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I am submitting herewith a dissertation written by Kenneth Raymond Tindall entitled "Analysis of Mutation in DNA Transformed Chinese Hamster Ovary Cells." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biomedical Sciences.

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ANALYSIS OF MUTATION IN DNA TRANSFORMED
CHINESE HAMSTER OVARY CELLS

A Dissertation
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Kenneth Raymond Tindall

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DEDICATION

To Susan

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ABSTRACT

DNA-mediated gene transfer was used to study mutation at the xanthine-guanine phosphoribosyl transferase (gpt) locus in hypoxanthine-guanine phosphoribosyl transferase (HGPRT)-deficient Chinese hamster ovary (CHO) cells transformed by the pSV2gpt plasmid vector. The pSV2gpt vector carries the E. coli gpt gene in an SV40-pBR322 recombinant plasmid and, through the use of the SV40 early promoter, expresses the gpt gene product, XGPRT, in pSV2gpt transformed mammalian cells. E. coli XGPRT is the bacterial equivalent of the mammalian purine salvage pathway enzyme, HGPRT. Selection protocols utilizing the purine analog, 6-thioguanine (TG), that have been developed for the isolation of mutants at the hgpert locus in CHO cells were shown to be applicable for the isolation of mutants at the gpt locus in several pSV2gpt transformed CHO cell lines.

The HGPRT-deficient CHO transformation host has been characterized and determined to carry a stable, nonrevertible mutation at the hgpert locus. Analyses of several isolated pSV2gpt transformed CHO clones have demonstrated E. coli XGPRT activity in cell free extracts of each line assayed. In addition, genomic DNA derived from several of these pSV2gpt transformed cell lines has been analyzed by Southern blot hybridization establishing the presence of one to several copies of the gpt gene integrated in the high molecular weight DNA. These data indicate the functional expression of the gpt gene in these transformed CHO cell lines.

One pSV2gpt transformant has a single copy of the gpt gene integrated in the CHO genomic DNA. Induction of TG-resistant (TG^r) mutants

of this cell line is quantitative following irradiation with ultraviolet light or X-rays. Both the spontaneous and induced TG^r mutants lack XGPRT activity. Furthermore, Southern blot hybridization analyses established specific deletions through gpt structural gene sequences in two of the TG^r mutants analyzed. These data indicate the utility of this gpt transformed cell line as an easily manipulated system for quantitative and molecular analyses of gene mutation in CHO cells.

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LIST OF ABBREVIATIONS

APRT	adenine phosphoribosyl transferase
AS	azaserine
AS 52, AS 61, AS 62, BA 21, CA 212	pSV2gpt transformed X3/5 CHO cell lines
CHO-K ₁ -BH ₄	Chinese hamster ovary cell line subclone with HGPRT activity
<u>dhfr</u>	dihydrofolate reductase locus
<u>E. coli</u>	<u>Escherischia coli</u>
EMS	ethylmethanesulfonate
F12FCM2.5	Ham's F12 medium lacking Hx containing 2.5% dialyzed and heat inactivated fetal bovine serum
F12FCM5	Ham's F12 medium containing 5% dialyzed and heat inactivated fetal bovine serum
F12FCS10	Ham's F12 medium containing 10% heat inactivated fetal bovine serum
GMP	guanine monophosphate
Hx	hypoxanthine
HGPRT	hypoxanthine-guanine phosphoribosyl transferase
IMP	inosine monophosphate
kb	kilobase pairs
λ	bacteriophage lambda
MPA	mycophenolic acid
SV40	Simian virus 40
TG	6-thioguanine
TK	thymidine kinase
UV	ultraviolet light

X3/5	an HGPRT-deficient subclone of CHO-K ₁ -BH ₄
XGPRT	xanthine-guanine phosphoribosyl transferase
XMP	xanthine monophosphate
52B5, 52XA6	TG-resistant subclones of the AS 52 cell line

CHAPTER I

INTRODUCTION

Until recently, the study of mutation in mammalian cells has lacked direct evidence of a genetic basis for altered mammalian cell phenotypes (3,7,9,69). Proponents of epigenetic mechanisms for these altered phenotypes have argued that biochemical studies of mammalian cell variants only indirectly indicate mutations and that, in many cases, an epigenetic basis for the variant cell phenotype cannot be excluded. Indeed, the best mammalian cell systems used to determine mutation have not examined the DNA directly but rather have extrapolated from an altered protein primary sequence (10,49), altered enzyme kinetics (21,22,68) or altered heat stability (21,26,68). While there is little doubt that mammalian cells undergo mutation and that specific locus mutation induction can be quantitated (37), there are no existing systems using mammalian cells that allow for the direct analysis of an altered DNA sequence. Developments in recombinant DNA techniques have provided a number of methods applicable to the molecular analysis of mutations in mammalian cells. These methods include the manipulation of cloned DNA fragments in plasmid and lambda phage vectors, DNA mediated gene transfer of specific DNA fragments to mammalian cells and analysis of these DNA fragments by Southern blot hybridization. DNA sequencing methods ultimately provide a means by which mutagen-induced specific alterations in a cloned gene may be analyzed.

Use of these techniques has met with considerable success in the analysis of mutations at selectable loci in bacteriophage, bacterial and yeast systems (42). Advances in our understanding of the molecular basis of mutation in these systems are due largely to the abundance of selectable genetic markers, many of which can be cloned and isolated directly in either the parental or mutant form. Fine structure mapping followed by inferred or direct sequence analysis of the mutation site has been one widely used approach (6,15,18,23,47,73). These techniques, however, are not as readily applicable to studies of gene mutation in mammalian cells.

There are relatively few loci in mammalian cells in which a similar pattern of mutant and revertant selection schemes can be applied (37). Isolation of specific genes for mutational analysis is made more difficult by the presence of intervening sequences and the quantities of DNA present in mammalian cells. Nevertheless, deletion mutations have been observed at the dihydrofolate reductase (dhfr) locus in Chinese hamster ovary (CHO) cells using blot hybridization of parental and mutant genomic sequences (29). Similar studies using cDNA or cloned DNA probes are in progress in the study of gene mutation at the X-linked hypoxanthine-guanine phosphoribosyl transferase (hgpert) locus and the autosomal adenine phosphoribosyl transferase (apert) locus (24,25,71). A limitation of these approaches is that only major genetic alternations such as deletions and transpositions are detectable. To date, rapid methods by which point mutations in mammalian loci can be localized and sequenced have not been devised. There are simply no methods by which large DNA

fragments derived from the mammalian genome can be readily manipulated for localization of a gene mutation for subsequent DNA sequence analysis.

Alternatively, use of dominant selectable markers such as the Herpes simplex virus thymidine kinase (tk) or the aprt genes in DNA-mediated gene transfer experiments have provided a useful approach in the study of gene expression and gene mutation in DNA transformed mammalian cell lines (40,62,64). The advantages of such an approach include the use of cloned and defined selectable genetic loci. Such genes are often cloned into plasmids carrying additional genes which express antibiotic resistance as in the commonly used plasmid vector, pBR322 (4). Often the gene of interest is a cDNA insert or a relatively small viral gene, thus providing a more easily manipulated DNA fragment for the ultimate goal of localization and DNA sequence analysis of point mutations. In addition, the associated antibiotic resistance genes present in the plasmid vector sequences are occasionally retained following DNA transformation of mammalian cells. These antibiotic resistance genes, in some cases, can be used to isolate the transforming DNA via a technique termed "plasmid rescue" (2,32,59). Integrated transforming DNA can be cleaved from within genomic sequences by restriction endonucleases, then circularized and ligated. The specific regions of interest can then be isolated following E. coli transformation and selection for antibiotic resistance. Such an approach is potentially useful in rescuing integrated DNA sequences in both the parental and mutant state, since one is not using the gene of interest for selection directly; but rather selection of the DNA fragment of interest is based on associated plasmid DNA sequences.

Thus, DNA-mediated gene transfer techniques have the potential to be quite useful in the analysis of gene mutation in mammalian cells; yet few studies in this area have been initiated. Most studies have focused on the ultimate fate and organization of transforming DNA sequences largely at the tk gene in TK-deficient transformed mouse L cells (40,64, 65,75). Relatively few other dominant selectable markers or sufficiently defined mammalian recipient cell lines are available for study.

The goals of this dissertation research were (i) to establish a system suitable for the direct molecular analysis of mutation in mammalian cells and (ii) to demonstrate the functional utility of such a system. This study describes such a system in DNA transformed Chinese hamster ovary (CHO) cells.

We have used the plasmid vector, pSV2gpt (50,52), in DNA-mediated gene transfer experiments using hypoxanthine-guanine phosphoribosyl transferase (HGPRT)-deficient CHO cells as the transformation host. The pSV2gpt vector carries the E. coli gpt gene in an SV40-pBR322 recombinant plasmid and, through the use of the SV40 early promoter, expresses the gpt gene product, xanthine-guanine phosphoribosyl transferase (XGPRT). This enzyme is the bacterial equivalent of the mammalian purine salvage enzyme, HGPRT which our laboratory has studied extensively in the analysis of specific locus mutations in CHO cells. By using specific inhibitors to block de novo purine biosynthesis in HGPRT-deficient CHO cells, selected subclones of transformed CHO cells have been shown to carry and express the E. coli gpt gene. E. coli XGPRT activity can be demonstrated electrophoretically in extracts from these cells and genomic DNA derived

from several stable gpt⁺ CHO lines has been analyzed by Southern blot hybridization establishing the presence of one to several copies of pSV2gpt sequences in the high molecular weight DNA.

Results presented in this study indicate the utility in quantitative and molecular mutagenesis studies of one isolated gpt⁺ transformed cell line, which carries a single integrated copy of the gpt gene. In addition, analysis of several other isolated gpt⁺ transformants are investigated and categorized with respect to their response following mutagen treatment.

CHAPTER II

ANALYSIS OF MUTATION IN pSV2gpt TRANSFORMED CHO CELLS

Introduction

Analysis of molecular mechanisms of mutation in mammalian cells has been facilitated by rapid advances in the development of recombinant DNA techniques. This approach has provided the means by which DNA sequence alterations in mammalian cells may be studied directly. The molecular cloning of genetic loci useful in mammalian mutagenesis studies such as the hypoxanthine-guanine phosphoribosyl transferase (hgprt), adenine phosphoribosyl transferase (aprt), and dihydrofolate reductase (dhfr) genes (5,12,43) have provided cloned DNA probes for the analysis of mutation at these loci. These probes have been used by several investigators to define deletion mutations at these three loci in mutant mammalian cells (25,29,71).

Another approach to the study of molecular mutagenesis involves the use of cloned dominant selectable genes in DNA-mediated gene transfer experiments. Several investigators have reported DNA sequence alterations associated with changes in thymidine kinase (tk) gene expression in TK-deficient mouse L cells transformed by the Herpes simplex virus tk gene (40,55). Mammalian cells transformed by the aprt gene have been studied with respect to mutation at the transformed aprt locus, as well (62,63,77).

We have used the technique of DNA-mediated gene transfer to study HGPRT-deficient Chinese hamster ovary (CHO) cells transformed by the

pSV2gpt plasmid vector (50,52). This mammalian expression vector carries the E. coli gpt gene in an SV40-pBR322 recombinant plasmid and, through the use of the SV40 early promoter, expresses the gpt gene product, xanthine-guanine phosphoribosyl transferase (XGPRT) in pSV2gpt transformed mammalian cells. E. coli XGPRT is the bacterial equivalent of the mammalian purine salvage pathway enzyme, HGPRT. Thus, using selection protocols developed for the isolation of HGPRT-deficient CHO cells, the mutability of isolated pSV2gpt transformed lines can be studied.

In this report, we characterize the HGPRT-deficient CHO transformation host biochemically and evaluate several pSV2gpt transformed CHO lines with regard to their spontaneous and induced mutant frequencies. In addition, all isolated mutant pSV2gpt transformed lines studied were demonstrated to lack XGPRT activity. These data support a mutational basis for the gpt⁻ phenotype observed in some of these transformed cell lines.

Materials and Methods

Cell Culture

The parental Chinese hamster ovary cell line, CHO-K₁-BH₄ (hgprt⁺) has been previously described (38). The X3/5 line (hgprt⁻) which served as the transformation host for the pSV2gpt gene transfer experiments was derived from the CHO-K₁-BH₄ line following X-irradiation with 500 rads and selection in 10 μ M 6-thioguanine (TG). A list of all cell lines used in this study including pertinent genotypic and phenotypic characteristics is included in Table I.

TABLE I. SPECIFIC ACTIVITY OF HGPRT OR XGPRT IN CELL EXTRACTS

Cell Line ^a	Class ^b	Genotype ^c	Phenotype ^d	Specific Activity ^e (nmol/min/mg protein)
CHO-K ₁ -BH ₄	Parental line	<u>hgp</u> rt ⁺	TG ^S	5.6
X3/5	Transformation host	<u>hgp</u> rt ⁻	TG ^r	ND ^f
CA212	I	<u>hgp</u> rt ⁻ , <u>gpt</u> ⁺	TG ^S	19.1
AS52	II	<u>hgp</u> rt ⁻ , <u>gpt</u> ⁺	TG ^S	19.5
BA21	III	<u>hgp</u> rt ⁻ , <u>gpt</u> ⁺	TG ^S	24.9
AS61	IV	<u>hgp</u> rt ⁻ , <u>gpt</u> ⁺	TG ^S	20.1
52B3	II	<u>hgp</u> rt ⁻ , <u>gpt</u> ⁻	TG ^r	ND
52XA6	II	<u>hgp</u> rt ⁻ , <u>gpt</u> ⁻	TG ^r	ND
21A1	III	<u>hgp</u> rt ⁻ , <u>gpt</u> ⁻	TG ^r	ND

^a52B5, 52XA6 and 21A1 are TG^r isolates of either the AS52 or BA21 cell lines.

^bClass as defined in Table II.

^cListed in the apparent genotype for each cell line.

^dPhenotype with respect to clonal growth in 10 μ M TG.

