls.

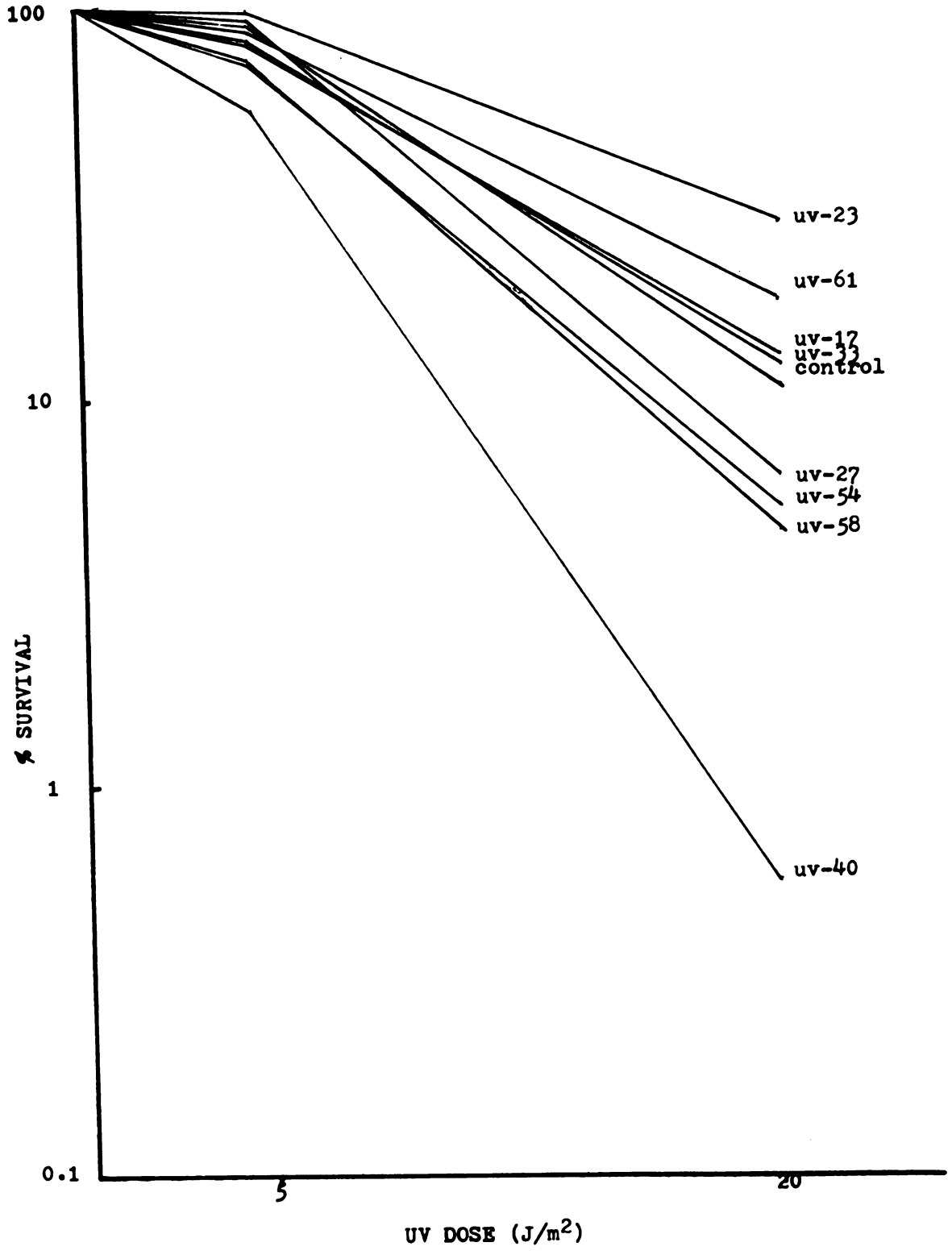


Figure 4. Preliminary Range of UV Sensitivity.

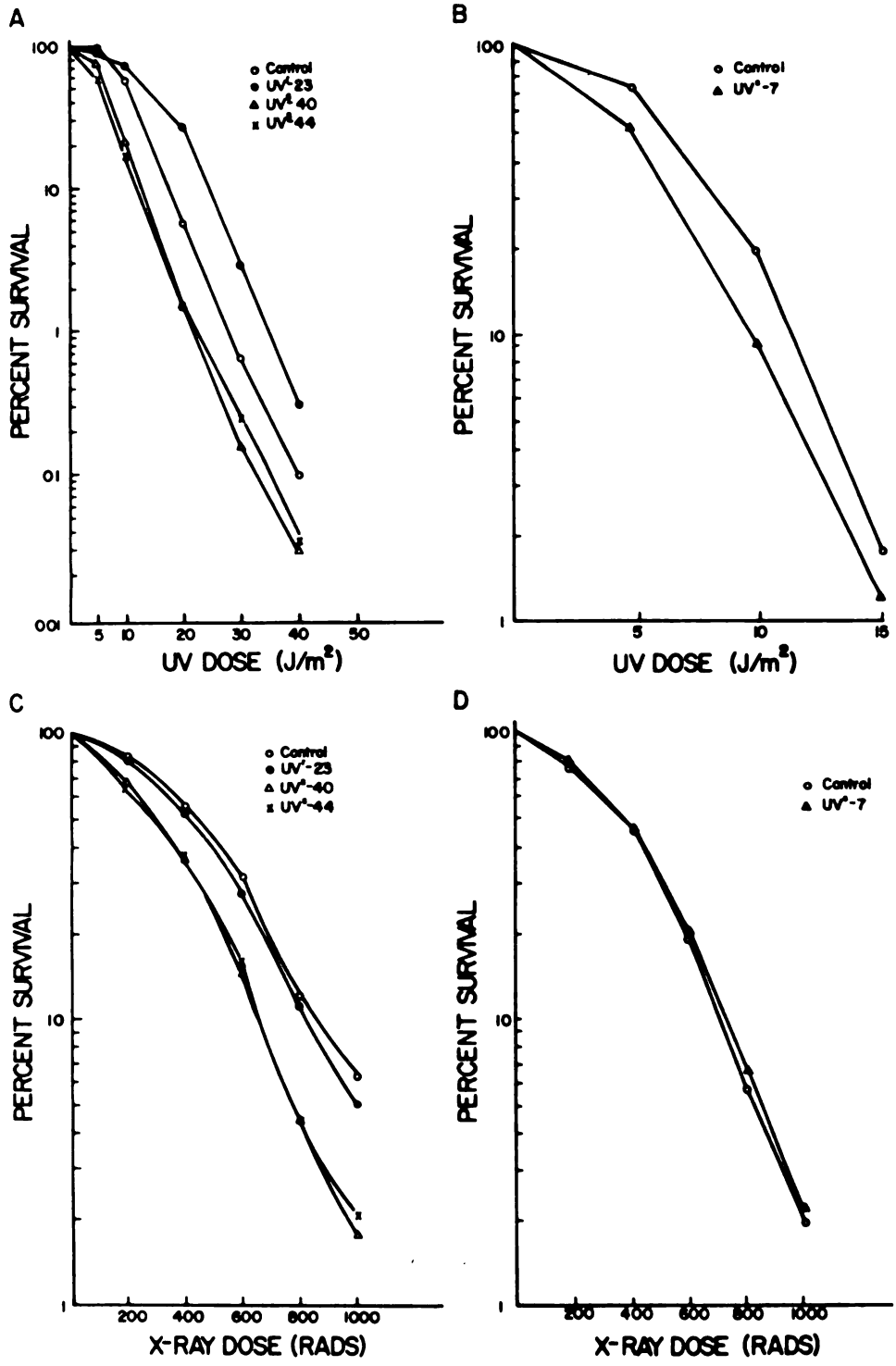


Figure 5. UV and X-Ray Survival for Selected Clones.

D_o is commonly defined as the increase in dose necessary to reduce survival by 1/e (.37) on the straight-line portion of the survival curve. N is the multiple of 100% to which the straight-line portion would extrapolate at 0 UV exposure. The values are presented in Table 5.

Table 5.--D_o Values for UV Exposure.

Cell Lines	Slope	D_o (ergs)	N
Control	-8.8×10^{-3}	49.1	3.34
UV^r-23	-8.0×10^{-3}	53.7	6.84
UV^s-40	-9.6×10^{-3}	43.9	1.93
UV^s-44	-9.8×10^{-3}	46.9	1.48
Control	-16.1×10^{-3}	26.7	43.64
UV^s-7	-16.2×10^{-3}	26.7	33.80

The differences observed between the control lines for the two experiments are a consequence of varying UV sources, inaccurate monitoring of dose (a consequence of faulty monitoring equipment) and cell growth conditions. The interexperimental differences are inconsequential.

The data suggest a minimum of intraexperimental variation in D_o values. The N values change considerably from line to line, presumably a consequence of the size of the shoulder portion of the survival curve. Detailed analysis at low doses has not been conducted to precisely

evaluate the shoulder of the survival curve for the various lines.

Sensitivity to Chemical Mutagens

The mutants were also tested for their sensitivity to two chemical mutagens; NAc-AAF and MNNG. As shown in Figure 6, UV^S-40 and UV^S-44 are very sensitive to NAc-AAF, whereas the sensitivity of UV^S-7 and UV^r-23 to this chemical is similar to the wild type. UV^S-40 and UV^S-44 are also sensitive to MNNG (Figure 7). UV^S-40 is more sensitive to the two chemical mutagens than UV^S-44.

Unscheduled DNA Synthesis (UDS)

UV-induced UDS for the four mutant cell lines and the wild type were determined in two experiments, presented together in Figure 8. The results clearly indicate that UV^S-7 is deficient in UDS. In contrast, UV^r-23 is more proficient in UDS. The other two mutants, UV^S-40 and UV^S-44, which are sensitive to both radiation and chemical mutagens, are only slightly reduced in their ability to conduct UDS.

Temperature Sensitivity Test

Since the potential for temperature sensitivity had been incorporated into the selection protocol, UV^S-40, UV^S-44, and UV^r-23 were examined for UV sensitivity and UV^r-23 and UV^S-7 for UDS at low temperature (34°C). The experiments were conducted in a manner similar to those previously described but in an incubator maintained at