^eSpecific activity (nmol [³H]-IMP or nmol [¹⁴C]-XMP formed/min/mg protein) was determined using [³H]-hypoxanthine as a substrate for the CHO-K₁-BH₄ line, [³H]-hypoxanthine and [¹⁴C]-xanthine for the X3/5 line and [¹⁴C]-xanthine for all other lines assayed.

^fND - no detectable [³H]-IMP or [¹⁴C]-XMP formed.

Cells were maintained in appropriate media at 5% CO₂, 37°C and 100% humidity. Both the CHO-K₁-BH₄ and X3/5 lines were maintained in monolayer in Ham's F12 medium supplemented with 5% dialyzed and heat inactivated fetal bovine serum (F12FCM5). All pSV2gpt transformed lines were subcultured in monolayer in MPA medium: F12FCM5 plus xanthine (250 µg/ml), adenine (25 µg/ml), thymidine (50 µM), aminopterin (3 µM) and mycophenolic acid (10 µg/ml), a modification of the selective medium described by Mulligan and Berg (52). Mycophenolic acid was a gift from R. L. Davis, Lilly Research Laboratories, Indianapolis, IN.

TG-resistant (TG^r) cell lines derived from pSV2gpt transformed lines were isolated and subcultured in Ham's F12 medium lacking hypoxanthine and supplemented with 2.5% dialyzed and heat inactivated fetal bovine serum (F12FCM2.5) plus 10 µM TG. When extracts for enzyme activity analyses were prepared from TG^r lines, cells were carried in nonselective medium (F12FCM5) four to ten population doublings prior to extract preparation.

Gene Transfer

The pSV2gpt plasmid in E. coli K12, strain HB101, was kindly supplied by P. Berg, Department of Biochemistry, Stanford University School of Medicine, Stanford, CA. The pSV2gpt plasmid shown in Figure 2.1, expresses the bacterial purine salvage enzyme, XGPRT, in mammalian cells using the SV40 early promoter, the SV40 small t-antigen splicing signals, and the SV40 early gene polyadenylation site (50,52). Supercoiled pSV2gpt plasmid was isolated by centrifugation to equilibrium in a cesium chloride ethidium bromide gradient. X3/5 cells were transformed by the

Fig. 2.1. Organization of the pSV2gpt vector. The pBR322 sequences in this vector extend from the PvuII to the EcoRI sites and include the plasmid origin of replication and the ampicillin resistance (AP^r) gene. SV40 sequences are numbered relative to the SV40 origin of replication. The E. coli gpt gene is positioned for expression in mammalian cells using the SV40 early promoter. Promoter sequences are contained between SV40 sequences, 5090 and 190. Intervening sequences of the small t-antigen and early polyadenylation site are included between SV40 sequences, 1701 and 4628 (adapted from ref. 50).

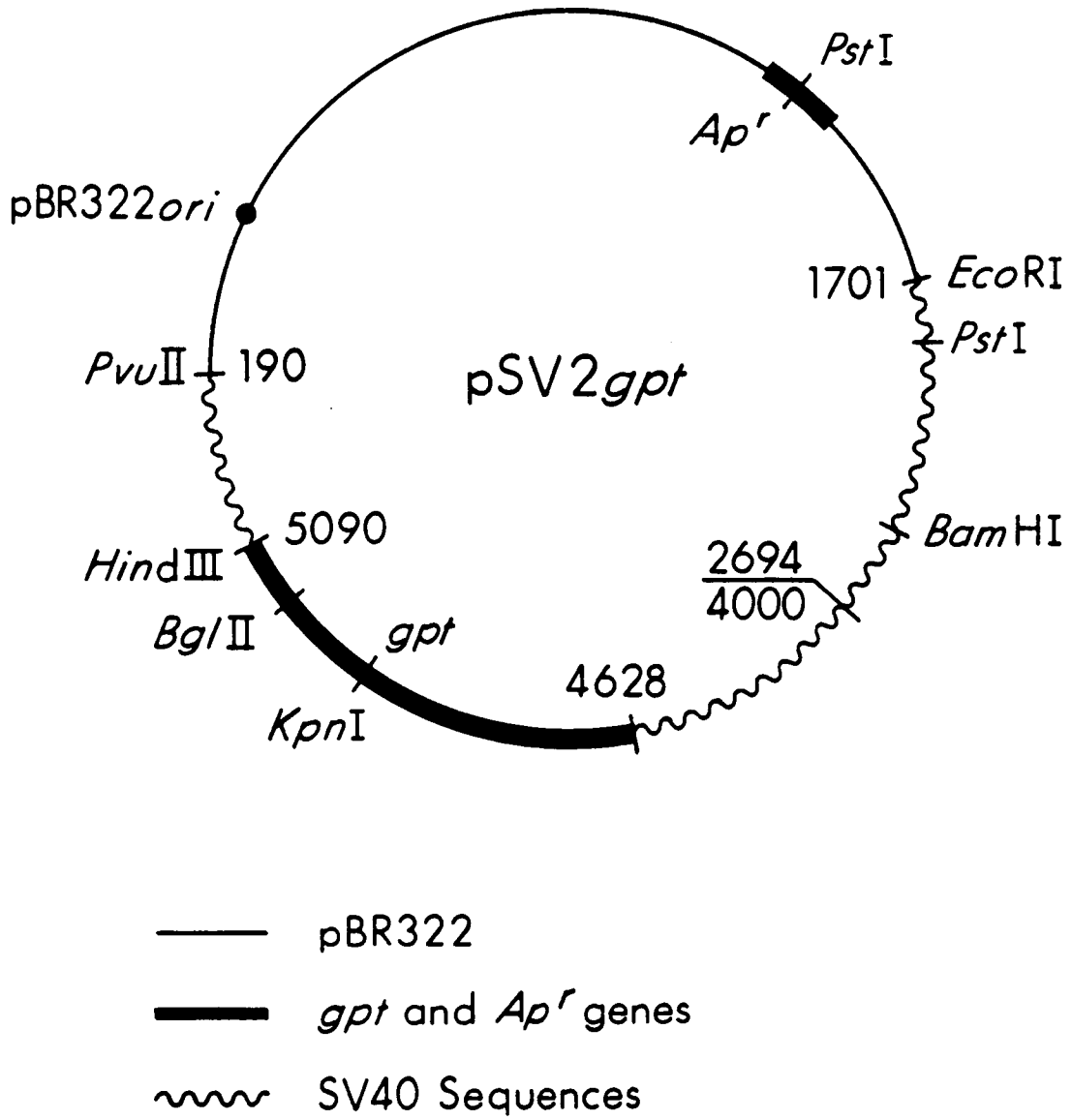


Fig. 2.1

pSV2gpt plasmid according to the method of Graham and van der Eb (30) as modified by Wigler (82), except that we did not use carrier DNA. Briefly, approximately 10^6 X3/5 cells per 100 mm dish (Corning) were treated with a DNA-calcium phosphate precipitate containing 1 to 20 μ g pSV2 DNA in F12FCM5. After 5 to 7 hrs incubation at 37°C the medium containing the DNA-calcium phosphate precipitate was removed and fresh prewarmed F12FCM5 was added back to the plates without washing the cells. Two to three days later when the plates were confluent, cells were plated at a density of 5×10^5 cells per plate in either MPA medium or Ham's F12 supplemented with 10% nondialyzed, heat inactivated fetal bovine serum and 10 μ M azaserine (AS) medium. Colonies arising were isolated 14 to 16 days later and subcultured in MPA medium.

Mutagenesis Experiments

Analyses of cytotoxicity and mutation to TG^r for all pSV2gpt transformed lines were determined as described for the CHO-K₁-BH₄ line (54). Cells were maintained in MPA medium until just prior to mutagen treatment. Approximately 10^6 cells per 100 mm tissue culture dish or 25 cm² flask (Corning) were washed 3 times in prewarmed saline G (60) without phenol red just prior to UV irradiation or chemical treatment. Cells were irradiated in 100 mm dishes in 5 ml saline G using a 15-Watt General Electric germicidal lamp (G15T8) positioned to deliver a dose of .325 J/M²/sec of predominately 254 nm light. Dose-rate was calibrated using a meter described by J. Jagger (41). Following UV-irradiation, saline G was removed and F12FCM5 medium added back to the treatment dish.

BA 21 cells were treated with ethylmethane sulfonate (EMS) (Kodak) in 5 ml F12FCM5 for 5 hrs at 37°C. F12FCM5 medium containing mutagen was removed, cells washed 3 times in prewarmed saline G and F12FCM5 medium added back to the treatment flask.

Twenty-four hours later, cytotoxicity was estimated by plating a sample of 200-1000 cells in triplicate in 60 mm dishes per treatment flask. Cells were incubated for 8 days at 37°C, the surviving colonies counted, and the relative cytotoxicity calculated as compared to the untreated control. The remaining cells at 24 hrs were subcultured for phenotypic expression for 14 days in F12FCM5 and then selected in 10 μ M TG in F12FCM2.5 at a density of 2×10^5 cells/100 mm dish.

HGPRT and XGPRT Enzyme Activity Analyses

(i) Preparation of cell-free extracts. Mammalian cell extracts containing HGPRT were prepared as previously described (7,26). Nearly confluent cultures were washed with saline G and drained of excess buffer. Extract buffer (10 mM Tris [pH 7], 10 mM $MgCl_2$, 30 mM KCL, 1 mM DTT and 1% Triton X-100) was added to each plate and incubated at 4°C for 20 min. Plates were tilted, drained, and the extract buffer collected. The extract was centrifuged at 15,000 x g for 25 minutes, the supernatant was frozen in liquid N_2 and stored at -70°C until use.

Cell-free extracts containing XGPRT from E. coli were prepared by methods previously described (36). E. coli, strain HB101, carrying the pSV2gpt plasmid was grown to late log phase, pelleted and washed in 30 mM sodium phosphate buffer, pH 7.4. Cells were pelleted again and resuspended in 2.5 ml of the same buffer. Cells were sonicated for 150

sec with cooling at 30 sec intervals using a Blackstone Ultrasonics, Inc. sonifier. The suspension was centrifuged 1 hr at 29,000 x g, the supernatant removed and dialyzed overnight at 4°C against 30 mM sodium phosphate, pH 7.4. The contents of the dialysis tube were frozen in liquid N₂ and stored at -70°C until use.

(ii) Activity analysis on gels. HGPRT or XGPRT activity was determined by a modification of the procedure described by Chasin and Urlaub (13). Cell extracts were subjected to electrophoresis through a 7.5% acrylamide gel in 0.2 M Tris (pH 8.5) buffer in an LKB Multiphor apparatus at 10°C. HGPRT or XGPRT activity in the gel was detected by applying a reaction mixture [0.1 M Tris (pH 7.4), 7 mM phosphoribosylpyrophosphate (PRPP), 10 mM MgCl₂, 4.8 mg/ml bovine serum albumin and either 20 μM [¹⁴C]-guanine (51.9 mCi/mmol) or 20 μM [¹⁴C]-xanthine (58.6 mCi/mmol)] directly to the gel surface and incubating at room temperature 1 hour. A pre-wet plastic backed polyethylenimine (PEI)-cellulose thin layer chromatography sheet was applied to the surface of the gel for 10 min. Sheets were rinsed, dried, coated with a solution of 2,5 diphenyloxazole (PPO) in ether (100 mg/ml) and exposed to Kodak (XAR-5 film for 3 to 7 days at -70°C.

(iii) Activity analysis in vitro. A binding assay to anion-exchange filters was used to determine HGPRT or XGPRT activity as previously described (7,26). Briefly, the assay was initiated by the addition of 20 to 40 μl enzyme extract to a 35°C reaction mix containing 20 μl of each of the following: 8.5 mM PRPP dissolved in Tris buffer [250 mM Tris (pH 7.5), 25 mM MgCl₂ and 50 mM KCl]; 5×10^{-4} M hypoxanthine or

5×10^{-4} M xanthine; and [^3H]-hypoxanthine (1 mCi/ml, 10 Ci/mmol) or [^{14}C]-xanthine (.1 mCi/ml, 58.6 mCi/mmol). At various time points, 10 μl samples were removed and spotted onto DEAE filter discs prespotted with 25 μl of 0.1 M EDTA (pH 7.0). The filters were air dried, then washed twice with 50% methanol. Bound radioactivity was determined by scintillation counting.

This assay measures the formation of either [^3H]-IMP or [^{14}C]-XMP by the reaction of either hypoxanthine or xanthine with PRPP in the presence of HGPRT or XGPRT. Only the phosphorylated product binds to the filter; the unreacted substrate is eluted. Specific activity is expressed as nmoles IMP or XMP formed/min/mg protein. Specific activity was calculated from the fitted linear regression of at least 5 reaction time points.

Immunoprecipitation of HGPRT

Antiserum against purified hamster HGPRT was a generous gift from C. T. Caskey, Department of Medicine, Baylor College of Medicine, Houston, TX. [^3H]-leu labeled HGPRT was prepared by growing CHO-K₁-BH₄ cells in [^3H]-leu (65 Ci/mmol) at a final concentration of 500 $\mu\text{Ci/ml}$ and 7.7 μM in F12FCM5. Labeling periods of 12 hrs were used in these studies. Immunoprecipitated [^3H]-HGPRT was isolated by previously described methods (7) except that the antiserum:HGPRT complex was precipitated using purified protein-A conjugated to Sepharose (33) (Pharmacia). The use of protein-A conjugated Sepharose significantly diminished nonspecific binding encountered with crude preparations of protein-A (data not shown).

Immuno-precipitable counts were separated in an 11.1% polyacrylamide disc gel. Gels were frozen, sliced and processed (7) for scintillation counting.

Blot Hybridization

Genomic DNA from the CA 212 and BA 21 gpt⁺ lines was isolated according to the method of Davis et al. (16). Restriction enzymes were purchased from either New England Biolabs or Bethesda Research Labs and used as recommended by the supplier. Each restriction reaction included 15 µg genomic DNA, 3 to 5 fold excess restriction enzyme, and RNase A (1 µg/ml). Following digestion, restricted DNA samples were extracted three times with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) then extracted twice with ether. DNA was precipitated with ethanol and the pellet was vacuum dried. Samples were resuspended in TE buffer [10 mM Tris (pH 7.5) - 1.0 mM EDTA] plus tracking dye [bromophenyl blue (.025%), xylene cyanol (.025%), Ficoll (2.5%) and sodium dodecyl sulfate (.1%)] for gel loading.