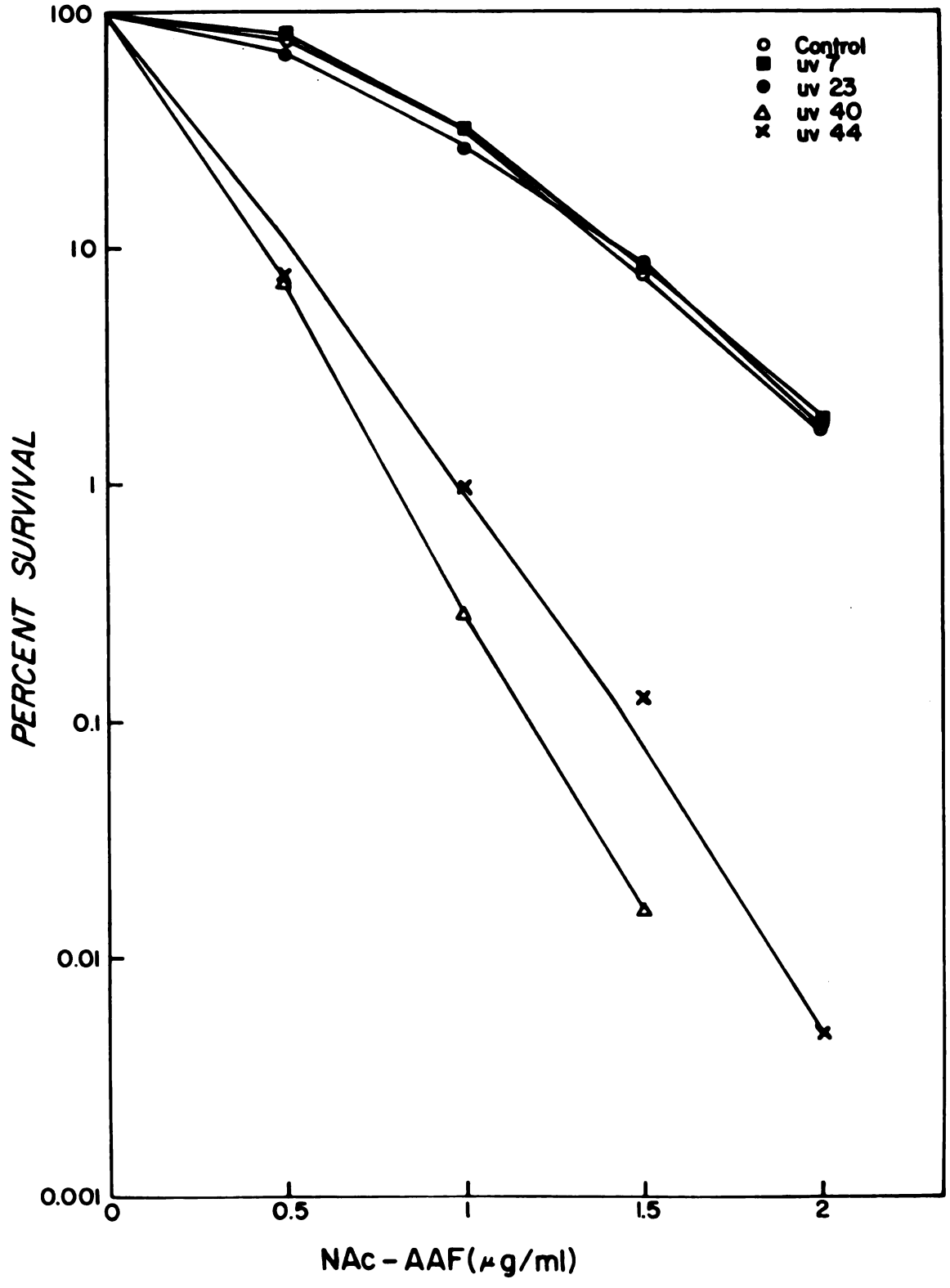


Figure 6. N-acetoxy-2-acetylaminofluorene Toxicity.

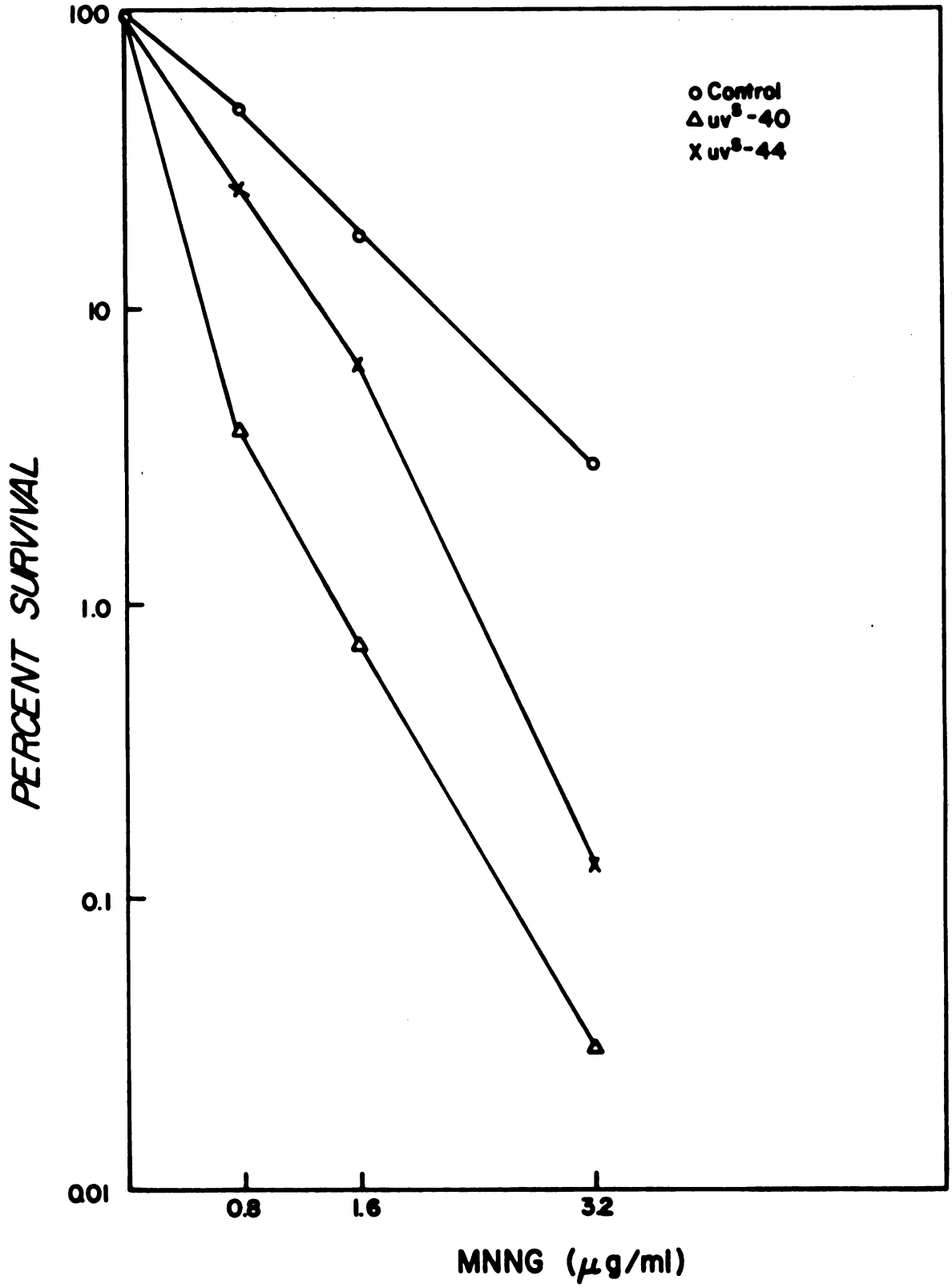


Figure 7. N-methyl-N-nitro-N-nitrosoguanidine Toxicity.

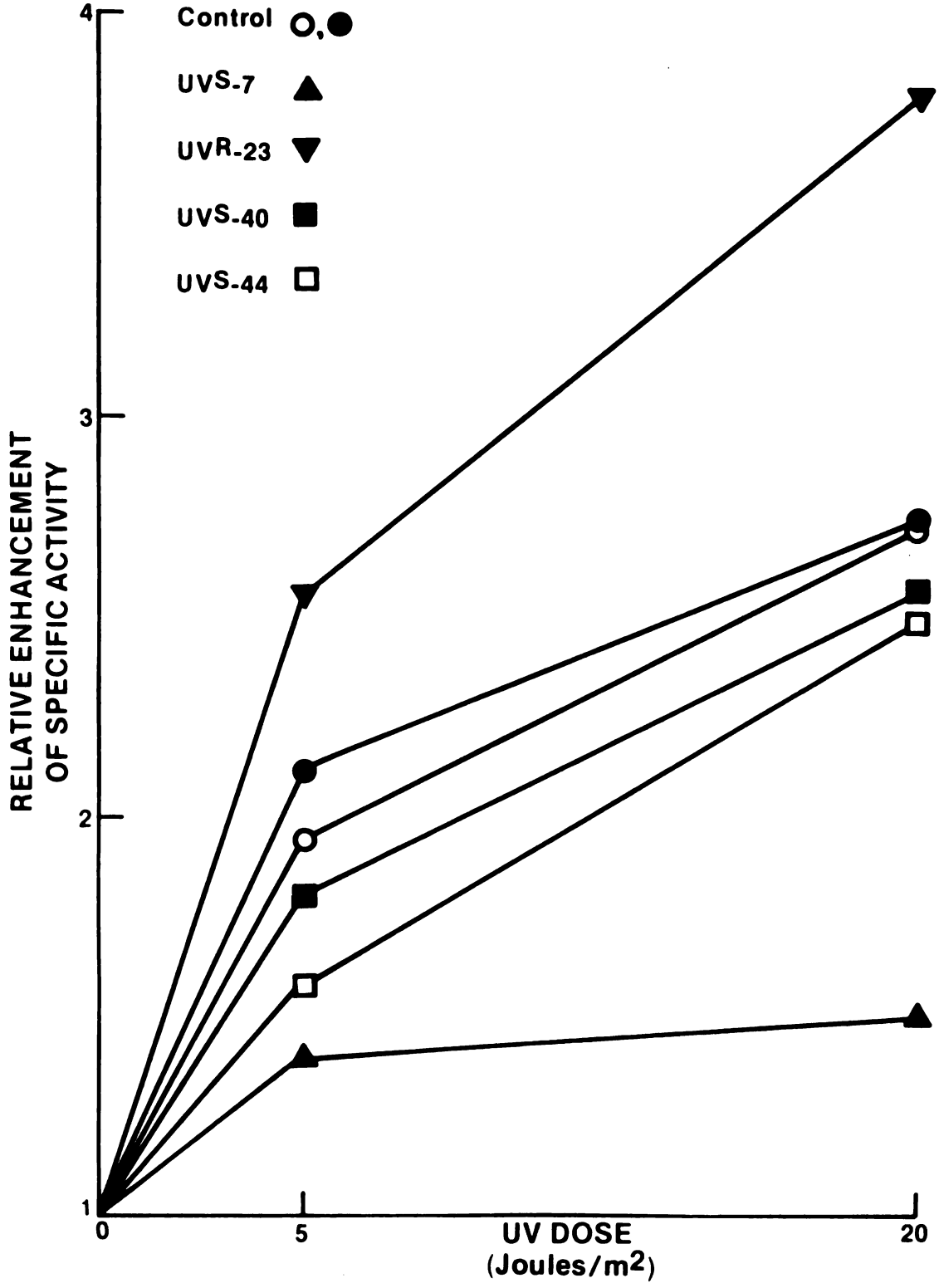


Figure 8. UV-Induced Unscheduled DNA Synthesis.

34°C. The experiments were intended to provide crude data relevant to the temperature sensitive nature of the mutant lines. Table 6 presents the data of an experiment examining the percent survival of mutants and control after exposure to one dose of UV (18 joules/m^2) at low temperature. At low temperature UV^s-40 and UV^s-44 remained highly sensitive and UV^r-23 was resistant to the lethal effects of UV.

Table 6.--Low Temperature Effects on UV-Survival.

Cell Line	Control	UV^r-23	UV^s-40	UV^s-44
% survival	13.2	30.1	2.3	2.6

Figure 9 presents the unscheduled DNA synthesis results at low temperature. The observed alteration in phenotype (UV^r-23 enhancement and UV^s-7 deficiency) persists at low temperature. The alterations responsible for the phenotypes of all four mutants, therefore, appear not to be temperature sensitive in nature.

Chromosome Examination

The examination of chromosomes from the parental and four mutant lines produced a modal number of twenty chromosomes for all lines. Representative preparations are shown in Figure 10. The chromosomes have not been analyzed in greater detail.