Restricted DNA samples were subjected to electrophoresis through 0.7% agarose gels in Tris-acetate buffer (40 mM Tris, 20 mM acetic acid, and 2 mM Na₂EDTA, pH 8.1). DNA was visualized on a long-wavelength UV transilluminator after staining with ethidium bromide (0.5 µg/ml). The gel was prepared for DNA transfer as described by Wahl et al. (78). DNA was transferred to nitrocellulose filters according to Southern (74). Filters were prehybridized at least 4 hours before the addition of [³²P] pSV2gpt probe. Nick translation of the pSV2gpt probe was performed as described by Rigby et al. (61). Specific activity of the [³²P]pSV2gpt

probe was greater than 1×10^8 cpm/ μ g DNA. Hybridization was allowed to continue for 40 hours at 42°C. Filters were washed 3 hours with 3 to 5 changes in 2X SSC - 0.1% sodium dodecyl sulfate (1X SSC is 150 mM NaCl, 15 mM sodium citrate). Air dried filters were exposed to Kodak XAR-5 film with Cronex Lighting-Plus (DuPont) intensifying screens at -70°C for 12 hrs to 3 days.

Results

Expression of XGPRT in pSV2gpt transformed lines

(i) Separation of HGPRT and XGPRT activities by gel analysis. Data shown in Figure 2.2 indicate the autoradiographic detection of HGPRT and XGPRT activities separated in a 7.5% polyacrylamide gel. The enzymatic conversion of [^{14} C]-guanine to [^{14} C]-GMP is catalyzed by either mammalian HGPRT or bacterial XGPRT. Panel A indicates the separation of mammalian HGPRT and bacterial XGPRT activities. The conversion of [^{14} C]-xanthine to [^{14} C]-XMP, however, is catalyzed only by XGPRT under these reaction conditions. The data presented in Panel B indicate that all four selected gpt⁺ transformants (CA 212, BA 21, AS 52, and AS 61) show activity migrating with the E. coli form of the enzyme. In addition, neither the X3/5 transformation host nor two TG^r gpt⁻ isolates of the AS 52 clone assayed have detectable HGPRT or XGPRT activity.

(ii) Analysis of HGPRT and XGPRT in vitro. The specific activities of either HGPRT or XGPRT in cell-free extracts of several cell lines were determined using either [^{3} H]-hypoxanthine or [^{14} C]-xanthine as a substrate and monitoring the formation of either [^{3} H]-IMP or [^{14} C]-XMP

Fig. 2.2. HGPRT and XGPRT activity in cell-free extracts of selected CHO lines. (A) Extracts were subjected to electrophoresis through a 7.5% polyacrylamide gel containing 0.2M Tris (pH 8.5) and the enzyme activity was determined in situ using [^{14}C]-guanine as a substrate. The reaction product was bound to PEI-cellulose chromatography plates and the relative sites of enzyme activity within the gel were detected by autoradiography of bound reaction product, in this case [^{14}C]-GMP. (B) Cell extracts were processed as in (A); however, [^{14}C]-xanthine was used as a substrate and the reaction product [^{14}C]-XMP was detected by autoradiography.

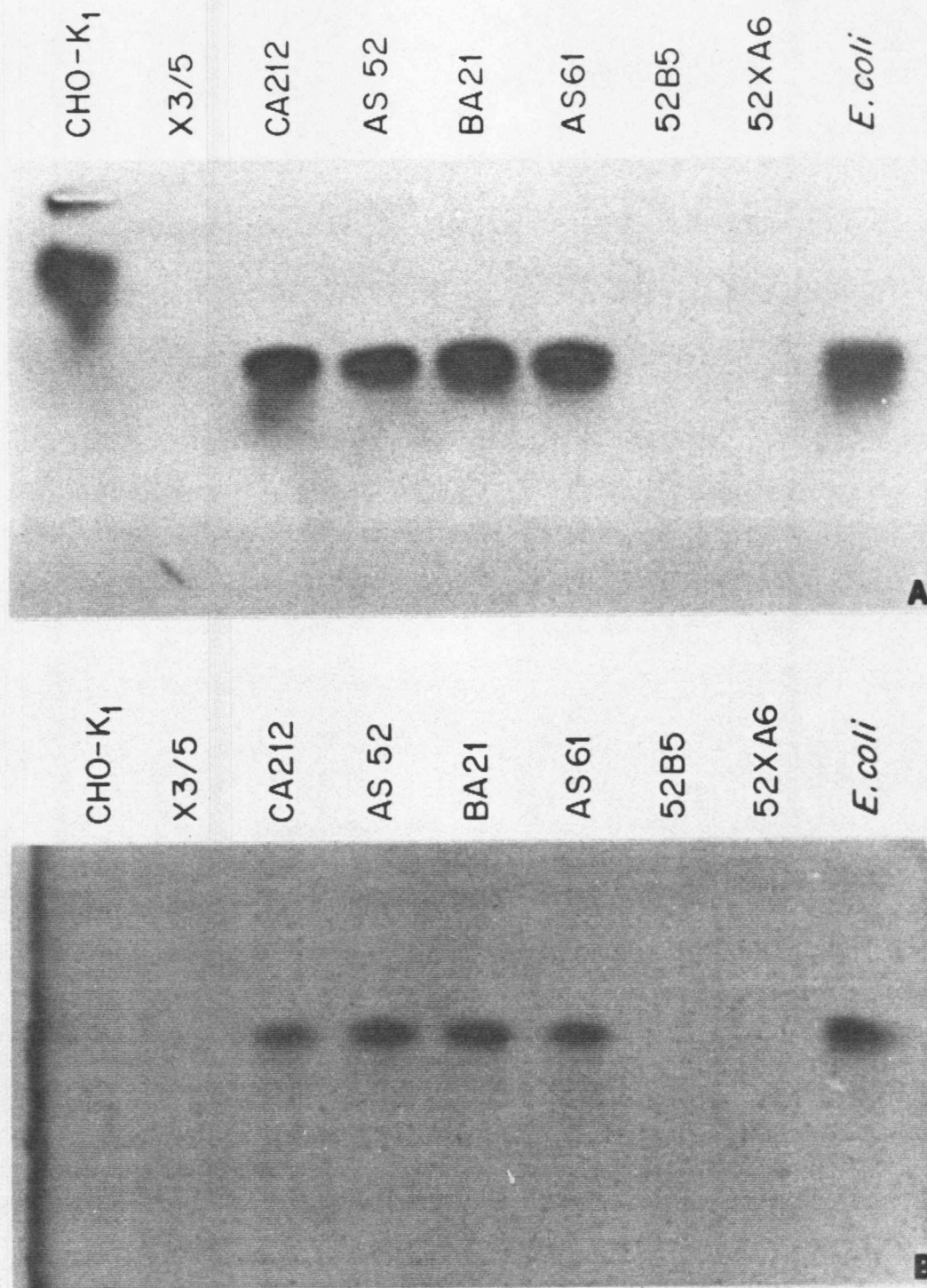


Fig. 2.2

vs time (Table I, p. 8). Since it was important to establish the presence or absence of HGPRT or XGPRT activity in the X3/5 line, as well as whether HGPRT derived from the CHO-K₁-BH₄ line had the ability to metabolize detectable levels of XMP under these reaction conditions, only extracts from the CHO-K₁-BH₄ and X3/5 lines were assayed using both [³H]-hypoxanthine and [¹⁴C]-xanthine as substrates in these reactions. Extracts containing HGPRT activity did not metabolize detectable levels of XMP when [¹⁴C]-xanthine was used as a substrate (data not shown); and extracts from the TG^r X3/5 line showed no detectable enzymatic activity with either substrate.

All four selected gpt⁺ clones metabolized xanthine with nearly the same specific activity. All of the gpt⁺ lines studied are sensitive to TG (TG^S) indicating that the bacterial XGPRT enzyme present in these cell lines functions similarly to the mammalian HGPRT enzyme in catalyzing the formation of the toxic reaction product, 6-thioguanine monophosphate. Indeed, analyses of fourteen TG^r isolates of either the AS 52 or BA 21 lines indicate the lack of any detectable XGPRT activity. Results obtained with three of these TG^r gpt transformed lines are reported in Table I, p. 8). Two of these clones, 52B5 and 52XA6, have been shown to carry deletions through the gpt structural gene, thus accounting for their TG^r phenotype (manuscript submitted).

Characterization of the X3/5 transformation host

Use of pSV2gpt transformed CHO cells for quantitative studies of mutation at the gpt locus requires use of an HGPRT deficient CHO line to assure selection of mutations at gpt rather than selection of hgp⁺

revertants. The X3/5 CHO line used as a host in these studies was isolated following an X-ray exposure of the CHO-K₁-BH₄ line of 500 rads. Several lines of evidence indicate the X3/5 line carries a stable mutation at hgprt. First, the X3/5 line has no detectable HGPRT activity in cell-free extracts using two methods of activity analyses as shown in Table I, p. 8 and Figure 2.2.

Second, reversion to hgprt⁺ by the X3/5 line has not been observed. In all DNA mediated gene transfer experiments using the X3/5 line as a recipient, colonies in MPA or AS medium were never observed on control plates containing cells which had received the CaPO₄-precipitation treatment without DNA. The frequency of reversion at the hgprt locus in the X3/5 line is estimated to be less than approximately 5×10^{-7} based on the combined results of the control plates of several independent gene transfer experiments.

Third, immuno-precipitation profiles on sodium dodecyl sulfate (SDS)-urea polyacrylamide gel electrophoresis using anti-HGPRT antibody on [³H]-leu labeled protein extracts from CHO-K₁-BH₄ and X3/5 cells indicate the absence of any immuno-precipitable HGPRT protein in the X3/5 line. These data are shown in Figure 2.3. Precipitation of the 27,000 dalton HGPRT subunit structure from HGPRT-proficient CHO-K₁-BH₄ cells is shown in panel A. Panel B shows the lack of any detectable immuno-precipitable HGPRT protein in extracts derived in the X3/5 line.

In short, the absence of detectable HGPRT enzyme activity, the apparent non-revertible nature of the hgprt gene mutation, and the lack of detectable immuno-precipitable HGPRT protein all serve to indicate

Fig. 2.3. SDS-Urea-polyacrylamide gel electrophoresis profile of immunoprecipitated HGPRT. (A) Immunopurified HGPRT from CHO-K₁-BH₄ cells in exponential growth labeled for 12 hours with [³H]-leu (solid line), 200 µg of cell extract were treated with 144 µg antiserum and 60 µg protein A-Sepharose, then denatured with SDS-urea. Gel profile for SDS-urea-PAGE is shown with predominant peaks of radioactivity (cpm/slice) at R_f values: 0.275, 0.65, and 0.72. HGPRT subunit is found at the R_f 0.65 peak at 2180 cpm, with a subunit molecular weight of 27,000 daltons. Calibration of the gel (broken line) is shown for the subunits of four calibration markers. R_f values and molecular weights in daltons are: (Δ) BSA, 0.20, 68,000; (▲) IgG H chain, 0.32, 50,000; (□) aldolase, 0.47, 40,000; and (■) IgG L chain, 0.75, 23,500. (B) Immunopurified HGPRT from X3/5 cells in exponential growth labeled for 12 hours with [³H]-leu and processed as in (A). This HGPRT-deficient mutant of CHO-K₁-BH₄ cells shows no peak of radioactivity at R_f 0.65.

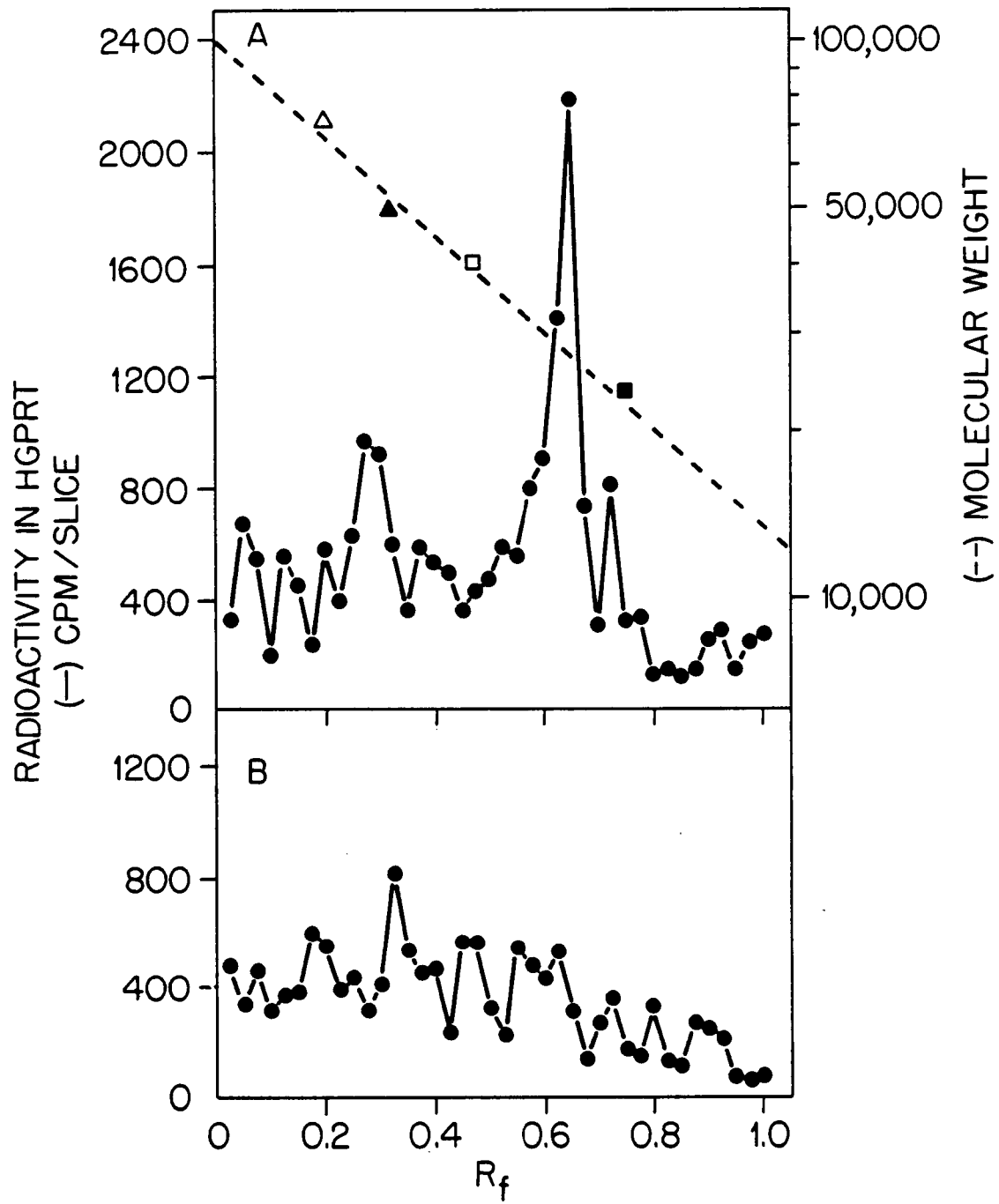


Fig. 2.3

the stringent nature of the hgprt mutation in the X3/5 line. These data support the use of the X3/5 line as a transformation host in studies of mutation at the gpt locus in pSV2gpt transformed cells.

Cytotoxicity and mutation studies

Cytotoxicity and mutation induction data derived from nine independently isolated pSV2gpt transformed X3/5 lines following exposure to UV-light were determined. All of the cytotoxicity curves were the same as previously reported UV-cytotoxicity in the parental CHO-K₁-BH₄ line within experimental error (53). Mutation response of the various gpt transformed cell lines to UV-irradiation, however, varied considerably between lines and as compared to the UV-induced mutation response of the parental line (53). These data provided a basis for the classification of these gpt transformed cell lines as indicated in Table II.

Each class of these gpt transformed cell lines has a distinct phenotype with respect to the mutation-induction response observed in individual lines following UV-irradiation. Detailed analyses of representative cell lines from two of these classes, Class I and Class III, are reported here. The Class II cell line carries a single copy of the gpt gene integrated into the X3/5 genomic DNA and is the subject of a separate manuscript (manuscript submitted). The Class IV cell line is unstable with respect to gpt gene expression. Following a 14 day subculture regimen in non-selective medium approximately 1% of the cells are TG^r as shown in Table II. Any increase in TG^r colonies arising following UV-irradiation was not determined due to the number of spontaneous mutants observed. These data are best explained by the

TABLE II. ANALYSIS OF MUTATION FREQUENCIES IN
pSV2gpt TRANSFORMED CHO CELLS

Class	Phenotype	Number of gpt Transformants Studied	Spontaneous TG ^r Frequency (mutants/10 ⁶ clonable cells)	Increase in TG ^r Clones with Mutagen Treatment ^a
I	Not mutable	4	< 1	0
II	Mutable	1	20-100	10 X
III	Mutable	3	500-1000	2-3 X
IV	Unstable	1	10,000	ND ^b

^aIncrease in TG^r mutants above the spontaneous mutant frequency at the highest point in the induced mutation curve.

^bNot determined.

extrachromosomal maintenance of the gpt gene under selective conditions in this transformed cell line. The Class IV line was not further examined in these studies.

The Class I lines do not yield cells with the gpt⁻ phenotype at detectable levels either spontaneously or following UV-irradiation. While no TG^r colonies were observed, all of the Class I lines indicated a normal cytotoxic response following UV-irradiation (data not shown).

By far, the most enigmatic class of gpt transformants studied in this manner are the Class III lines. All Class III lines show a two to three-fold increase in TG^r colonies over a spontaneous mutation frequency ranging as high as 1×10^{-3} per clonable cell (Table II). Figure 2.4 indicates the relative survival and induced mutation curves of two Class III cell lines, BA 21 (panel A) and AS 62 (panel B). These data are included to provide an indication of both the cytotoxic response as well as the spontaneous and induced mutant frequencies observed in typical UV-mutagenesis experiments using these Class III lines. Note that the mutation induction curves of the BA 21 and AS 62 lines both indicate a dose-dependent increase in TG^r colonies following UV-irradiation. However, while the total mutant yield is significantly different for each line, the increase over the observed spontaneous mutant frequency is consistently two to three-fold at the highest point in the mutation induction curve in both lines.

One of these lines, BA 21, was further analyzed for cytotoxicity and mutation using the chemical mutagen, EMS. Combined results of several EMS and UV experiments are reported in Figure 2.5. Since the

Fig. 2.4. Cytotoxicity and mutation induction response of Class III lines BA 21 (A) and AS 62 (B) to UV-irradiation. A typical experiment with each line is indicated here. Survival was determined 24 hrs following UV-exposure and reported as the cloning efficiency relative to the untreated control. Cells were selected for mutation to TG^R following subculture for 14 days in nonselective medium to allow for phenotypic expression. Reported mutant frequencies are corrected for cloning efficiencies. The absolute cloning efficiencies for the untreated spontaneous control for (A) and (B) were 78% and 68%, respectively. Cytotoxicity and mutation response lines were fit by eye.

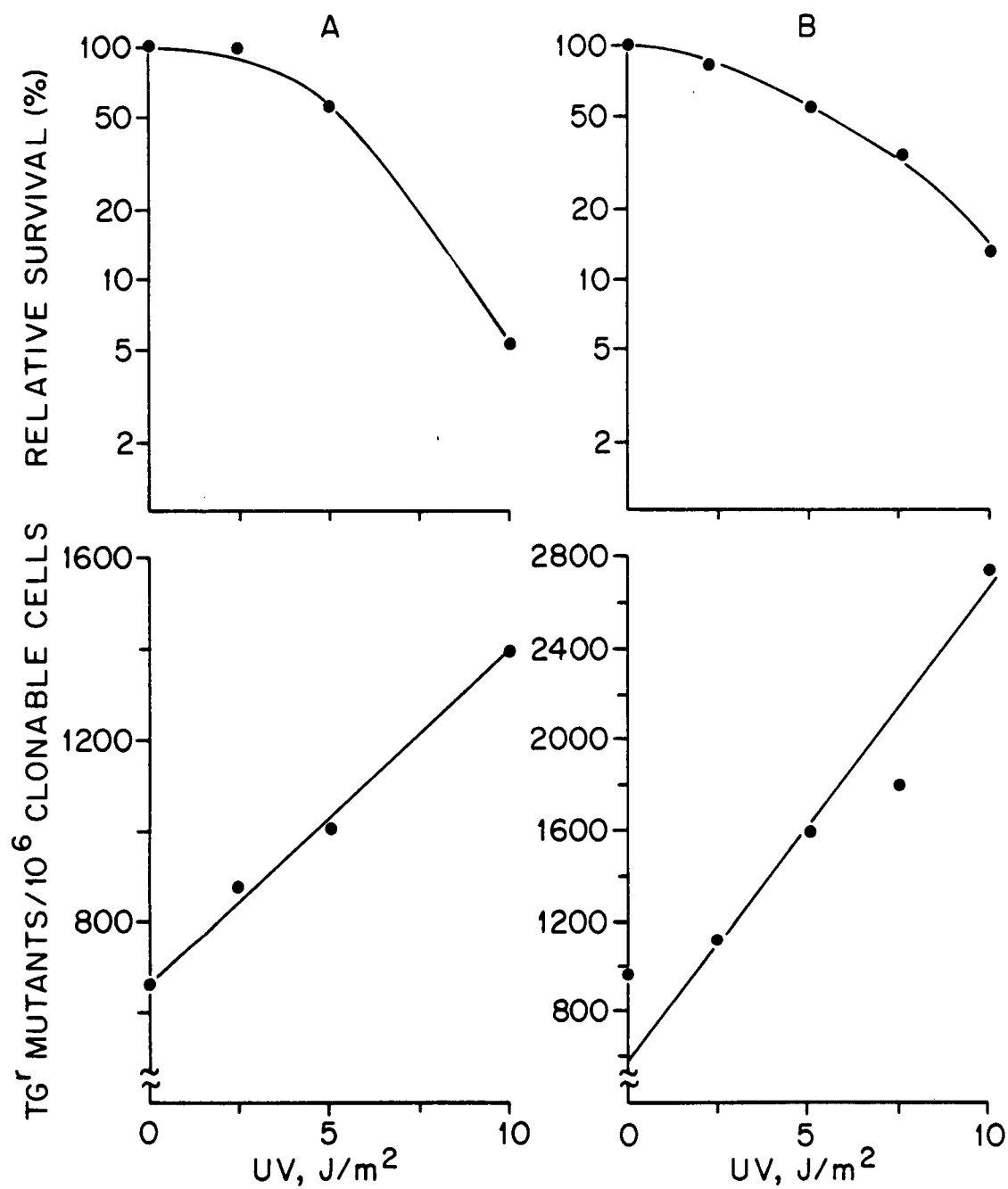


Fig. 2.4

Fig. 2.5. Cytotoxicity and mutation induction response of BA 21 line following exposure to EMS (A) or UV-irradiation (B). The results of three independent experiments with each mutagen are reported. Relative survival and TG^r mutant frequencies were determined as described in Fig. 2.4 except that the spontaneous mutant frequency from each individual experiment was subtracted from the induced mutant frequencies observed in the same experiment. Reported mutant frequencies then represent induced TG^r mutants over spontaneous per 10^6 clonable cells. Reported mutant frequencies are corrected for cloning efficiencies. Spontaneous TG^r mutant frequencies observed in (A) were 590×10^{-6} ($\bullet$), 660×10^{-6} ($\circ$), and 1150×10^{-6} (Δ). Spontaneous TG^r mutant frequencies observed in (B) were 660×10^{-6} (Δ), 1190×10^{-6} ($\circ$), and 1150×10^{-6} ($\bullet$). The absolute cloning efficiencies for the untreated controls were 78%, 63% and 62% for (A) and 78%, 68% and 65% for (B). Cytotoxicity and mutation response lines were fit by eye.

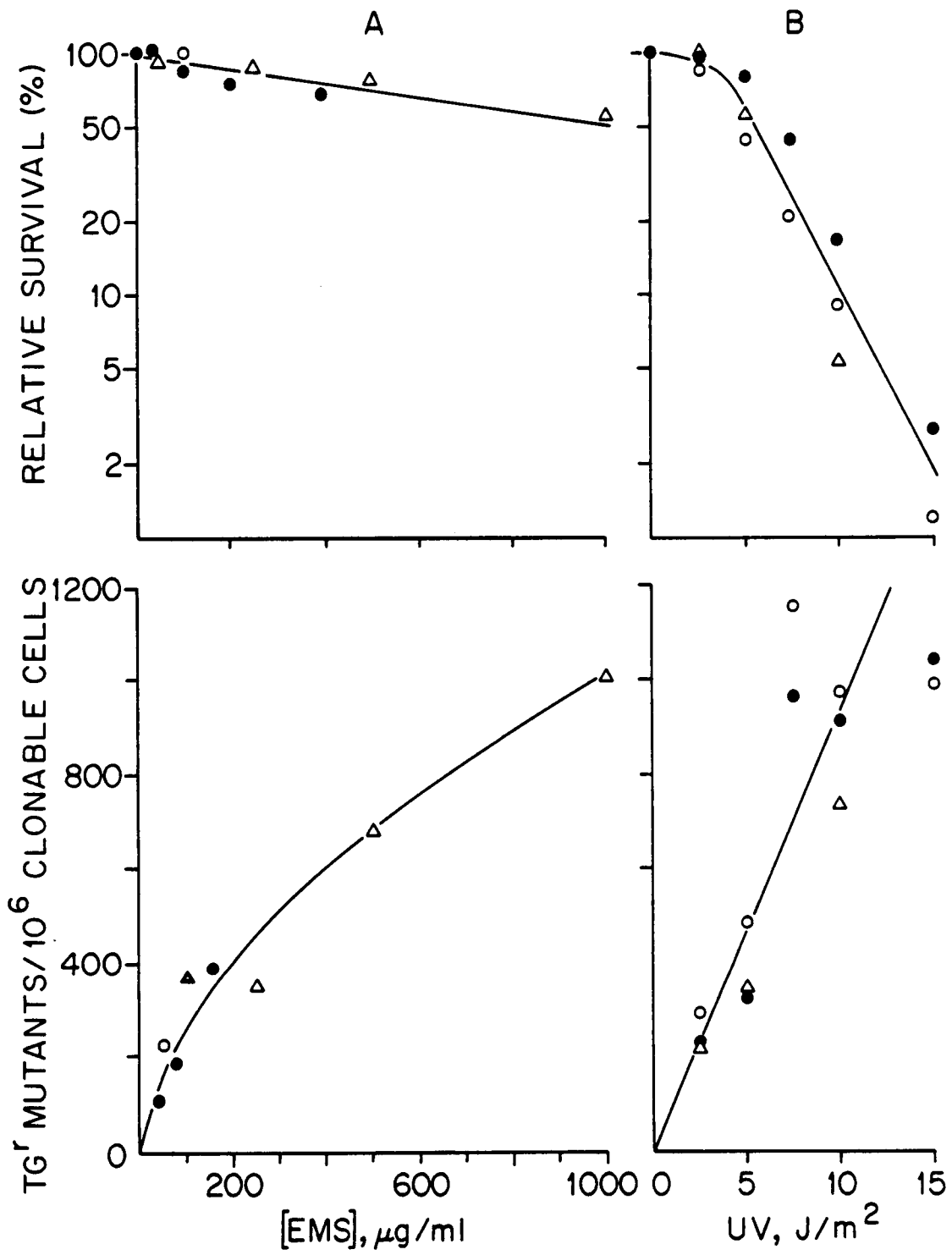


Fig. 2.5

spontaneous frequency varied considerably between experiments, induced mutant frequencies are plotted with the individual experimental spontaneous mutant frequency subtracted. Both EMS (Panel A) and UV-light (Panel B) exhibit consistent dose-dependent cytotoxic and mutagenic response in the BA 21 line. Once again, EMS cytotoxicity is not significantly different than EMS cytotoxicity previously observed in CHO-K₁-BH₄ cells (38).