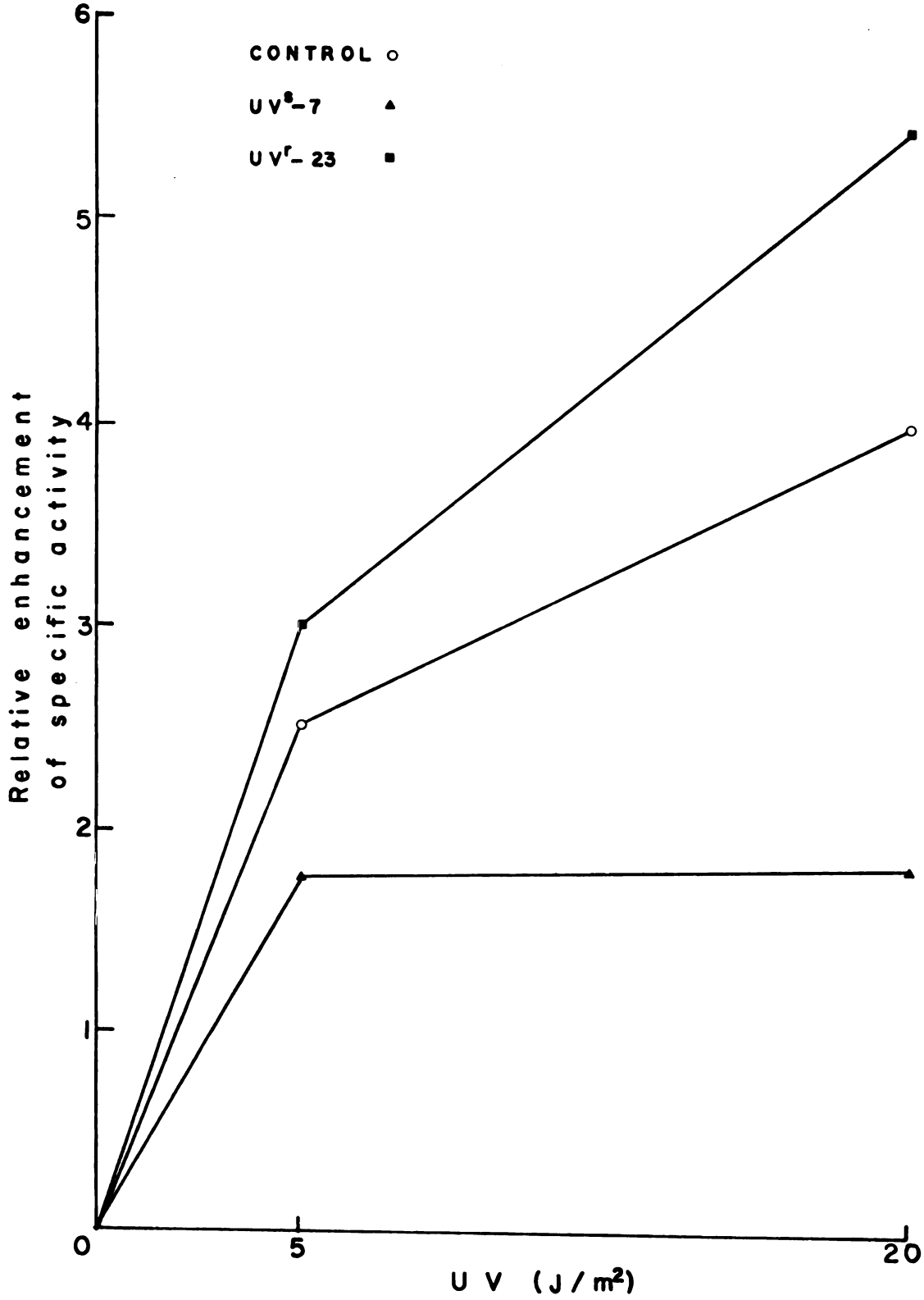
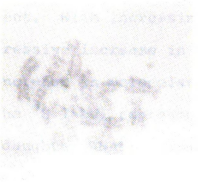
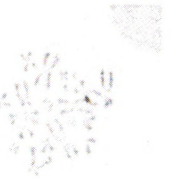


Figure 9. Low Temperature Repair.

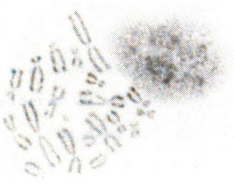


UV^S-7



UV^r-23

V79



UV^S-40

UV^S-44

Figure 10. Chromosomes.

Recovery to High Molecular Weight DNA Following UV-Irradiation

Figure 11 presents the data for the first experiment. With increasing chase time, most lines show a progressive increase in distance sedimented indicating an increase in molecular weight of ^{3}H-thymidine labeled DNA. The ^{3}H label represents DNA synthesized after UV-irradiation (daughter DNA). Disagreement exists in the literature as to whether small weight DNA following UV irradiation is a consequence of postreplication "gaps" or alternatively, a delay in normal DNA synthesis. Progressive recovery either represents the filling of gaps (postreplication repair) or recovery from a delay in normal synthesis. UV^{S}-44 is the only line examined that does not show a progressive increase in the size of the DNA with increasing chase time after irradiation. The distance of ^{3}H-TdR peaks sedimentation in these experiments is defined as:

$$\frac{\text{(Total Fraction \# - Peak } ^{3}\text{H-TdR Fraction \#)}}{\text{Total Fraction \#}}$$

It is a crude estimation of relative size and should not be considered an accurate estimate of molecular weight.

The data for the second experiment are presented in Figure 12. It can be seen that for control cells, a 45 minute chase following either a single or split dose was sufficient to permit recovery of the DNA to unirradiated

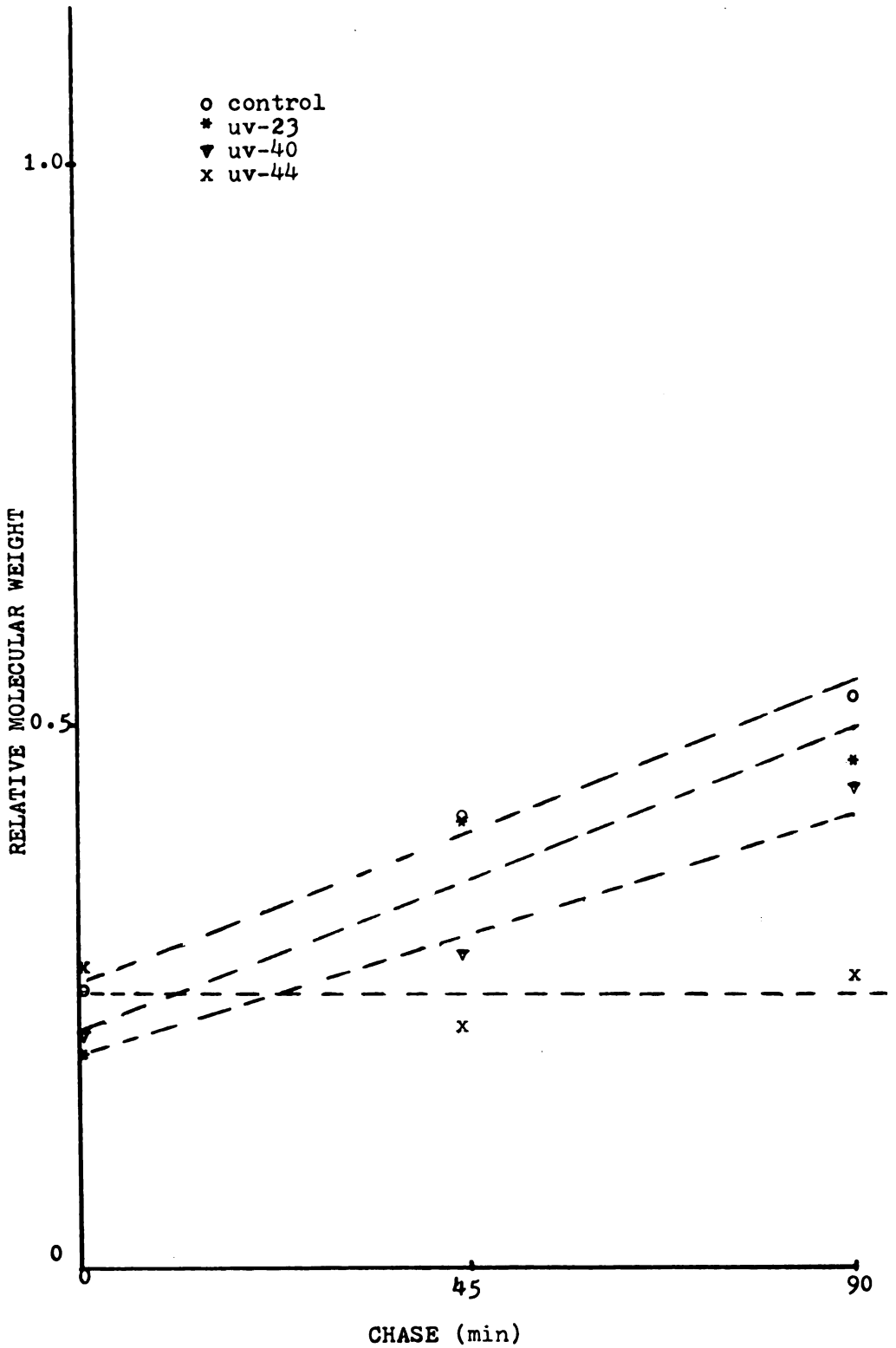


Figure 11. Relative Size of Irradiated DNA.

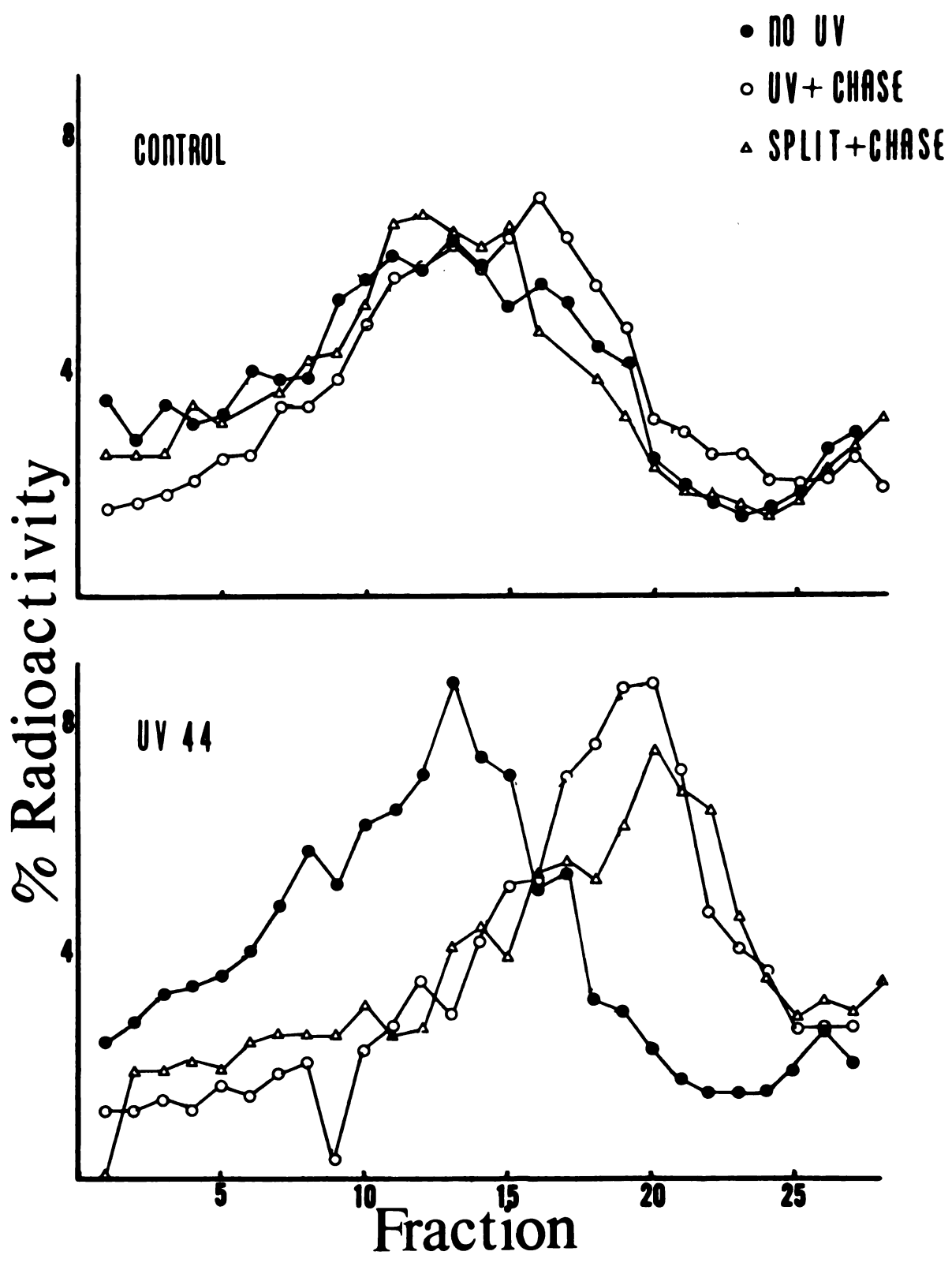


Figure 12. Gradiant Sedimentation Profiles.

size. For UV^S-44, neither condition permitted the DNA to recover to full size.

Cell Growth Rate

The growth curves for the parental line and four mutant lines are shown in Figure 13. All mutants grew at a rate slower than the parental line. The cell division times have been estimated at 16.0, 20.8, 21.5, 22.5, and 26.6 hours for the parental line, UV^r-23, UV^S-44, UV^S-40, and UV^S-7 respectively.

UV-Induced Mutation Frequencies--UV^S-7

Table 7 summarizes the data from three different experiments examining the UV-induced mutability of UV^S-7 at the Na^+/K^+ ATPase and HGPRT loci. In the first experiment, cells were plated at 1×10^5 cells/plate for spontaneous frequency determination and 2×10^5 cells/plate in plates which were irradiated with 18 joules/m^2 UV after 3.5 hours for cell attachment. Cells for spontaneous frequency determination were allowed one day of *in situ* growth before selection with 1 mM ouabain. UV-irradiated cells were selected after three days *in situ* growth, a period of time previously demonstrated to be sufficient for maximal expression of wild type UV-induced ouabain-resistant mutants (97). Under these conditions, the UV-induced ouabain-resistant mutation frequency for UV^S-7 was three times that of the parental line.

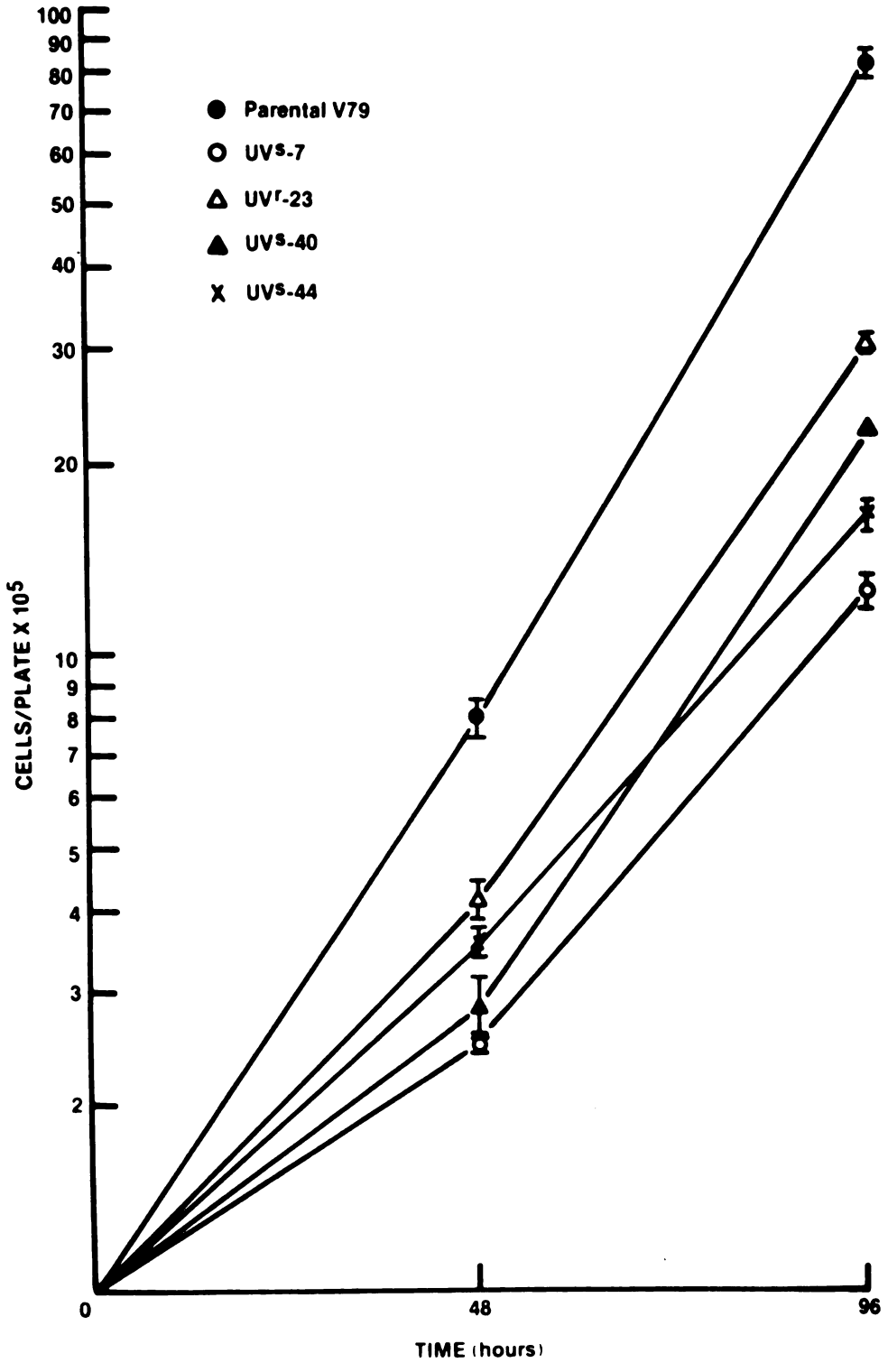


Figure 13. Mutant Growth Rates.

Table 7.--UV-Induced Mutagenesis for UV^S-7 and Its Parental V79 Cells.

Experiment	Cell Line	UV Dose (J/m^2)	Survival %	Expression Time (Days)	Total # of cells assayed $x10^5$	Mutation Frequency / 10^6 Survivors
	V79	0	100	1	30.8	3.3
#1	UV^S-7	0	100	1	23.0	1.3
OUA^R (A)	V79	18	7.4	3	4.1	448.8
	UV^S-7	18	5.6	3	2.4	1529.6†
	V79	0	100	2	19.9	8.5
#2	UV^S-7	0	100	4	19.1	2.6
OUA^R (A)	V79	20	2.0	2.5	0.8	1093.3
	UV^S-7 (B)	20	1.1	5	0.3	29444.4†
	V79	0	100	6	9.9	9.1
	UV^S-7	0	100	6	10.3	10.3
	V79	0	100	9	7.1	29.6
	UV^S-7	0	100	9	7.8	10.3
	V79	0	100	12	10.4	12.5
	UV^S-7	0	100	12	8.1	2.5
#3	V79	0	100	15	7.7	29.9
$6\text{-}TG^R$ (C)	UV^S-7	0	100	15	12.4	7.3
	V79	14	11.2	6	7.7	163.6
	UV^S-7	14	10.0	6	8.6	174.4
	V79	14	11.2	9	5.1	309.8
	UV^S-7	14	10.0	9	7.3	574.0†
	V79	14	11.2	12	9.2	220.7
	UV^S-7	14	10.0	12	8.0	411.3†
	V79	14	11.2	15	7.5	240.0
	UV^S-7	14	10.0	15	11.6	424.1†

No. of cells used per plate (9cm) was A= $1x10^5$ for spontaneous and $2x10^5$ for UV-induced except for B= $5x10^4$. C= $5x10^4$ for all plates.

† Mutation frequencies for UV^S-7 are significantly higher than those for V79 control with equivalent dose of UV, $P < 1\%$.

Due to the slow growth of UV^S-7, three days *in situ* does not result in colony size equal to the parental control at the time of selection. This factor was taken into account in the second experiment, which also employed the *in situ* ouabain selection technique. After exposure to a high dose of UV (20 joules/m^2), five days growth permitted surviving UV^S-7 cells to achieve a predominance of 16-cell colonies. The parental line was noted to achieve such colonies in 2.5 days. Employing such a variable expression protocol resulted in an induced mutation frequency for UV^S-7 nearly 27 times that of the parental line.

In the third experiment, the spontaneous and UV-induced 6-thioguanine resistance frequencies were examined with an *in situ* protocol, using one moderate dose of UV (14 joules/m^2) and multiple expression times of 6, 9, 12, and 15 days. Plating 5×10^4 cells/plate has previously been shown to be of sufficiently low density to permit the recovery of virtually all resistant cells for the control line (127). The results indicate that for both the parental control (V79) and mutant (UV^S-7) lines, maximal expression was not fully achieved by six days, but was by nine days. In addition, further expression did not result in an increase in the mutation frequency for UV^S-7, suggesting that the expression period for both the control and mutant line are similar for 6-thioguanine resistance. Most importantly, with this moderate dose of UV, the mutant line demonstrated

a slight increase in UV-sensitivity and a nearly twofold increase in mutability.

The mutability of UV^S-7 was also examined at the diphtheria toxin (DT) resistance locus (elongation factor II). The experiment was conducted concurrently with the 6-thioguanine experiment presented in Table 7. The same spontaneous and UV-induced (14 joules/m^2) populations were used to determine the frequency of DT resistance as were used to examine 6-TG resistance. The same expression times of 6, 9, 12, and 15 days were examined. The DT results are presented in Table 8 and summarized in Figure 14. The results demonstrate that both the parental control and mutant line share equivalent UV-induced maximal expression times of approximately nine days. Both lines demonstrate a sharp decline in the frequency of mutants after the maximal expression time, indicating a strong selective disadvantage for DT resistant cells in the population. At the maximal expression period of nine days, the UV-induced mutation frequency for UV^S-7 is seven times that of the wild type cells.

UV-Induced Mutation Frequencies--UV^S-40

The UV-induction of ouabain resistance for UV^S-40 was examined in two separate experiments. The first, presented in Table 9, examines the frequency of ouabain resistance following 0, 5, 10, or 20 joules/m^2 of UV. The *in situ* expression technique was used, with the variations in

Table 8.--UV-Induced Diphtheria Toxin Resistance for UV^S-7 and Its Parental Control.

Cell Line	UV Dose (J/m^2)	Survival (%)	Expression Time (Days)	Total Number* of Cells Assayed x 10^5	Mutation Frequency / 10^6 Survivors
V79	0	100	6	19.8	30.8
UV^S-7	0	100	6	20.7	116.5
V79	0	100	9	14.2	19.0
UV^S-7	0	100	9	15.5	119.7
V79	0	100	12	20.8	5.8
UV^S-7	0	100	12	16.3	3.9
V79	0	100	15	15.3	11.1
UV^S-7	0	100	15	24.8	31.0
V79	14	11.2	6	15.4	167.5
UV^S-7	14	10.0	6	17.2	1325.0
V79	14	11.2	9	10.1	245.2
UV^S-7	14	10.0	9	14.7	1757.3
V79	14	11.2	12	18.3	14.2
UV^S-7	14	10.0	12	16.0	722.7
V79	14	11.2	15	14.9	44.0
UV^S-7	14	10.0	15	21.0	656.8

*Number of cells used per plate (9 cm) was 1 x 10^5.