Southern Blot Hybridization Analyses

Analysis of the genomic arrangement of pSV2gpt sequences in representative Class I and Class III cell lines were performed using blot hybridization (74). Since all gene transfer studies were carried out in the absence of carrier DNA (see Materials and Methods) an indication of genomic integration can be obtained using restriction endonucleases such as SstI which do not restrict the pSV2gpt plasmid. If pSV2gpt sequences are integrated into the X3/5 genomic DNA, SstI will cut genomic DNA surrounding any pSV2gpt sequence integrations. Barring independent integrations within similarly sized SstI fragments, which would appear as a single band, the number of sites in which pSVgpt sequences have integrated can be estimated by the number of hybridization bands following SstI digestion.

Further analyses can be performed using restriction endonucleases such as HindIII which cuts the pSV2gpt plasmid only once. HindIII digests the pSV2gpt plasmid between the SV40 early promoter and the gpt structural gene. This site should be retained in any gpt integration which expresses XGPRT in mammalian cells. One can use the number of hybridization bands observed following HindIII digestion and hybridization with

a nick-translated [^{32} P]-pSV2gpt probe to estimate the number of gpt gene copies present in these lines. Multiple HindIII hybridization bands are a likely indicator of multiple copies of DNA sequences on either side of the HindIII site. These sequences include the gpt gene and the SV40 early promoter region. Therefore, assuming intramolecular recombination has not extensively altered the pSV2gpt sequences, a rough estimate of the number of potentially functional gpt genes can be obtained from the number of HindIII bands observed.

Figure 2.6 shows the results obtained following hybridization of SstI or HindIII digested BA 21 (Class III) and CA 212 (Class I) DNA with nick-translated [^{32} P]-pSV2gpt probe. Both lines exhibit pSV2gpt sequence integration within the high molecular weight genomic DNA.

The BA 21 Class III line indicates a single integration site of pSV2gpt sequences within an SstI fragment of approximately 20 kb. At least five hybridization bands are observed in the BA 21 line following HindIII digestion, indicating the probable presence of multiple copies of the gpt gene within the BA 21 integration site. The CA 212 Class I line indicates at least two sites of pSV2gpt sequence integration in the high molecular weight DNA. SstI digestion yields two fragments which hybridize to the [^{32} P]-pSV2gpt probe at approximately 8.7 and 6.8 kb. Thus, pSV2gpt sequences are integrated into at least two sites within the CA 212 genome. HindIII digestion produces at least four hybridization bands again indicating the probable presence of multiple gpt gene copies in the CA 212 line.

Fig. 2.6. Determination of pSV2gpt sequences in BA 21 (Class III) and CA 212 (Class I) lines. Genomic DNA (15 μ g) was digested with either SstI or HindIII, subjected to electrophoresis through a 0.7% agarose gel, transferred to a nitrocellulose filter, and hybridized against a [^{32} P]-nick-translated pSV2gpt probe. Molecular weight markers correspond to HindIII, EcoRI double digests of bacteriophage, λ (cI857). All values are in kilobases.

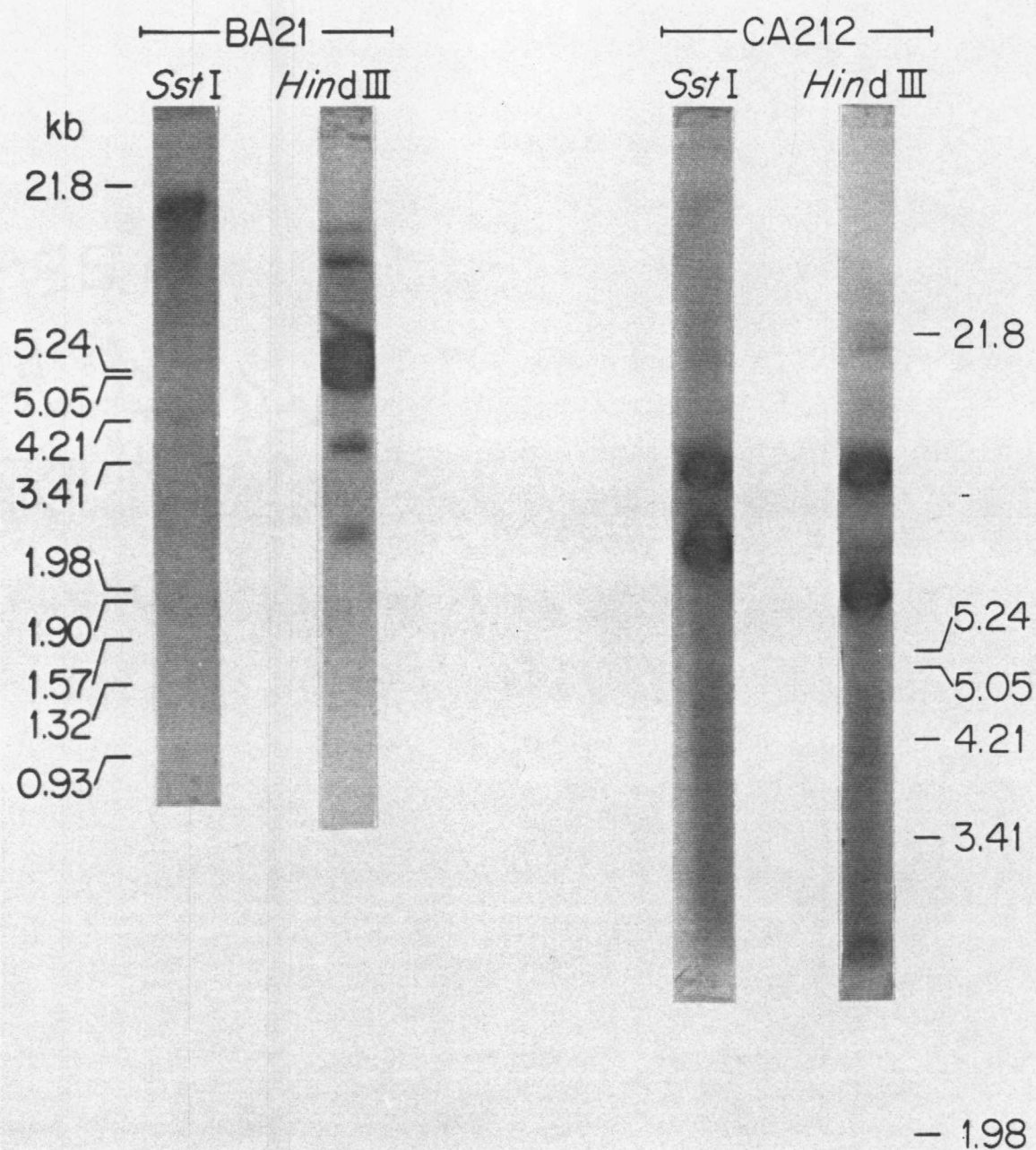


Fig. 2.6

Discussion

These studies were initiated to determine biochemical and genetic parameters of pSV2gpt transformed CHO cells for the purpose of using selected transformed cell lines for the molecular analysis of mutation at the gpt locus. The data presented in both Figure 2.2, p. 18 and Table II, p. 25 indicate functional expression of the gpt sequences in all pSV2gpt transformed lines assayed. In addition, the TG^r phenotype of several spontaneous or induced gpt⁻ transformed lines can be correlated with the absence of detectable XGPRT activity. Given the demonstrated stringency of defined mutant selection protocols in selective HGPRT-deficient CHO cells (54), these results are not too surprising. Earlier studies have shown that greater than 98% of TG^r CHO-K₁-BH₄ cells are deficient in HGPRT activity when selected under these conditions (54). Although a relatively small sample size was studied here, all 14 gpt transformed TG^r isolates assayed lacked detectable XGPRT activity. We presume that the vast majority of TG^r colonies observed in this study reflect gene alterations at the gpt locus in these transformed lines.

It was important to establish that the X3/5 line carries a "non-leaky" mutation at the hgp locus, since isolated gpt transformants were to be studied for mutation at the gpt locus. Data in Table II, p. 25 and Figures 2.2, p. 18 and 2.3, p. 22 provide support for use of the X3/5 line in these pSV2gpt gene transfer experiments. Most likely, the X3/5 hgp gene carries a deletion mutation or a point mutation with an extremely low reversion frequency. We suspect that most, if not all, of

the colonies arising in MPA or AS selective medium following pSV2gpt transformation are expressing XGPRT and do not arise as a function of hgprt gene reversion in the X3/5 line.

Mutation studies involving nine independently isolated pSV2gpt transformed X3/5 cell lines provided a basis for the classification of these transformed cell lines as presented in Table II, p. 25. Blot hybridization data (Figure 2.6, panel B) of the Class I transformant studied, CA 212, indicate the presence of pSV2gpt sequences integrated into genomic DNA in at least two sites based on SstI digestion and observed hybridization bands. HindIII digestion of CA 212 DNA yields four hybridization bands indicating the probable presence of multiple functional copies of the gpt gene in this cell line.

The blot hybridization data of the CA 212 line (Figure 2.6, panel B) and the relative stability of the Class I gpt transformants in non-selective medium are consistent with the integration of multiple functional copies of the gpt gene in the CHO lines. If two or more copies of the gpt gene are functionally integrated into the genomic DNA of Class I lines, possibly at different integration sites, then mutation to TG^r would require multiple mutations affecting all functional gpt genes. Multiple mutations ultimately giving rise to TG^r would be expected to be very low frequency events, thus, accounting for the lack of spontaneous or induced mutation observed in the Class I lines.

The Class II line, AS 52, has been demonstrated to contain a single functional gpt gene integrated in the genomic DNA (manuscript submitted). This line is mutable and can be used to study mutation at the

gpt locus much the same as any selectable hemizygous locus is studied in cultured somatic cells.

The molecular basis of spontaneous and induced mutation in the Class III lines remains obscure. Blot hybridization analysis of one Class III line, BA 21 (Figure 2.6, panel A), indicates the probable presence of multiple functional copies of the gpt gene integrated at a single site within the BA 21 genome. If multiple gpt genes are integrated at a single site in Class III lines, then mutation and selection of TG^r mutants in these lines is only possible, (i) if only one gpt gene present in the genome is functional, or (ii) if the process yielding mutations affects all functionally integrated gpt genes within the single integration site.

The elimination of all functioning gpt sequences except one functional copy under selective conditions may occur as a result of hypermethylation at the 5-position of cytosine residues in the CpG configuration. Hypermethylation of these sites has been shown to be associated with diminished or absent gene activity at several loci in mammalian cells (76,80). At least two studies have detected 5-methyl cytosine residues associated with the loss of tk gene expression in tk transformed mouse L cells (14,40,55). Thus, one explanation for the high spontaneous frequency of TG^r mutants arising in the Class III lines may involve hypermethylation of CpG sequences in one or more functional gpt genes once the selection pressure for gpt function is removed. In this case, it is presumed that all gpt gene copies except one are methylated and, therefore, not expressing XGPRT since mutation induction in these lines will

be observed only if one gpt gene is functioning. The nominal two to three-fold increase in induced mutation frequencies in the Class III lines could reflect a masking effect by the significant spontaneous frequencies.

Alternatively, a high frequency deletion of all functional gpt sequences may account for the observed spontaneous and induced frequency of TG^R colonies associated with the Class III lines. Deletion mutations in E. coli have recently been shown to occur as a result of slipped mispairing of short homologous regions on either side of deleted sequences during DNA replication (1). Since deletions observed in the human β -globin family have implicated the involvement of homologous sequences in the formation of these deletions (72), mammalian cells may produce deletions, in some cases, by a mechanism similar to E. coli. Multiple integration of gpt sequences within one integration site will produce a stretch of homologous sequences. Under non-selective conditions slipped mispairing and deletion of functional gpt sequences during replication may ultimately be detected as TG^R colonies. If this is the case, then the frequency with which these deletions occur spontaneously in non-selective medium is very high, ranging from approximately 5×10^{-4} to 1×10^{-3} per conable cell. Assuming that several functional gpt genes are integrated in these lines, dose-dependent mutation induction will be observed only if mutagen-treatment increases the proposed recombinational activity leading to deletion of functional gpt sequences resulting in the production of TG^R clones.

It is noteworthy that the frequency of gpt mutation in the Class III lines is similar to reported frequencies of phenotypic switching and genetic instability in tk and aprt transformed mouse L cells (40,55,64, 75,77). These reports cite spontaneous mutation frequencies in these tk and aprt transformed lines to be much higher than expected and in a similar range (10^{-4} to 10^{-3} mutants per clonable cell) as observed in these reported Class III CHO transformants. Support for a deletion mechanism in producing the observed mutant phenotype is derived from reports of genomic rearrangements which occur in accordance with the high frequency spontaneous tk and aprt phenotypic switch (40,62,63,64,75). At present, we cannot distinguish between the two possibilities presented above or any other unforeseen pathway by which the gpt⁻ phenotype arises in the Class III lines.

Finally, the Class IV line which is very unstable with respect to gpt gene expression was found to yield a spontaneous frequency of TG^r mutants in the range of 10^{-2} per clonable cell. One explanation for the unstable phenotype of this line involves segregation during subculture in nonselective medium of an extrachromosomal DNA fragment that carries the selectable gene. Such DNA fragments are generally not associated with centromeric sequences and, therefore, will not be segregated normally under nonselective conditions. Thus, loss of the selectable marker (gpt⁺) will yield cells of the TG^r mutant phenotype. No detailed studies of the Class IV line were performed.

Thus, the apparent status of the gpt gene in Class I, II, and IV cell lines fits a predictable model of integrated or extrachromosomal

gpt sequences and the expected response of each of these configurations to UV-irradiation. The precise nature of the gpt sequences in Class III lines is less well understood. Yet the Class III lines seem to respond in a manner similar to some tk and aprt transformed mammalian cells with respect to spontaneous mutation frequency (40,55,62,64,75). These results indicate that at least some DNA transformed mammalian cell lines have extremely high mutation frequencies within the transformed loci. Such results may have significant effects on experiments involving gene transfer to mammalian cells and are worthy of further study.

CHAPTER III

DETECTION OF DELETION MUTATIONS IN pSV2gpt TRANSFORMED CHO CELLS

Introduction

Molecular analysis of gene mutation in mammalian cells has most often been indirect, relying upon studies of variant proteins (10,49), immunological variants (22,27,79) complementation by microinjection of suppressor tRNAs (8,10,11) or genetic reversion (22,26,34,35) to imply the nature of the gene alterations. More recently, deletion mutations of the hgprt, aprt, and dhfr loci in mammalian cells have been studied directly using blot hybridization analyses (74) and [³²P] nick-translated cDNA probes (24,25,29,71).

Since numerous studies have established that transformation of mammalian cells may result in the stable integration of transforming DNA within genomic sequences (34,64,66,81), mutational effects at an integrated locus may be studied in such a cell provided appropriate selection techniques are available for the isolation of mutations at the transformed locus. Blot hybridization analysis has provided a basis for the study of mutation in mammalian cells transformed by a cloned dominant selectable marker such as the Herpes simplex virus thymidine kinase (TK) gene (40,55). Several studies (40,55,64,75) have identified specific changes in integrated tk sequences associated with cells selected to express the TK phenotype.

We have used this approach to study deletion mutations which affect gpt gene expression in hgp^rt⁻ Chinese hamster ovary (CHO) cells which have been transformed by the plasmid vector, pSV2gpt (50,52). The pSV2gpt plasmid carries the E. coli gpt gene and through the use of the SV40 early promoter expresses the bacterial enzyme, xanthine-guanine phosphoribosyl transferase (XGPRT) in mammalian cells. One isolated CHO transformant carries a single copy of the gpt gene stably integrated into the high molecular weight DNA. Since bacterial XGPRT and mammalian hypoxanthine-guanine phosphoribosyl transferase (HGPRT) are functionally analogous except that XGPRT catalyzes the formation of XMP from xanthine while HGPRT does not, selection protocols utilizing the purine analog 6-thioguanine (TG) derived for the study of mutation at the hgp^rt locus in CHO cells (54) can also be used to select for mutations at the single gpt gene in the pSV2gpt transformed CHO line.

In this study, we show that mutation induction at the gpt locus in pSV2gpt transformed CHO cells can be quantified and that specific deletion mutations which affect gpt gene function are the basis for the observed 6-thioguanine resistant (TG^r) phenotype in mutants derived from this transformed line.

Materials and Methods

Cell Lines and Cell Culture Methods

The parental line, CHO-K₁-BH₄ (hgp^rt⁺), has been previously described (38). The X3/5 line (hgp^rt⁻) which served as the transformation host for the pSV2gpt gene transfer experiments was derived from the

CHO-K₁-BH₄ line following X-irradiation of 500 rads and selection in 10 μ M TG. Both the CHO-K₁-BH₄ and X3/5 lines were maintained in monolayer in Ham's F12 medium supplemented with 5% dialyzed and heat inactivated fetal bovine serum (F12FCM5) at 5% CO₂, 37°C and 100% humidity.

The AS 52 line is a gpt⁺ transformant of X3/5 which carries a single copy of the gpt gene integrated in the genomic DNA. This line was maintained in monolayer in MPA medium: F12FCM5 plus xanthine (250 μ g/ml), adenine (25 μ g/ml), thymidine (50 μ M), aminopterin (3 μ M) and mycophenolic acid (10 μ g/ml), a modification of the selection medium described by Mulligan and Berg (52).

Geimsa stained metaphase spreads of the AS 52 line indicate a near diploid modal chromosome number of 20 chromosomes/metaphase with 8% tetraploid cells. The modal chromosome number for the parental CHO-K₁-BH₄ line is also 20 chromosomes/metaphase with 3 to 5% tetraploid cells (J. R. San Sebastian and K. R. Tindall, unpublished observations).

Both 52B5 and 52XA6 are TG resistant (TG^r) clones derived from the AS 52 line. 52B5 is a spontaneous TG^r isolate of AS 52. 52XA6 was isolated from the AS 52 line following an X-ray exposure of 400 rads. These TG^r lines were isolated and subcultured in Ham's F12 lacking hypoxanthine and supplemented with 2.5% dialyzed fetal bovine serum (F12FCM2.5) plus 10 μ M TG. When extracts for enzyme activity analyses were prepared from the 52B5 and 52XA6 lines, cells were carried in nonselective medium (F12FCM5) four to ten population doublings before enzyme extracts were prepared.

Gene Transfer

The pSV2gpt plasmid in E. coli K12, strain HB101, was kindly supplied by P. Berg, Department of Biochemistry, Stanford University School of Medicine, Stanford, CA. Supercoiled pSV2gpt plasmid was isolated by centrifugation to equilibrium in a cesium chloride ethidium bromide gradient. X3/5 cells were transformed by the pSV2gpt plasmid according to the method of Graham and van der Eb (30) as modified by Wigler (82) except that carrier DNA was not used. Briefly, 10^6 X3/5 cells per 100 mm plastic tissue culture dish (Corning) were treated with a DNA-calcium phosphate precipitate containing 1 to 20 μ g pSV2 DNA in F12FCM5. After 5 to 7 hrs incubation at 37°C the medium containing the DNA-calcium phosphate precipitate was removed and fresh prewarmed F12FCM5 was added back to the plates without washing the cells. Two to three days later when the plates were confluent, cells were plated at a density of 5×10^5 cells/plate in either MPA medium or Ham's F12 plus 10% nondialyzed fetal bovine serum and 10 μ M azaserine (AS), medium. Colonies arising were isolated 14 to 16 days later and subcultured in MPA medium.

Mutagenesis Experiments

Cytotoxicity and mutation to TG^r for the AS 52 line was determined as described for the CHO-K₁-BH₄ line (54). Cells were maintained in MPA medium until just prior to irradiation. Approximately 10^6 cells per 25 cm² tissue culture flask (Corning) were washed 3 times in prewarmed saline G (60) without phenol red just prior to treatment and were irradiated in 5 ml saline G. Cells were irradiated with a 320 KV Gemini Industrial X-ray Unit (TFI Corp., New Haven, CT), and the dosimetry was

calibrated using a condenser R-meter (Victoreen Instruments, Cleveland, OH). Following irradiation, saline G was removed and prewarmed F12FCM5 was added back to the treatment flask. Cytotoxicity was estimated 24 hrs later by plating an aliquot of 200-1000 cells in triplicate in 60 mm dishes per treatment flask. Cells were incubated for 8 days at 37°C, the surviving colonies counted, and the relative cytotoxicity calculated as compared to the untreated control. The remaining cells at 24 hrs were subcultured for phenotypic expression for 14 days in F12FCM5 and then selected in 10 μ M TG in F12FCM2.5 at a density of 2×10^5 cells/100 mm dish.

Southern Blot Analysis

Genomic DNA from all cell lines was isolated according to the method of Davis et al. (16). Restriction enzymes were purchased from either New England Biolabs or Bethesda Research Labs and used as recommended by the supplier. Each restriction reaction included 15 μ g genomic DNA, 3 to 5 fold excess restriction enzyme, and RNase A (1 μ g/ml).

Following digestion, restricted DNA samples were extracted three times with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) then extracted twice with ether. DNA was precipitated with ethanol and the pellet was vacuum dried. For double restriction enzyme digestions, the pellet was resuspended in the appropriate buffer and processed as described above. Otherwise, the samples were resuspended in TE buffer [10 mM Tris (pH 7.5) - 1.0 mM EDTA] plus tracking dye [bromophenyl blue (.025%), xylene cyanol (.025%), Ficoll (2.5%) and sodium dodecyl sulfate (.1%)] for gel loading.

Restricted DNA samples were subjected to electrophoresis through 0.7% agarose gels in Tris-acetate buffer (40 mM tris, 20 mM acetic acid, and 2 mM Na₂EDTA, pH 8.1). DNA was visualized on a long-wavelength UV transilluminator after staining with ethidium bromide (0.5 µg/ml). The gel was prepared for DNA transfer as described by Wahl et al. (78). DNA was transferred to nitrocellulose filters according to Southern (74). Filters were prehybridized at least 4 hours before the addition of [³²P] pSV2gpt probe. Nick translation of the pSV2gpt probe was performed as described by Rigby et al. (61). Specific activity of the [³²P]pSV2gpt probe routinely ranged from 5×10^7 to 5×10^8 cpm/µg. Hybridization was allowed to continue 40 hours at 42°C. Filters were then washed 3 hours with 3 to 5 changes in 2X SSC - 0.1% sodium dodecyl sulfate (1X SSC is 150 mM NaCl, 15 mM sodium citrate). Air dried filters were exposed to Kodak XAR-5 film with Cronex Lighting-Plus intensifying screens at -70°C for 1 to 7 days.

Analysis of HGPRT and XGPRT Activity

Cell-free extracts from mammalian cell lines were prepared as previously described (7,26). Nearly confluent cultures were washed twice with saline G and drained of excess buffer. Extract buffer (10 mM Tris [pH 7], 10 mM MgCl₂, 30 mM KCl, 1 mM DTT and 1% Triton X-100) was added to each plate and incubated at 4°C for 20 min. Plates were tilted, drained, and the extract collected. The extract was centrifuged 15,000 x g for 15 minutes, the supernatant aliquoted, frozen in liquid N₂ and stored at -70°C until use.

Cell-free extracts containing XGPRT from E. coli were prepared as described by Holden et al. (36). E. coli, strain HB101, carrying the pSV2gpt plasmid was grown to late log phase, pelleted and washed in 30 mM sodium phosphate buffer, pH 7.4. Cells were pelleted again, resuspended in 2.5 ml of the same buffer, and sonicated for 150 sec with cooling at 30 sec intervals using a Blackstone Ultrasonics, Inc. sonifier. The sonicated extract was centrifuged 1 hr at 29,000 x g, the supernatant removed and dialyzed overnight at 4°C against 30 mM sodium phosphate, pH 7.4. The contents of the dialysis tube were frozen in liquid N₂ and stored at -70°C until use.

HGPRT and XGPRT activity was determined by a modification of the procedure described by Chasin and Urlaub (13). Cell extracts were subjected to electrophoreses through a 7.5% acrylamide gel in 0.2M Tris (pH 8.5) buffer in an LKB Multiphor apparatus at 10°C. HGPRT or XGPRT activity in the gel was detected by applying a reaction mixture [0.1 M Tris (pH 7.4), 17 mM phosphoribosylpyrophosphate (PRPP), 10 mM MgCl₂, 4.8 mg/ml bovine serum albumin and either 20 μM [¹⁴C]-guanine (51.9 mCi/mmol) or 20 μM [¹⁴C]-xanthine (58.6 mCi/mmol)] directly to the gel surface and incubating at room temperature 1 hour. A pre-wet plastic backed polyethyleneimine (PEI)-cellulose thin layer chromatography sheet was applied to the surface of the gel for 15 min. Sheets were rinsed, dried, coated with a solution of 2,5 diphenyloxazole (PPO) in ether (100 mg/ml) and exposed to Kodak XAR-5 film for 3 to 7 days at -70°C.

Under the reaction conditions described, either HGPRT or XGPRT will metabolize [¹⁴C]-guanine to [¹⁴C]-GMP; however, only XGPRT will

metabolize [^{14}C]-xanthine to [^{14}C]-XMP. The phosphorylated product is bound to the PEI-cellulose sheet. Thus, the relative sites of HGPRT or XGPRT activity within the gel can be detected by autoradiography of bound [^{14}C]-GMP or [^{14}C]-XMP.

Results

Transformation and Selection Rationale

The plasmid vector, pSV2gpt shown in Figure 3.1, carries the E. coli gpt gene. This plasmid utilizes the SV40 early promoter, SV40 small t-antigen intervening sequences and SV40 early gene polyadenylation site to express the gpt gene product, XGPRT, in pSV2gpt transformed mammalian cells (50,51,52). Since only XGPRT and not HGPRT metabolizes xanthine under physiological conditions, transformation and selection of a functioning gpt gene in either HGPRT proficient or HGPRT deficient mammalian cells can be achieved by using metabolic blockers of de novo purine biosynthesis such as AS or MPA in conjunction with exogenous xanthine as a purine source (52).

For studies of mutation at the gpt locus in pSV2gpt transformed CHO cells, a HGPRT deficient isolate of the CHO-K₁-BH₄ line (38) was selected to serve as the host for the gene transfer experiments. This cell line, designated X3/5, was isolated following an X-ray exposure of 500 rads and appears to carry a stable, non-revertible mutation at hgprt (data not shown).

Mutation studies at the gpt locus in pSV2gpt transformed X3/5 cells were carried out as described for mutation selection at the hgprt locus

Fig. 3.1. Organization of the pSV2gpt vector. The pBR322 sequences in this vector extend from the PvuII to the EcoRI sites and include the plasmid origin of replication and the ampicillin resistance (Ap^r) gene. SV40 sequences are numbered relative to the SV40 origin of replication. The *E. coli* gpt gene is positioned for expression in mammalian cells using the SV40 early promoter. Promoter sequences are contained between SV40 sequences, 5090 and 190. Intervening sequences of the small t-antigen and early polyadenylation site are included between SV40 sequences, 1701 and 4628 (adapted from ref. 50).