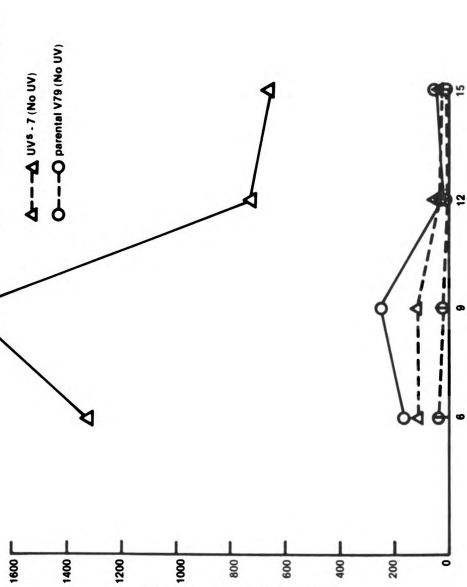


Figure 14. Diphtheria Toxin Resistance for UV^5-7 and Its Parental Control.

Table 9.--UV-Induced Mutagenesis for UV^S-40 and Its Parental V79 Cells.

Cell Line	UV Dose (J/M^2)	Survival (%)	Expression Time (Days)	Total # of cells assayed $x10^5$	OUA^R Mutation Frequency 10^6 Survivors
V79	0	100	1	20.7 (B)	1.5
UV^S-40	0	100	1	11.7 (B)	7.7
V79	5	91.6	2	9.5 (A)	77.9
UV^S-40	5	71.9	2	8.0 (B)	58.8
V79	10	65.7	2	6.1 (A)	75.4
V79	10	65.7	3	6.8 (A)	117.6
UV^S-40	10	16.6	3	3.7 (C)	127.0
UV^S-40	10	16.6	4	3.9 (C)	146.2
V79	20	14.0	2	5.8 (C)	89.7
V79	20	14.0	3	5.8 (C)	243.1
V79	20	14.0	4	2.9 (B)	410.3
UV^S-40	20	1.1	3	1.3 (D)	$346.2^{\dagger\ \dagger\dagger}$
UV^S-40	20	1.1	4	1.3 (D)	$584.6^{\dagger\ \dagger\dagger}$
UV^S-40	20	1.1	5	1.1 (D)	$1145.5^{\dagger\dagger}$

No. of cells used per plate (9cm) was
A= $5x10^4$, B= $1x10^5$, C= $2x10^5$, D= $8x10^5$

† Mutation frequencies for UV^S-40 that are significantly higher than those for V79 control at equivalent dose of UV and equivalent expression time. $P < 1\%$

†† Mutation frequencies for UV^S-40 that are significantly higher than those for V79 control at equivalent dose of UV but one day less expression time for V79 control, $P < 1\%$.

expression time as indicated. As UV^S-40 divides slower than the V79 control, equivalent or longer expression times were commonly examined for UV^S-40 to permit the development of equivalent colony size before selection. The data indicate no difference in the resistance frequencies at a low UV dose of 5 joules/m^2 and only a slight, but statistically insignificant, hypermutability of UV^S-40 at a moderate UV dose of 10 joules/m^2. At 20 joules/m^2, the hypermutability of UV^S-40 is quite evident.

A second UV-induced ouabain resistant experiment was conducted, examining the frequency of resistant colonies at 2, 3, 4, and 5 days in situ expression, A single UV dose of 20 joules/m^2 was used. The data are presented in Table 10 and summarized in Figure 15. UV^S-40 is clearly hypermutable compared to the V79 parental control with 20 joules/m^2 of UV-irradiation.

UV-Induced Mutation Frequencies--UV^r-23

Table 11 presents the data derived from two separate experiments examining the UV induction of ouabain resistant colonies for UV^r-23 and the parental control line. Expression for both experiments was achieved via in situ colony growth following the various UV doses indicated. The data suggest that UV^r-23 may be hypomutable, as the frequency of ouabain-resistant mutants is less than that for the parental control at all UV doses. However, only at the highest dose (20 joules/m^2) does that difference appear significant.

Table 10.--Expression of UV-Induced Mutagenesis for UV^S-40 and Its Parental Control.

Cell Line	UV Dose (J/m^2)	Survival (%)	Expression Time (Days)	Total Number of Cells Assayed x 10^5	Mutation Frequency / 10^6 Survivors
V79	0	100	2.5	22.1 (A)	1.4
UV^S-40	0	100	2.5	18.5 (A)	28.1
V79	20	10.9	2	4.8 (B)	81.2
UV^S-40	20	1.1	2	1.3 (D)	339.5
V79	20	10.9	3	4.6 (B)	142.1
UV^S-40	20	1.1	3	1.3 (D)	534.9
V79	20	10.9	4	2.4 (A)	212.5
UV^S-40	20	1.1	4	1.2 (D)	686.7
V79	20	10.9	5	1.1 (C)	244.8
UV^S-40	20	1.1	5	1.3 (D)	728.1

Number of cells used per plate (9 cm) was: A = 1×10^5, B = 2×10^5, C = 5×10^4, D = 5.2×10^5.

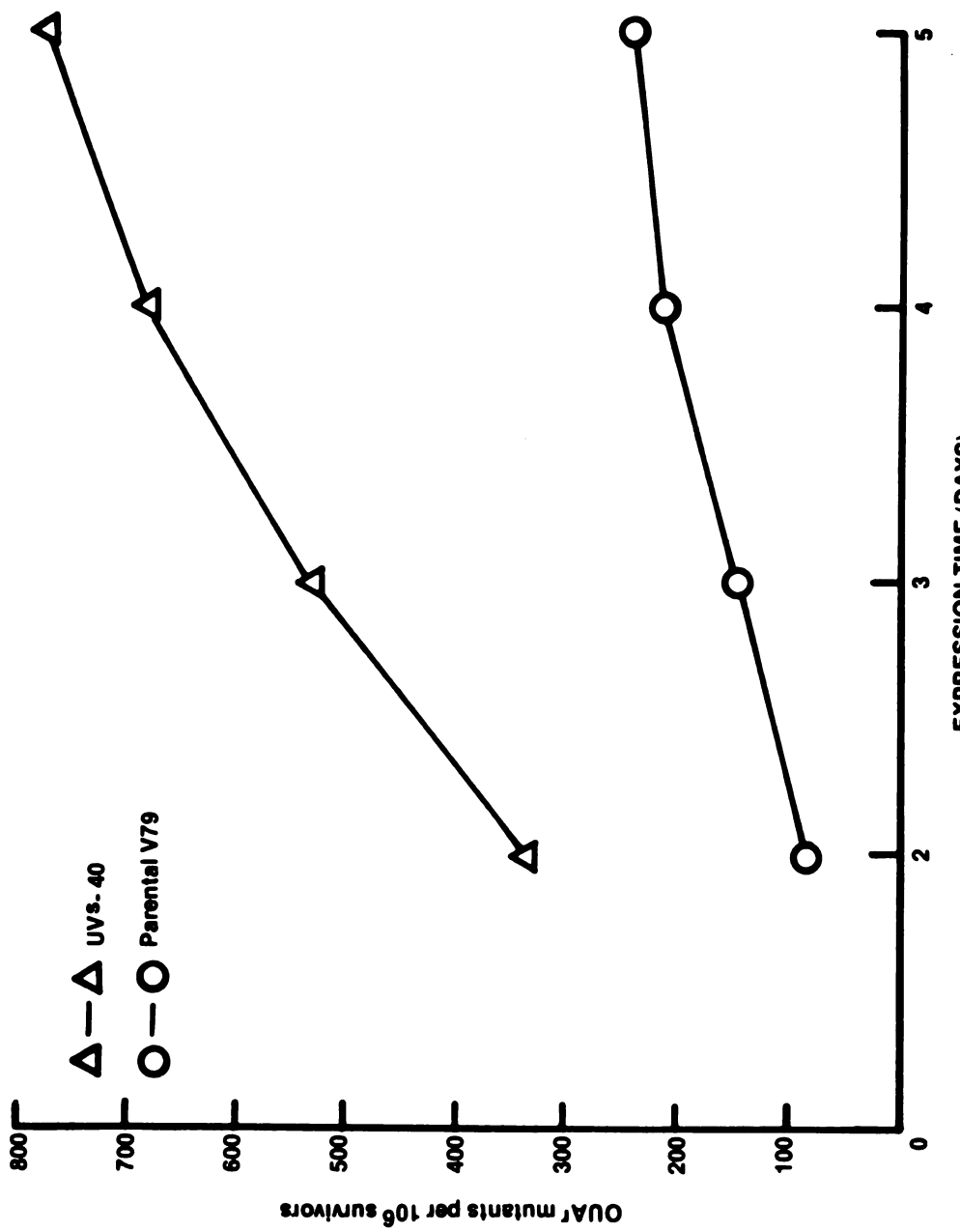


Figure 15. Expression of UV-Induced Mutagenesis for UV^S-40 and Its Parental Control.

Table 11.--UV-Induced Mutagenesis for UV^r-23 and Its Parental V79 Cells--*In Situ* Expression.

Experiment	Cell Line	UV Dose (J/m^2)	Survival (%)	Expression Time (Days)	Total Number of Cells Assayed x 10^5	Mutation Frequency 10^6 Survivors
	V79	0	100	1.25	11.0 (C)	17.4
#1	UV^r-23	0	100	1.25	18.4 (D)	0.5
OUA	V79	18	7.2	3	1.6 (C)	286.6
	UV^r-23	18	25.3	3	2.8 (B)	229.4
	V79	0	100	1	19.5 (C)	4.1
	UV^r-23	0	100	1	50.9 (D)	2.0
#2	V79	10	38.8	2.5	3.6 (A)	32.8
OUA	UV^r-23	10	74.0	2.5	9.0 (A)	20.4
	V79	20	1.8	3	0.7 (D)	2060.2
	UV^r-23	20	15.7	3	2.0 (A)	793.5

Number of cells used per plate (9 cm) was: A = 5×10^4, B = 6×10^4, C = 1×10^5, D = 2×10^5.

It was suggested for both UV^s-7 and UV^s-40 that slower division time might necessitate longer expression periods than the control cells to achieve a maximal value. This is particularly important when utilizing *in situ* selection techniques, where one hopes to achieve equivalent colony size for selection. This hypothesis, however, might be used to argue against the hypomutability of UV^r-23, which also grows slower than the parental control, and may not have fully expressed at the time of selection. Therefore, a third experiment was conducted, examining the UV-induction of both ouabain-resistant and 6-thioguanine-resistant colonies for UV^r-23 and the parental control using the replating technique for expression. One dose of UV (16.8 joules/m^2) and multiple expression times of 3, 6, 9, and 12 days were used. The results are presented in Table 12 and the ouabain and 6-thioguanine portions summarized in Figure 16. The previous tendency for UV^r-23 to appear hypomutable was confirmed. For the expression periods examined, no delay in ouabain or 6-thioguanine expression was noted for UV^r-23 cells.

A sharp reduction in the frequency of UV^s-23 ouabain-resistant cells was noted between the third and sixth days of replating. This observation suggests a great selective disadvantage for UV^r-23 ouabain-resistant cells. These findings coincide with the qualitative observation that both 6-thioguanine- and ouabain-resistant clones derived

Table 12.--UV-Induced Mutability of UV^r-23 and Its Parental Control with Replated Expression.

Cell Line	UV Dose (J/m^2)	Survival (%)	Expression Time (Days)	Total Number of Cells Assayed x 10^5	Mutation Frequency / 10^6 Survivors	Total Number of Cells Assayed x 10^5	Mutation Frequency / 10^6 Survivors
				Ouabain		6-Thioguanine	
V79	0	100	3	18.1	0.6	8.0	74.2
UV^r-23	0	100	3	19.0	0	8.3	56.4
V79	0	100	6	10.6	0	4.6	140.1
UV^r-23	0	100	6	19.6	0.5	8.6	63.9
V79	0	100	9	13.0	0	5.7	121.3
UV^r-23	0	100	9	17.4	0.6	7.6	88.0
V79	0	100	12	16.8	0.6	7.3	148.7
UV^r-23	0	100	12	17.8	0	7.8	53.8
V79	16.8	14.8	3	15.5	47.7	7.0	223.9
UV^r-23	16.8	21.8	3	18.3	40.4	8.0	227.1
V79	16.8	14.8	6	11.9	47.1	5.2	598.1
UV^r-23	16.8	21.8	6	11.9	33.6	5.2	442.5
V79	16.8	14.8	9	9.6	16.7	4.2	557.1
UV^r-23	16.8	21.8	9	16.0	11.9	7.0	457.1
V79	16.8	14.8	12	10.5	19.0	4.6	602.2
UV^r-23	16.8	21.8	12	17.6	21.0	7.0	459.0

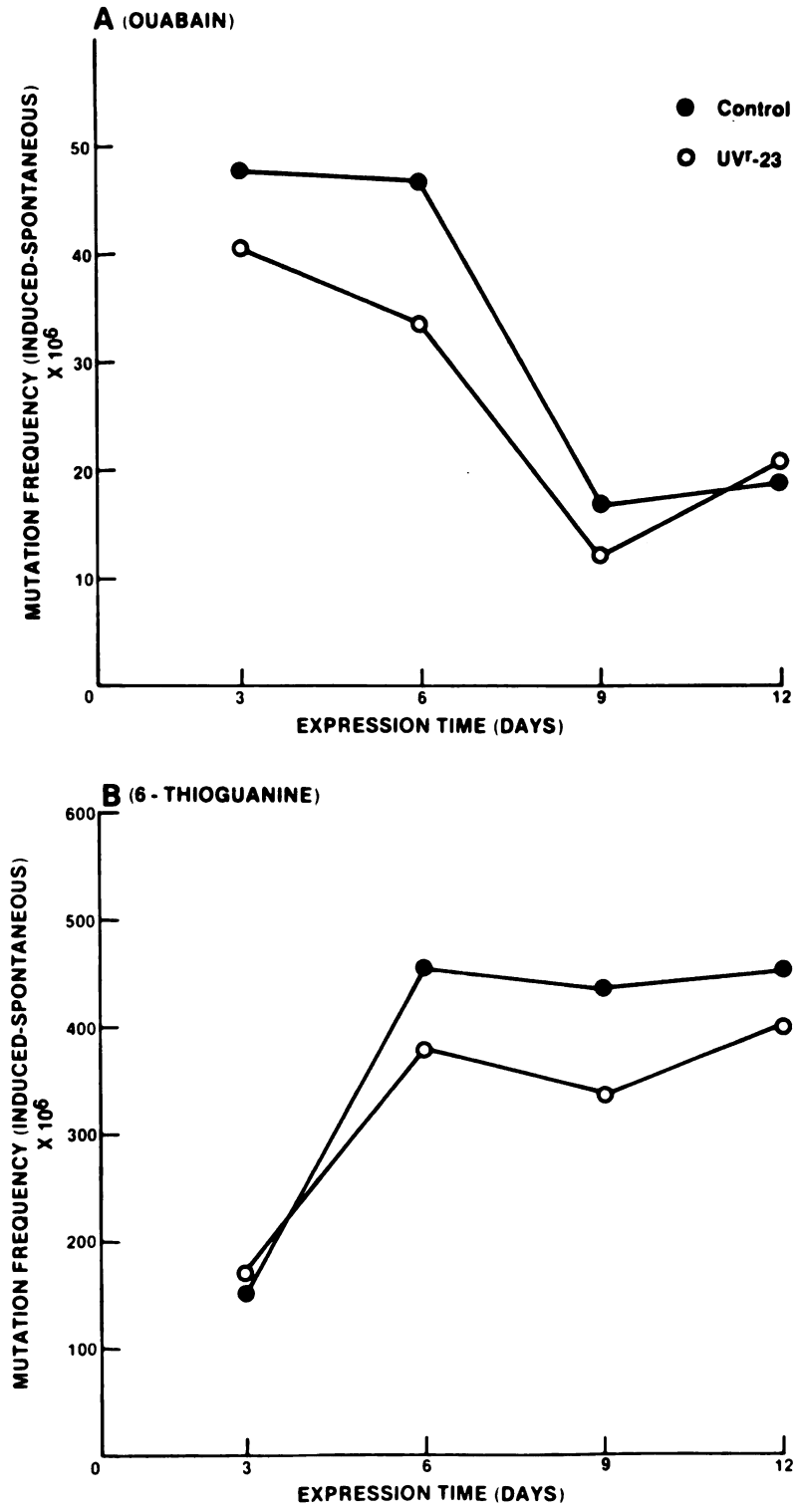


Figure 16. Expression of Ouabain and 6-thioguanine Resistance for UV^S-7 and Parental Control Cultures.

from UV^r-23 cells appeared less healthy than their parental equivalents, even though UV^r-23 cells generally appeared as healthy as the parental cells when grown as clones or in mass culture.

UV-Induced Mutation Frequencies--UV^S-44

The UV-induced mutability of UV^S-44 was examined in three experiments, the results of which are presented in Table 13. With a single UV dose of 18.2 joules/m^2, and replating expression times of 8 and 13 days, the 6-thioguanine-resistant mutation frequency for UV^S-44 was found to be comparable to that for the parental control cells. The two ouabain experiments were conducted utilizing *in situ* expression and 18 joules/m^2 of UV. In both cases, UV^S-44 was provided a single *in situ* expression time of three days. No significant difference was noted between the mutability of UV^S-44 or parental control cells. It was, however, noted that UV^S-44 ouabain-resistant colonies exhibit poor growth and colony formation.

Retesting of Induced Mutants

UV-induced ouabain-resistant mutants from UV^S-7 were isolated (from Table 7, Experiment #2) and reselected in 1 mM ouabain. Of thirty clones retested all were confirmed as ouabain-resistant mutants. Following the diphtheria toxin experiment (Figure 14), UV-induced DT^r mutants of UV^S-7 were isolated and tested for sensitivity

Table 13.--UV-Induced Mutagenesis of UV^S-44 and Its Parental Control.

Experiment	Cell Line	UV Dose (J/m^2)	Survival (%)	Expression Time (Days)	Total Number* of Cells Assayed x 10^5	Mutation Frequency / 10^6 Survivors
	V79	0	100	1.25	11.0	17.4
#1	UV^S-44	0	100	1.25	11.4	0.9
OUA	V79	18	7.2	3	1.6	286.6
	UV^S-44	18	2.2	3	3.3	253.9
	V79	0	100	1.5	18.9	7.9
#2	UV^S-44	0	100	1.5	3.2	1.0
OUA	V79	18	6.1	3	2.6	316.6
	UV^S-44	18	2.0	3	4.3	318.6
	V79	0	100	8	12.2	23.0
	UV^S-44	0	100	8	8.4	0
	V79	0	100	13	8.4	12.0
#3	UV^S-44	0	100	13	8.2	0
6-TG	V79	18.2	9.0	8	5.2	396.3
	UV^S-44	18.2	1.8	8	6.3	353.8
	V79	18.2	9.0	13	8.5	352.7
	UV^S-44	18.2	1.8	13	7.0	374.0

*Number of cells used per plate (9 cm) was 5 x 10^4.

to both diphtheria toxin and pseudomonas exotoxin. Of twenty-four clones retested all were found to be resistant to both toxins. The results lend support to the frequencies reported and to the hypermutability of UV^S-7.

Spontaneous Mutation Rates

UV^S-7 demonstrated UV-induced hypermutability at three loci, a slightly increased sensitivity to UV, and a greatly reduced capacity to conduct unscheduled DNA synthesis when stimulated by UV. This mutant cell line, however, shows no increased sensitivity to x-ray or NAc-AAF. As all abnormal phenotypes appeared to occur in response to UV, it was of particular interest to examine the spontaneous mutability of UV^S-7 relative to the parental control.

As previously described, to evaluate the spontaneous mutation rate, it is necessary to determine the change in mutation frequency relative to cell growth. Populations of UV^S-7 and parental control cells which had been preselected in HAT medium were maintained for a period of 35 days with frequent samplings of mutation frequencies. Figure 17 presents the results obtained with these frequency determinations. Both cell lines showed a consistent increase in the frequency of 6-thioguanine-resistant cells. This increase was faster in the control line than the UV^S-7 mutant, a result anticipated since UV^S-7 grows slower and thereby accumulates new mutants less quickly. Frequency determinations were also made at later times as the two cultures

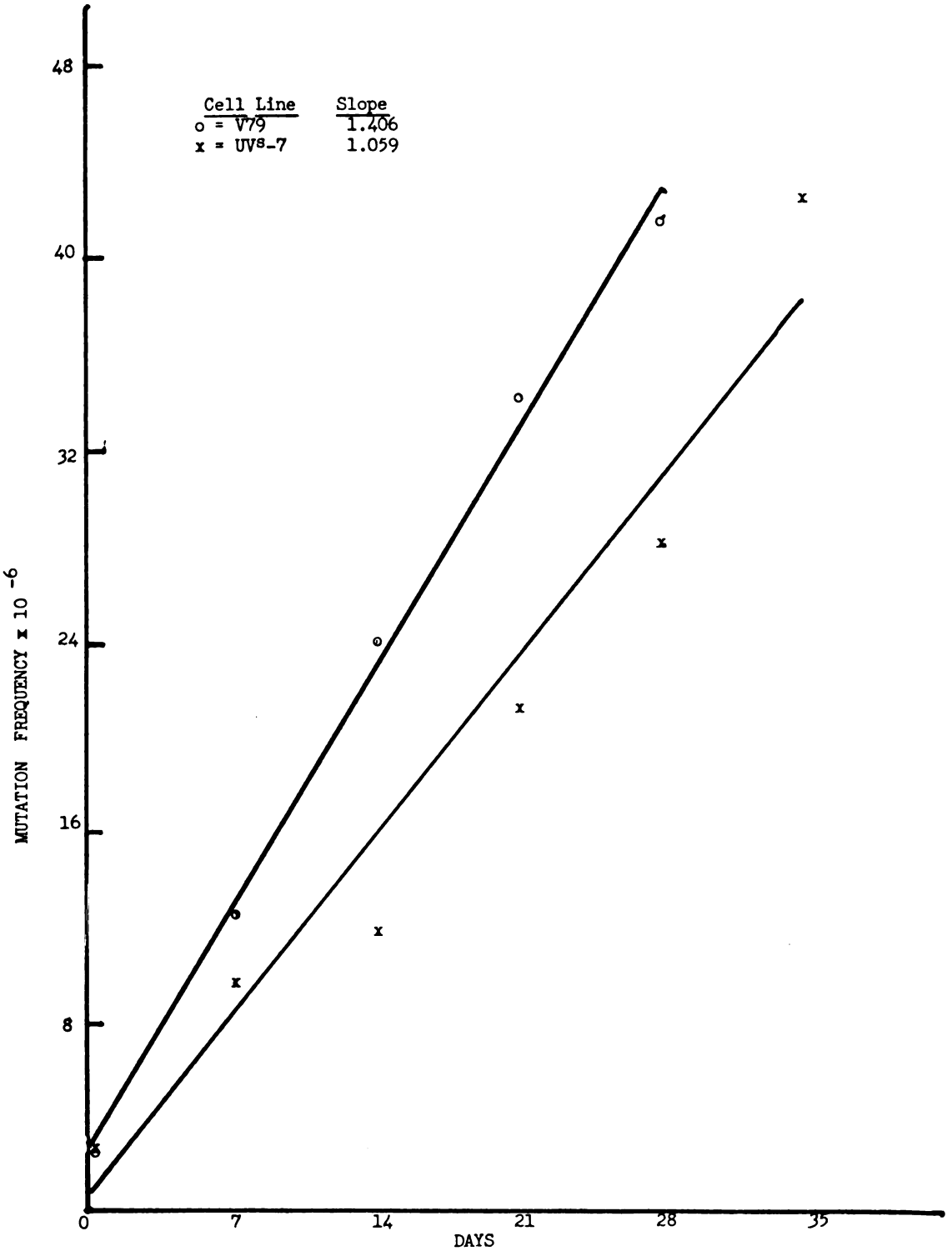


Figure 17. Spontaneous Mutation Frequencies in UV^S-7 and Parental Control Cultures.

were maintained for a total period of eighty days. At various intervals throughout this eighty day period, the increase in population size was monitored between frequency platings to permit the calculations of spontaneous mutation rates. The data are presented in Table 14. A standard Student t-test indicates that no significant difference exists between the average spontaneous mutation rate for UV^S-7 and that for the parental control.