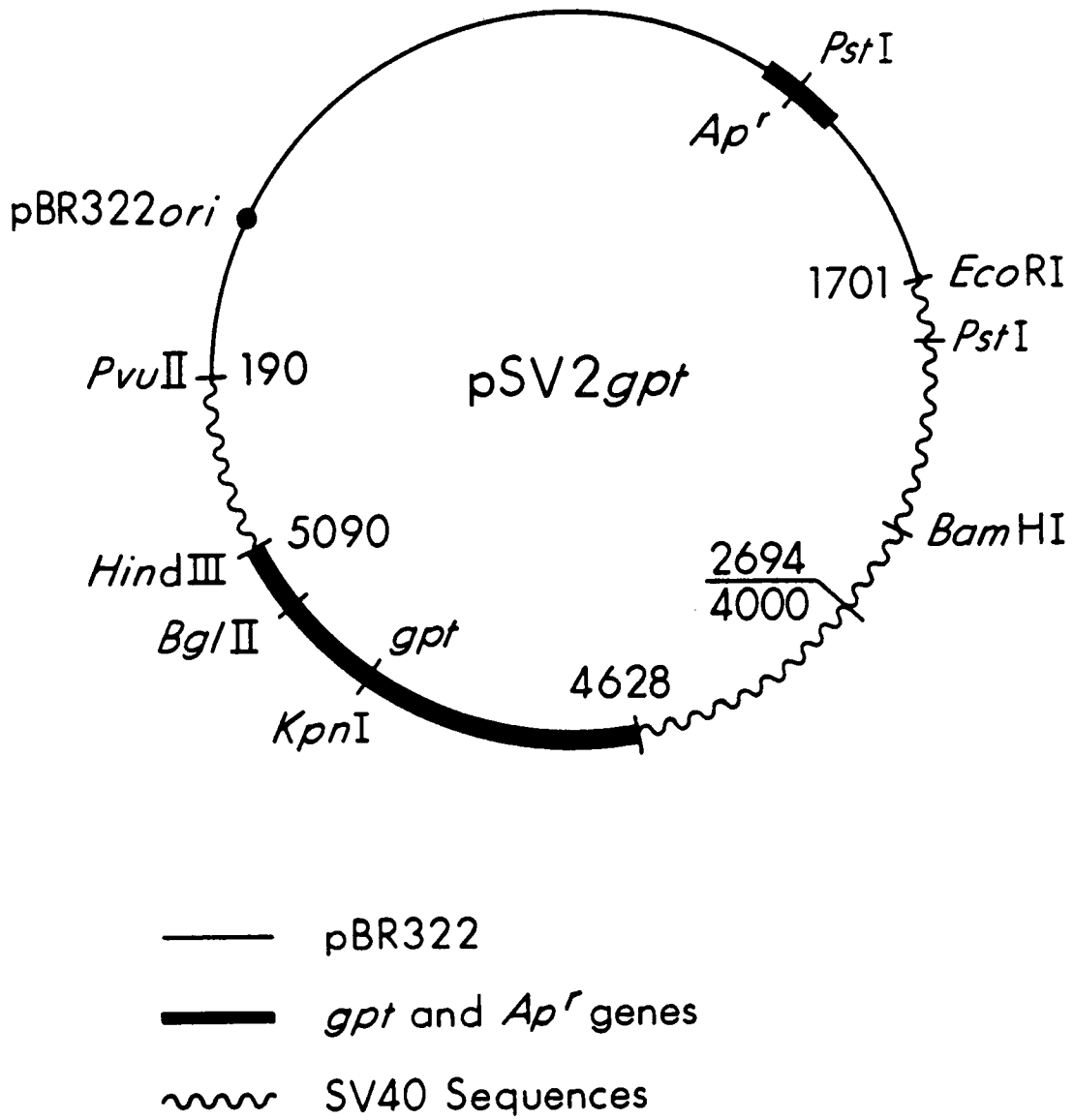


Fig. 3.1

in CHO cells (54). Since the purine analog, TG, is metabolized to the toxic analog, 6-thioguanine monophosphate, by XGPRT with about the same efficiency as HGPRT (48), gpt transformed cells deficient in XGPRT activity can be selected in medium containing TG following a period of subculture in nonselective medium to allow for expression of the mutant phenotype. All experiments described allowed a phenotypic expression time of 14 days when selecting for mutation at the gpt locus.

Single Copy Integration of pSV2gpt Sequences

Southern blot analysis (74) of pSV2gpt sequences in one X3/5 transformed line, AS 52, which was isolated following transformation without carrier DNA is shown in Figure 3.2. Because DNA concatamers including undefined carrier DNA sequences may be found in mammalian cells during transformation (58), transformation of the X3/5 line was performed without carrier DNA to provide a defined fragment of DNA for hybridization analysis of the structure of integrated pSV2gpt sequences.

The restriction enzymes SstI and XbaI do not cut the pSV2gpt plasmid and therefore will cut genomic DNA sequences adjacent to any integrated pSV2gpt sequences. Lanes 2 and 3 show the results of digestion of AS 52 genomic DNA by SstI and XbaI followed by hybridization with a [³²P]pSV2gpt nick-translated probe. Single bands of hybridization are observed in the high molecular weight DNA corresponding to about 12.0 and 10.4 kb, respectively. Digestion of AS 52 genomic DNA by restriction enzymes which cut the pSV2gpt plasmid once within or near the gpt structural gene indicated two bands of hybridization as expected for a single gpt copy integration. Figure 3.2 lanes 4 through 6 show the

Fig. 3.2. Determination of the molecular arrangement of pSV2gpt sequences in the AS52 cell line. Genomic DNA (15 μ g) was digested with the indicated restriction enzyme, subjected to electrophoresis through a 0.7% agarose gel, transferred to a nitrocellulose filter, and hybridized against a [^{32} P]-nick-translated pSV2gpt probe. All double restriction enzyme digests were performed sequentially. Internal size standards used were linearized pSV2gpt (5.2 kb) and pSV2gpt plasmid sequentially digested with BamHI and PvuII (3.0 and 2.2 kb). Approximately, 50 pg of each of the purified standards were added to calf thymus DNA (15 μ g) for electrophoretic separation. All values are in kilobases.

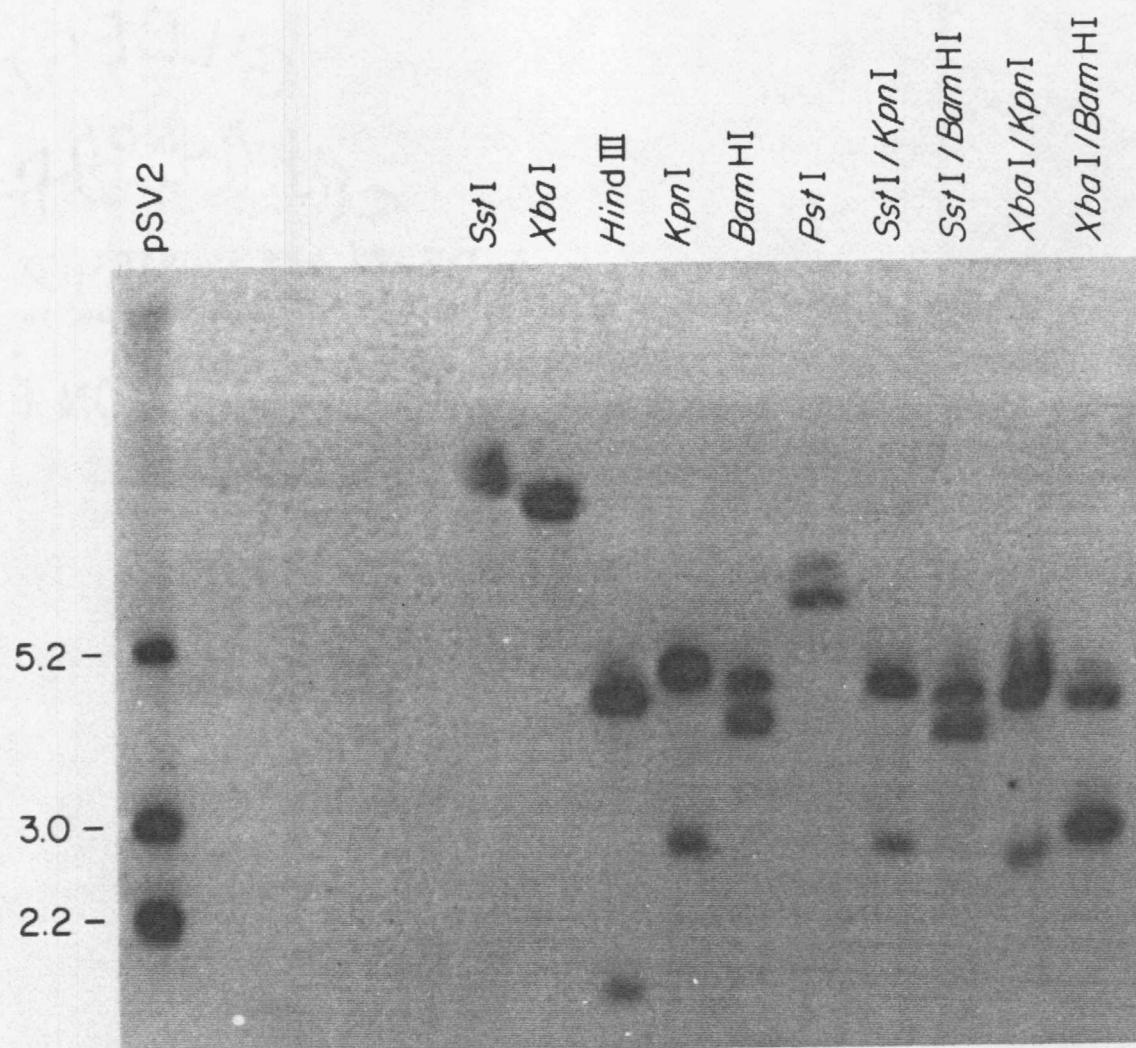


Fig. 3.2

expected results following HindIII, KpnI, and BamHI digestion of AS 52 DNA. HindIII digestion yields two bands 4.7 and 1.9 kb; KpnI digestion yields two bands 5.2 and 2.8 kb; and digestion with BamHI yields two bands 4.9 and 4.3 kb.

PstI restricts the pSV2gpt plasmid at two sites producing two restriction fragments. Depending upon the integration site, three hybridization bands should be observed if both PstI sites are retained. Figure 3.2 lane 7 indicates the loss of one of the PstI sites in the integrated structure, since only two bands hybridized to the nick-translated [^{32}P] pSV2gpt probe. These data, in addition to the results of sequential double digestions of AS 52 genomic DNA by selected restriction enzymes as shown in lines 8 through 11 of Figure 3.2, as well as other hybridization analyses (data not shown) provide a basis for the map of the pSV2gpt integration site in the AS 52 line illustrated in Figure 3.3.

The pSV2gpt plasmid has integrated into the AS 52 genomic DNA at one end very near the PstI site of the ampicillin resistance gene of the associated pBR322 sequences and at the other end near the single BamHI site of the pSV2gpt vector. Such an integration deletes approximately 1.2 kb of the pSV2gpt plasmid DNA including some sequences derived from the SV40 early polyadenylation site. This map was constructed entirely from hybridization blot data and is considered to be accurate to about 0.2 kb. This map also assumes no intramolecular recombination between intracellular pSV2gpt molecules prior to integration.

Fig. 3.3. Map of the integrated pSV2gpt sequences in the AS 52 line.

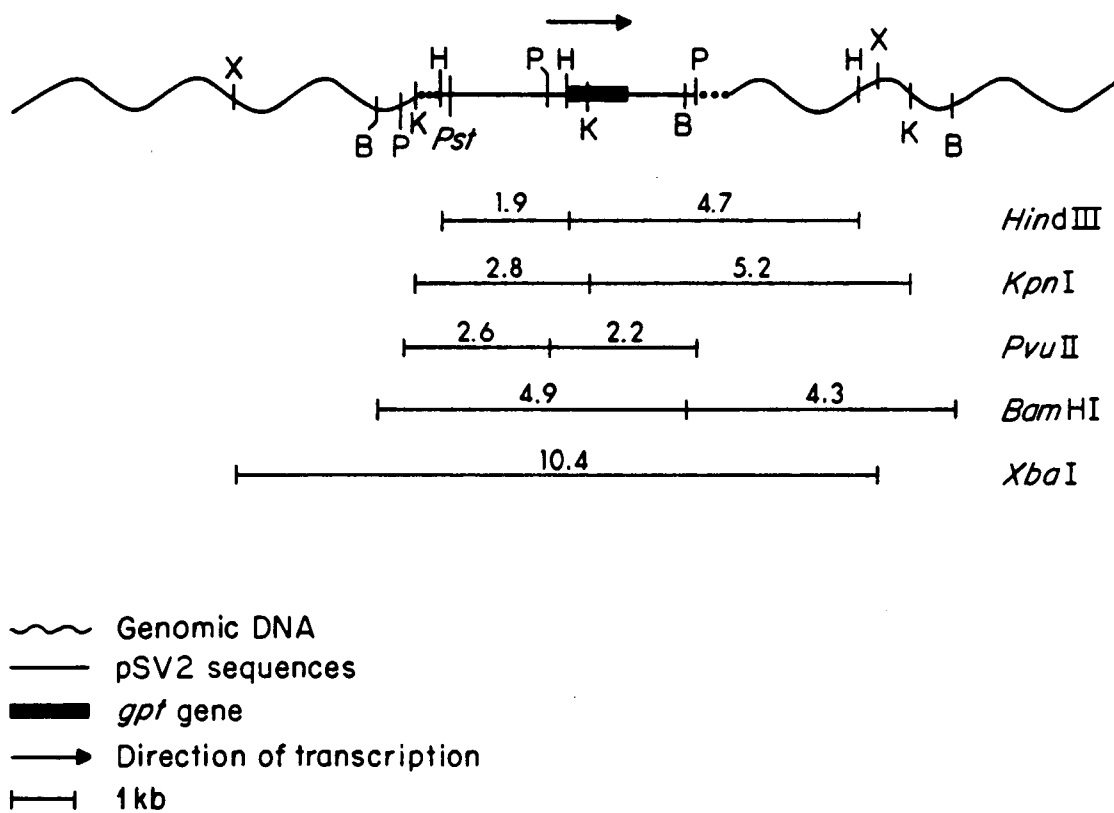


Fig. 3.3

Mutation Induced at the gpt Locus by X-Irradiation

The effects of X-irradiation on survival and mutation induction in the AS 52 line are shown in Figure 3.4. Survival of the CHO-K₁-BH₄ (hgprt⁺) parental (53) and the AS 52 (gpt⁺) lines are similar following X-ray exposure. For example, relative survival of the AS 52 line as shown in Figure 3.4 at 100, 300, and 700 rads is approximately 85%, 40% and 4%, respectively. Comparatively, previously reported (53) survival of the CHO-K₁-BH₄ line at 100, 300, and 700 rads exposure is approximately 90%, 55%, and 9%, respectively.