Stability of Mutants

The four mutant cell lines that have been most extensively studied throughout this research appear to be quite stable. Each line was maintained continuously in culture for a period of time in excess of thirty days and in the two and a half years since their initial isolation, no reversion of any altered phenotype has been observed.

Table 14.--Spontaneous Mutation Rates.

Day Plated	V79			UV^S-7		
	Mutation Frequency / 10^6 Cells	Total Cells in Population	Mutation Rate $\times 10^{-6}$	Mutation Frequency / 10^6 Cells	Total Cells in Population	Mutation Rate $\times 10^{-6}$
0	2.59	2.0×10^7	2.135	2.87	2.0×10^7	2.888
7	12.63	1.4×10^{10}		9.86	5.7×10^8	0.779
14	23.98	2.0×10^7	2.328	11.95	2.4×10^{10}	3.256
21	34.11	8.4×10^9		21.34	1.3×10^{12}	2.175
28	41.07	7.1×10^{12}	1.431	27.90	8.4×10^{13}	4.116
35	--	--		42.06	4.9×10^{15}	
69	43.29	2.7×10^{28}	3.016	45.70	7.2×10^{24}	2.360
80	62.51	1.9×10^{32}		60.00	3.2×10^{28}	
		$*\bar{X}_{V79} = 2.228 \times 10^{-6}$			$*\bar{X}_{UV^S-7} = 2.596 \times 10^{-6}$	

*Means are not significantly different ($P > 0.01$).

DISCUSSION

The Isolation Protocol and Its Success

A new isolation protocol (Figure 1) was designed and implemented to aid the selection of mammalian cell mutants with defects relevant to the mechanisms of DNA repair. The first phase of this protocol specifically focused on the generation of new mutants. It has long been recognized that most new mutations are recessive (128). On the assumption that wild type cells in culture are homozygous for normal alleles at repair related loci, the likelihood of generating a homozygous recessive mutant with a point mutagen such as UV, would be exceedingly small. The generation of a hemizygous state through the deletion of one of the homologues, and subsequent point mutagenesis of the remaining homologue should provide an alternative theoretical approach and an adequate means for the generation of temperature sensitive mutants. In the studies reported here, an attempt to generate hemizygosity involved the incorporation of BrdU for approximately two rounds of replication followed by exposure to black light. Such treatment has previously been shown to induce strand breaks (128) and is also capable of producing temperature sensitive mutants (130). Subsequent exposure to UV, primarily

resulting in the formation of pyrimidine dimers, could result in the formation of a point mutation in the hemizygous locus. This induction technique should permit the generation of either complete deficiencies or partial reductions in repair related functions.

The two-step mutation induction is relatively harsh, and may result in the generation of mutants with alterations at a variety of loci in addition to those related to repair. From the chromosomal observations reported, it does not appear that the induction or selection procedures encouraged the isolation of mutants with altered ploidy.

The induction procedure appears to have been affective in generating a variety of mutant phenotypes. Although unmutagenized populations have not been tested in the selection portion of the isolation protocol, it is unlikely that such a variety of mutants would readily exist in the general population. Isomura and colleagues attempted to isolate UV-sensitive mutants from populations of unmutagenized HeLa S3 cells without success (114). Furthermore, the data presented here and the personal experience of this researcher indicate that the mutants exhibit poorer plating efficiencies, slower growth characteristics, and a general inability to effectively tolerate medium deficiencies and depletions as compared to wild type cells. Such mutants are likely to experience a strong selective disadvantage

in the general population. This hypothesis is testable and may be of considerable importance in future considerations of somatic cell population genetics.

The selection portion of the isolation protocol is a modification of the tritium suicide technique previously reported to be an effective selection procedure in Chinese hamster ovary cells (131). Modifications included the growth of cells in reduced levels of fetal calf serum (1%) to accumulate cells in non-S phase, thereby avoiding ^{3}H-TdR incorporation due to normal DNA synthesis. The 10 joules/m^{2} dose of UV just prior to ^{3}H-TdR exposure was designed to encourage the uptake of tritium by excision repair proficient cells. Following the accumulation of tritium damage at low temperature, cells which survived as a consequence of culture dish shadowing were eliminated by scrapping and those which survived because of an inability to utilize exogenous thymidine (thymidine kinase deficient) were eliminated by growth in HAT medium.

The selection scheme is also harsh. Although the lethal effects of selection were not quantitated, significant killing was observed. Various treatments, and in particular the 10 joule/m^{2} dose of UV, may have selectively eliminated desirable mutants, including those highly sensitive to UV. For this reason, Kuroki and Miyashita (103) have criticized the use of harsh selection and have focused on replica plating as a means of mutant identification.

Replica plating, however, involves no means of enrichment and thereby proves tedious, particularly if attention is focused on repair deficiency, which cannot be easily identified. It was, therefore, beneficial to employ some form of selection protocol with the risk of eliminating some mutants. It can be noted that UV^S-7 would be difficult to detect via replica plating, except by virtue of its slow growth.

Since 20 of the 78 isolated clones appeared to have altered UV sensitivity or DNA repair, the isolation protocol appears to have functioned as originally expected. This has been further verified by the detailed characterization of a few selected isolated mutants, and a second attempt at the isolation protocol, which also produced a number of clones qualitatively identified as UV sensitive. Although greater consideration may have been focused on improvements in the isolation technique, it seemed most beneficial to concentrate attention on the characterization of the specific isolated cell lines.

A great many loci influence the quality and quantity of DNA repair, as has been extensively demonstrated in Escherichia coli. It was, therefore, not surprising to find such extensive diversity among the isolated mammalian mutants. This diversity is illustrated in Figure 4 and Table 4. The four mutants most extensively characterized, UV^S-7, UV^S-40, UV^S-44, and UV^r-23, illustrate the extreme

examples of this variation. Although the observed diversity was not surprising, the recovery of large numbers of clones, exemplified by UV^r-23, with resistance to UV and/or enhancement of unscheduled DNA synthesis was unexpected. Such clones may have survived as a consequence of their resistance to UV. The isolation procedure utilizes several exposures to UV. These exposures may select for resistant cells and enrich their proportion in the general population. This would increase the likelihood that several colonies surviving the tritium selection would actually be resistant to UV, with normal or enhanced UDS.

UV^s-7 is precisely the type of mutant that the isolation protocol was intended to select. Greatly reduced UDS would facilitate survival through the lethal growth selection. As UV^s-7 is only slightly sensitive to UV, the 10 joule/m^2 exposure prior to ^{3}H-TdR incorporation would not have been excessively lethal. Had UV^s-7 been highly sensitive to UV, comparable to complementation group D XP cells (52), a 10 joule/m^2 dose would have killed the mutants.

UV^s-40 and UV^s-44 are moderately sensitive to a variety of mutagens and only slightly depressed in their ability to conduct UDS. The selection of UV sensitive mutants with relatively normal UDS was expected, as the laboratories of both Isomura (114) and Randtke (113) have

previously used UV in conjunction with letal growth techniques to select for cell lines which were UV sensitive.

All four of the mutants examined in detail exhibit varying degrees of slow cell division. This factor may also have encouraged their selection, as residual incorporation through normal synthesis would be lower in such lines. Each of the mutants, however, expresses characteristics that suggest selection was enhanced by features less trivial in nature.

As the isolated mutants were found to be of significant interest and worthy of detailed studies, the isolation procedure was not characterized in more complete detail. Future considerations for the use of such a procedure might focus on better quantitation and on the isolation of temperature sensitive mutants.

Comparative Evaluation of UV Sensitivity and Unscheduled DNA Synthesis

The data for UV^s-7 and UV^r-23 correlate with what one would expect of mutants with altered nucleotide excision repair. UV^r-23 exhibits enhanced UDS and corresponding resistance to UV. UV^s-7 is deficient in its ability to conduct UDS and is sensitive to UV. The degree of UV^s-7 sensitivity is not as great as that observed for the XP complementation group D, which exhibits approximately equivalent reduction in UDS. One must consider, however, that while Chinese hamster and normal human fibroblasts are

roughly equisensitive to UV exposure, the human cells possess an ability to conduct UV-induced UDS many times that of the hamster cells. A reduction in UDS in hamster cells might, therefore, contribute less to UV sensitivity than in human cells.

The UV sensitivity of UV^S-40 and UV^S-44 was greater than that for UV^S-7, however, their UDS was only slightly reduced. The data suggest that the basic defect in both of these cell lines is associated with a mechanism other than nucleotide excision repair. Similar UV sensitivity without a corresponding reduction in UDS has been observed in the cells from patients with many of the radiation sensitive syndromes listed in Table 2.

The UV survival curves for all the new mutants were used to generate D_o and N values. As the data indicate, only slight differences were observed between the D_o values for the various mutants and the parental control. The major differences in UV survival appeared to be a consequence of the size of the shoulder portion of the curve. A theoretical explanation for this observation is as yet not ascertained. If the size of the shoulder reflects a measure of excision repair, this would be consistent with the apparent shoulder extension for UV^r-23 and reduction for UV^S-7. UV^S-40 and UV^S-44, however, have virtually no shoulder, and yet have near normal excision repair. Studies of the various complementation groups of XP

demonstrate a lack of correlation between D_o and excision repair. It appears that both the shoulder and straight-line portions of UV survival curves are influenced by factors in addition to excision repair.

The Damage of UV Versus That of N-acetoxy-2-acetylaminofluorene

The controversy in the literature regarding the similarities and differences in the repair of damage produced by UV and NAc-AAF was eluded to in the LITERATURE REVIEW. The present data suggest that while the mechanisms of repairing damage caused by these two mutagens show similarities, specific differences must also exist. UV^s-40 and UV^s-44 were found to be sensitive to both mutagens, a result previously demonstrated for XP fibroblasts (52). Though the defect in XP cells is specific to nucleotide excision repair, the defects of UV^s-40 and UV^s-44 appear to be of a more general nature, as the mutants also exhibit sensitivity to the mutagens MNNG and x-ray. The latter are believed to produce damage repaired by mechanisms other than nucleotide excision repair. The results observed for UV^s-7 and UV^r-23 emphasize differences that exist between the mechanisms of repair for UV and NAc-AAF damage. UV^s-7 is slightly sensitive to UV, but consistently shows no sensitivity to NAc-AAF. UV^r-23 is resistant to UV, but exhibits no resistance to NAc-AAF, as compared to control cells. The results presented have focused solely on survival. Additional

experiments could be considered examining NAc-AAF induced UDS or mutagenesis for UV^S-7 and UV^r-23. In addition, modifications in the isolation procedure may lead to the selection of mutants altered in their response to NAc-AAF, but not to UV.

UV-Induced Mutagenesis

The UV-induced hypermutability of UV^S-7 was clearly demonstrated at three loci, which have previously been quantitatively characterized in Chinese hamster fibroblasts. These results are consistent with the observations that UV^S-7 is deficient in UDS and sensitive to UV. Similar correlations have been observed with the classical XP fibroblasts (52), which inferred that excision-repair is an error-free process. Deficiencies in excision-repair could lead to the saturation of error-free processes at lower doses of UV, resulting in increased UV sensitivity and hypermutability through error-prone mechanisms or error-prone physiological states. The biological realities are clearly more complicated than this simplistic explanation, as evidenced by the previously discussed lack of correlation between UV sensitivity and UDS among the various XP complementation groups.

The degree of hypermutability for UV^S-7 varies from locus to locus. The most dramatic effect occurs at the ouabain-resistant locus, where expression of four cell divisions for both control and mutant cells (Table 7, Experiment #2) results in a UV^S-7 induced frequency nearly

27 times control values. At the diphtheria toxin-resistant locus, UV^S-7 mutability is as much as seven times control values, while the inducibility of 6-thioguanine resistance is only two- to three-fold higher than the control.

An important consideration in evaluating hypermutability is the type of mutagenic event that can be detected at a given locus. UV-induced damage results primarily in the production of pyrimidine dimers which yield a predominance of point mutations. As both ouabain (Na^+/K^+ ATPase) and diphtheria toxin (elongation factor-2) resistance require functional products for cell survival, resistance is thought to be rendered through minor base changes, such as point mutations. The observation in hamster cells that ouabain resistance is inducible by UV but not x-ray has been taken as confirmation that the induction of ouabain resistance is dependent on point mutagenesis. The induction of diphtheria toxin resistance in hamster cells is possible with both UV and x-ray, suggesting that small deletions may result in a functional product with resistance. 6-thioguanine resistance, however, requires the complete loss of HGPRT activity (124). A variety of mutational changes have been identified in 6-thioguanine resistant clones (124), including point mutations.

Although resistance at all three loci can be induced by UV, factors including the size of the gene, availability of damage to repair enzymes, and expression

of mutated genes may influence the degree of hypermutability observed for UV^S-7.

UV^S-40 was examined for UV-induced resistance to ouabain in two experiments (Table 9 and Table 10). The first experiment examined the relationship between UV dose and mutability. Hypermutability of UV^S-40 was clear only at high doses. Data at a moderate dose of UV (10 joules/m^2), stimulates some interesting questions regarding the hypermutability. When UV^S-40 and the parental control were permitted equivalent expression in units of time (three days), the UV^S-40 and parental control mutation frequencies were 127.0 and 117.6, respectively. The difference was clearly insignificant. When mutation frequencies were examined with expression in units of equal cell divisions, for which UV^S-40 requires four days to achieve the same colony size as three days growth for the parental control, the differences appeared more significant with observed frequencies of 146.2 and 117.6 for UV^S-40 and the parental control, respectively. The data in the second experiment indicate that both the mutant and control mutation frequencies can be influenced by increasing <u>in situ</u> expression. UV^S-40 ouabain-resistant recovery appears to be affected to a slightly greater extent. Ouabain expression will be considered in greater detail later in this dissertation. Both of the experiments illustrate the hypermutability of UV^S-40

at higher doses, but differences at moderate doses are minimal.

The experiments for UV^S-40 were not designed to permit the evaluation of mutation frequencies per unit survival. From the data in Table 9 and Table 10, one cannot deduce if control and mutant cell lines share approximately equivalent mutability at equitoxic doses of UV. Such a relationship had not been demonstrated for XP variant cells (60). This observation was interpreted to infer that repair in the XP variant is more error-prone than that in control cells. Such error-prone repair may be a consequence of greater dependence on normal error-prone tolerance function, alterations in enzymes which are normally error-free, or modified physiological states generating error-prone conditions for error-prone enzymes.

The UV-induced mutability of UV^r-23 was examined with both ouabain and 6-thioguanine resistance. Ouabain experiments employed both <u>in situ</u> and replating expression techniques. The data for ouabain and 6-thioguanine suggest a slight hypomutability for UV^r-23. Lower mutation frequencies were noted for UV^r-23 in all three experiments reported. The frequency differences observed between control and UV^r-23 are not large for any single comparison, but the consistency of observed hypomutability throughout the experiments supports the concept that UV^r-23 is hypomutable.

Both ouabain and 6-thioguanine resistant clones derived from UV^r-23 were found to be unusually sensitive to modifications in growth conditions. If these clones are at a greater selective disadvantage in the population, the apparent hypomutability observed may have been an artifact. This possibility is supported by a number of reports in the literature, demonstrating the dependence of the recovery of ouabain-resistant mutants on factors which influence membranes and/or Na^+/K^+ ATPase, including caffeine (132), 12-o-tetradecanoylphorbol-13-acetate (133), P,P-diclorodiphenyl-tricloroethane (134), and adenocine 3',5'-cyclic phosphoric acid (cAMP) (135). High levels of cAMP have been associated with both slow growth and a decrease in ouabain-resistant mutant recovery (136). The slow growing UV^r-23 may exhibit enhanced cAMP levels and thereby be at a selective disadvantage to express ouabain resistance. Therefore, although the data suggest hypomutability, such a phenomenon appears difficult to demonstrate, particularly when the magnitude is small.

The mutability of UV^s-44 was examined and UV-induced frequencies were found not to be at variance with control values. UV^s-44 was found sensitive to a variety of mutagens, exhibited near normal levels of UDS, and demonstrated a reduced ability to chase low molecular weight DNA into high molecular weight DNA following UV irradiation. The molecular observations for UV^s-44 suggest the mutant possesses a

defect in ligation or suffers from an extended delay in normal synthesis following UV exposure. In either case, the alteration appears to influence survival, but not mutagenesis following UV damage.

In addition to the specific details relevant to the individual mutants, the induced mutagenesis studies have provided some important details concerning the utility of the various mutation assays employed. The importance of the type of mutagenic event (base change, deletion, etc.) has already been eluded to. In addition, the studies reported here confirm earlier speculations regarding mutation expression. Experiments examining 6-thioguanine resistance indicate that all cell lines achieve maximal expression of UV-induced resistance at approximately seven days. As the cell lines grow at different rates, it can be deduced that expression is not dependent on cell division. This finding confirms earlier observations suggesting independence of 6-thioguanine expression on cell division (136). The independence of this assay on divisions is particularly attractive for comparative studies examining cell lines or experimental treatments that exhibit variations in division time.

Studies reported here examining the induction of ouabain resistance, particularly those with UV^S-7, indicate a dependence of expression on cell division. As Na^+/K^+ ATPase is a membrane-bound function, the elimination of

sensitive molecules could require dilution through the production of new membranes during cell division.

The data for UV^S-40 also support the dependence of ouabain-resistant expression on cell division. The increase in mutation frequency for the slower growing UV^S-40 cells over four days in situ expression, in conjunction with a less dramatic increase in parental control frequencies over the same period, suggests resistance for UV^S-40 does not express as quickly as control cells. The observed increase in control may be a consequence of new spontaneous mutations and colony selection factors previously described for diphtheria toxin resistance in hamster cells.

In regard to the diphtheria toxin resistance assay, equivalent maximal expression occurred for both mutant and control lines at approximately nine days. As UV^S-7 would have experienced considerably less cell divisions than the control cells in equivalent periods of time, expression appears not to be dependent on cell division.

Spontaneous Mutation Rate

Modifications of both the fluctuation analysis of Luria and Delbruck (137) and the replating technique of Newcombe (126) have been used to determine spontaneous mutation rates in mammalian cells. Most of these studies have been conducted to provide inferential evidence that an observed phenotypic change is a true mutation (i.e., demonstration of a mutation rate in the range of 10^{-6} to 10^{-9}),

with a de-emphasis on significance. The use of less plates and simple estimations for calculations is common. Comparative spontaneous mutagenesis, however, requires greater emphasis on significance and consideration of all aspects which effect the calculated rate. Three such aspects which have commonly been ignored in the past are metabolic cooperation, expression time, and selective advantage.

The first two of these aspects may lead to a gross underestimation of mutation rate when the fluctuation analysis is used. While metabolic cooperation can be compensated for with low cell density at selection, expression time is a major complication that cannot be eliminated from fluctuation type experiments. Starting populations are far too small to expect the existence of occurred, but unexpressed mutants, which might compensate for those which are not yet expressed at the time of selection.

With a modified Newcombe technique one examines the change in mutation frequencies over time, keeping track of the increase in the population. Through the generation of theoretical data, it has been determined that this relationship may be expressed as:

$$a = \frac{2(F_2 - F_1)}{\ln(N_2/N_1)} \ln 2$$

where $\underline{a}$ is the mutation rate, F_1 and F_2 are mutation frequencies at different times, and N_1 and N_2 are the total

number of cells in the population at the time of F_1 and F_2 determination, respectively (Stephen Warren, personal communication). Since the technique permits a large initial population and division over long periods of time, expression time is less of a problem than it is with the fluctuation analysis. As with the fluctuation test, appropriate compensation can avoid problems with metabolic cooperation. A major drawback of the Newcombe technique comes through the examination of a locus at which selective disadvantage exists for the phenotype being selected. DT^r and OUA^r mutants, for example, appear to be at a selective disadvantage as compared to wild type cells. Resistant cells would continuously be eliminated from the population, prohibiting an adequate estimation of mutation rate.

As there is no apparent selective disadvantage to 6-thioguanine resistant cells in the population, this locus was selected to examine the mutation rate in UV^S-7 and control cells using a modified Newcombe technique. The technique proved easy to employ and permitted the determination of several rate values for each cell line. No significant difference was detected between the mean values of 2.228×10^{-6} and 2.596×10^{-6} mutations/cell/division. The data once again confirm the UV-specific nature of UV^S-7's defect.

SUMMARY

An immense contribution has been made to the current understanding of prokaryotic repair function through the isolation and characterization of relevant bacterial mutants. Despite the success of these efforts and similar efforts in lower eukaryotes, little attention has been focused on attempts to isolate new mutants in mammalian systems. The present study reports the use of a new protocol to isolate mammalian cell mutants with alterations in DNA repair and/or UV sensitivity. The employment of this protocol was successful in generating a variety of Chinese hamster fibroblast clones of potential interest. These clones exhibited phenotypes including UV sensitivity, with or without a reduction in the capacity to conduct unscheduled DNA synthesis, and UV resistance with enhanced unscheduled DNA synthesis.

Four specific clones were selected for detailed studies, including an examination of UV-induced mutability. One clone was found to represent a new class of mutants, exhibiting reduced unscheduled DNA synthesis and UV-induced hypermutability similar to classical xeroderma pigmentosum cells, but only a slight sensitivity to UV. The spontaneous mutation rate for this clone was not found

to be significantly different from control values. The defect appears to be specific for UV-induced damage. Two additional clones have demonstrated moderate sensitivity to a variety of mutagens and only slightly reduced unscheduled DNA synthesis. One of these cell lines proved hypermutable to UV, while the other, a cell line which also exhibited poor "postreplication repair," showed no alteration in mutability. The fourth clone characterized in detail exhibited a resistance specific for UV, conducted enhanced unscheduled DNA synthesis and appeared slightly hypomutable.

The characterized clones demonstrate the wide variety of mutants that may be isolated through the implementation of the isolation protocol. Their relevance to the study of DNA repair and mutagenesis has been clearly shown. Additional characterization should prove useful in evaluating the specific mechanisms and elaborate subtleties of mammalian DNA repair and mutagenesis. Furthermore, modifications of the isolation protocol should prove useful in selecting additional mutants with defects relevant to these studies.

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