Mutation induction curves at the gpt locus in AS 52 cells and at the hgprt locus in CHO-K₁-BH₄ cells, however, differ significantly. Data presented in Figure 3.4 indicate a dose-dependent increase of induced mutation at the gpt locus in the AS 52 line of approximately 110×10^{-6} , 340×10^{-6} , and 1000×10^{-6} following an X-ray exposure of 100, 300, and 700 rads, respectively. Mutation at the hgprt locus following X-irradiation of CHO-K₁-BH₄ cells has been previously reported (53). Mutation frequencies in the CHO-K₁-BH₄ line of approximately 25×10^{-6} , 40×10^{-6} and 90×10^{-6} were observed following an X-ray exposure of 100, 300, and 700 rads, respectively.

These significant differences in apparent induced mutation are not unique to X-irradiated cells. AS 52 cells exposed to 254 nm UV-light again shows a similar cytotoxic response to the UV exposure as CHO-K₁-BH₄ cells; however, the slope of induced mutation is once again higher in the AS 52 line as compared to the CHO-K₁-BH₄ line (KRT, unpublished observations). Both spontaneous TG^r AS 52 mutants and TG^r mutants

Fig. 3.4. Cytotoxicity and mutation induction response of the AS 52 line to X-irradiation. The results of two independent experiments are reported. Survival was determined 24 hrs following X-ray exposure and reported as the cloning efficiency relative to the untreated control. Cells were selected for mutation to TGR following subculture for 14 days in nonselective medium to allow for phenotypic expression. Reported mutant frequencies are corrected for cloning efficiencies. The absolute cloning efficiencies for the untreated spontaneous controls were 73% (●) and 61% (○). Cytotoxicity and mutation response lines were fit by eye.

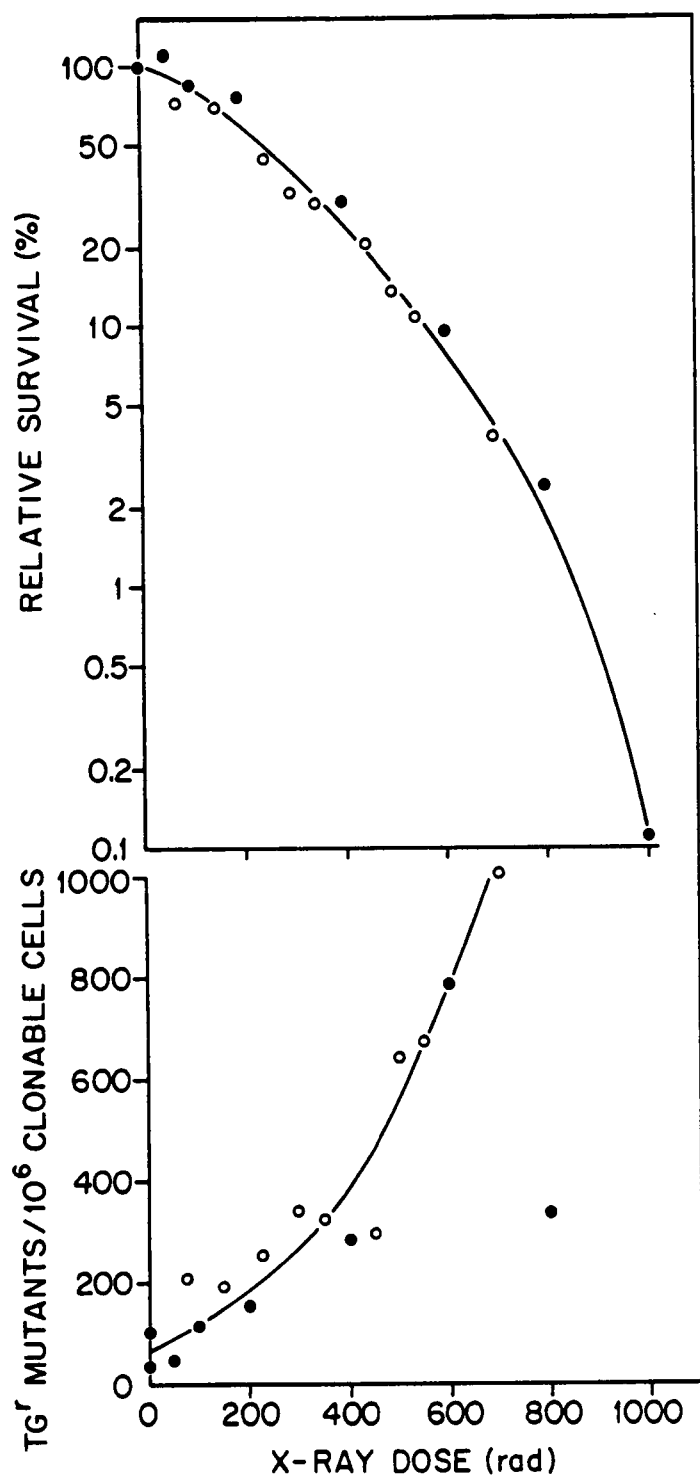


Fig. 3.4

arising following X-irradiation of the AS 52 line were isolated for further analysis.

HGPRT and XGPRT Activity in Cell-Free Extracts

Cell-free extracts were prepared (7,26) for analysis of HGPRT and XGPRT enzymatic activity in TG^r and 6-thioguanine sensitive (TG^S) cell lines in a 7.5% polyacrylamide gel (Figure 3.5). Use of [^{14}C]-guanine as a substrate (panel A), which both mammalian HGPRT and *E. coli* XGPRT can metabolize to form [^{14}C]-GMP, indicates a clear separation of mammalian and bacterial enzymatic activity. Panel B indicates only bacterial XGPRT metabolized [^{14}C]-xanthine to [^{14}C]-XMP, while CHO HGPRT will not metabolize xanthine at detectable levels.

Since cell extracts from the AS 52 line show activity migrating with the *E. coli* XGPRT (Figure 3.5, panels A and B), integrated sequences corresponding to the *gpt* gene (Figure 3.2, p. 52) were concluded to be expressing XGPRT in this AS 52 line. Note that cell extracts from the TG^r transformation host, X3/5, and two TG^r isolates derived from AS 52, all lack detectable HGPRT or XGPRT activity.

Demonstration of Deletion Mutations

The 52B5 and 52XA6 TG^r derivatives of the AS 52 line were further analyzed by Southern blot hybridization against a [^{32}P] nick-translated pSV[*gpt*] probe as shown in Figure 3.6. Panel A compares the hybridization pattern of AS 52 and 52B5 *Xba*I digested genomic DNA in lanes 2 and 4. Lanes 3 and 5 compare the hybridization pattern of *Hind*III digested genomic DNA from AS 52 and 52B5, respectively. A difference in migration

Fig. 3.5. HGPRT and XGPRT activity in cell-free extracts of selected CHO lines. (A) Extracts were subjected to electrophoresis through a 7.5% polyacrylamide gel containing 0.2M Tris (pH 8.5) and the enzyme activity was determined in situ using [^{14}C]-guanine as a substrate. The reaction product was bound to PEI-cellulose chromatography plates and the relative sites of enzyme activity within the gel were detected by autoradiography of bound reaction product, in this case [^{14}C]-GMP. (B) Cell extracts were processed as in (A); however, [^{14}C]-xanthine was used as a substrate and the reaction product [^{14}C]-XMP was detected by autoradiography.

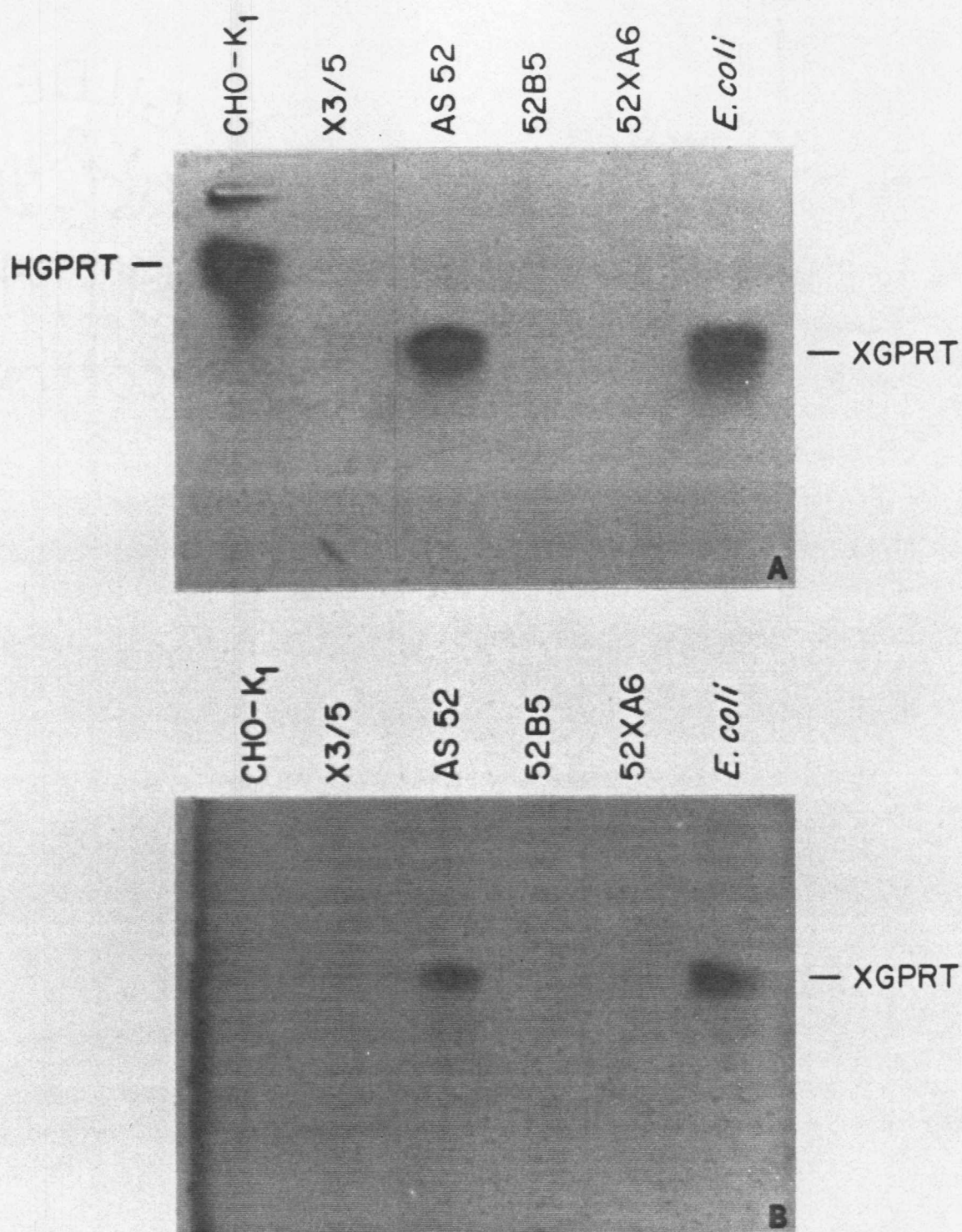


Fig. 3.5

of the single XbaI band indicates a deletion of sequences within the XbaI fragment of the 52B5. Similarly, the large HindIII fragment of the 52B5 line migrates at about 3.7 kb, compared to the 4.7 kb HindIII fragment in the parental AS 52 line. The 1.9 kb HindIII fragment was observed in both the AS 52 and 52B5 lines, thus localizing a deletion of approximately 1.0 kb to within the 4.7 kb HindIII fragment.

Southern blot hybridization analysis of the 52XA6 line was compared to the AS 52 parental line as shown in Figure 3.6, panel B. Differences in migration of the XbaI fragment (lanes 2 and 5) indicate a deletion of approximately 3.1 kb. The HindIII 4.7 kb fragment visible following HindIII digestion of AS 52 DNA and which includes the gpt structural gene is completely deleted in the 52XA6 line, while the 1.9 kb HindIII fragment (circled) remains intact. Digestion of the 52XA6 genomic DNA by PvuII indicates deletion of the 2.2 kb PvuII fragment (lane 7) as compared to the hybridization pattern observed with PvuII digestion of AS 52 shown in lane 4.

Although a precise localization of the deleted pSV2gpt sequences observed in the 52B5 and 52XA6 lines cannot be determined from these data, it is possible to conclude that both deletions must include some gpt structural gene sequences. The 52B5 line exhibits a loss of approximately 1.0 kb from within the HindIII fragment which includes the gpt structure gene (Figure 3.3, p. 55 and Figure 3.6). Hybridization analysis of HindIII - BamHI double digests of 52B5 DNA indicate retention of the BamHI site which normally exists 1.87 kb from the pSV2 HindIII site through the gpt structural gene (data not shown). Since the BamHI site

Fig. 3.6. Detection of deleted pSV2gpt sequences in TG^r isolates of the AS 52 line. Genomic DNA (15 µg) isolated from either the AS 52 line or the TG^r derivatives of AS 52, 52B5 or 52XA6, was digested with the indicated restriction enzyme and subjected to electrophoresis through a 0.7% agarose gel, transferred to a nitrocellulose filter and hybridized against a [³²P]-nick-translated pSV2gpt probe. Internal size standards used were linearized pSV2gpt (5.2 kb) and pSV2gpt plasmid sequentially digested with BamHI and PvuII (3.0 and 2.2 kb). Approximately 50 pg of each of the purified standards were added to calf thymus DNA (15 µg) for electrophoretic separation. All values are in kilobases. The 1.9 kb HindIII fragment is circled on these blots. These bands are more visible on the original autoradiogram and this fragment is clearly seen in Figure 3.2, p. 52. (A) Arrows indicate the altered migration of the XbaI and 4.7 kb HindIII fragments in the TG^r 52B5 line as compared to the parental AS 52 hybridization pattern. (B) Arrows indicate the loss of the 4.7 kb HindIII and the 2.2 kb PvuII fragments in the TG^r 52XA6 line as compared to the AS 52 hybridization patterns. Altered migration of the XbaI fragment in the 52XA6 line is also shown.

Normal + 1

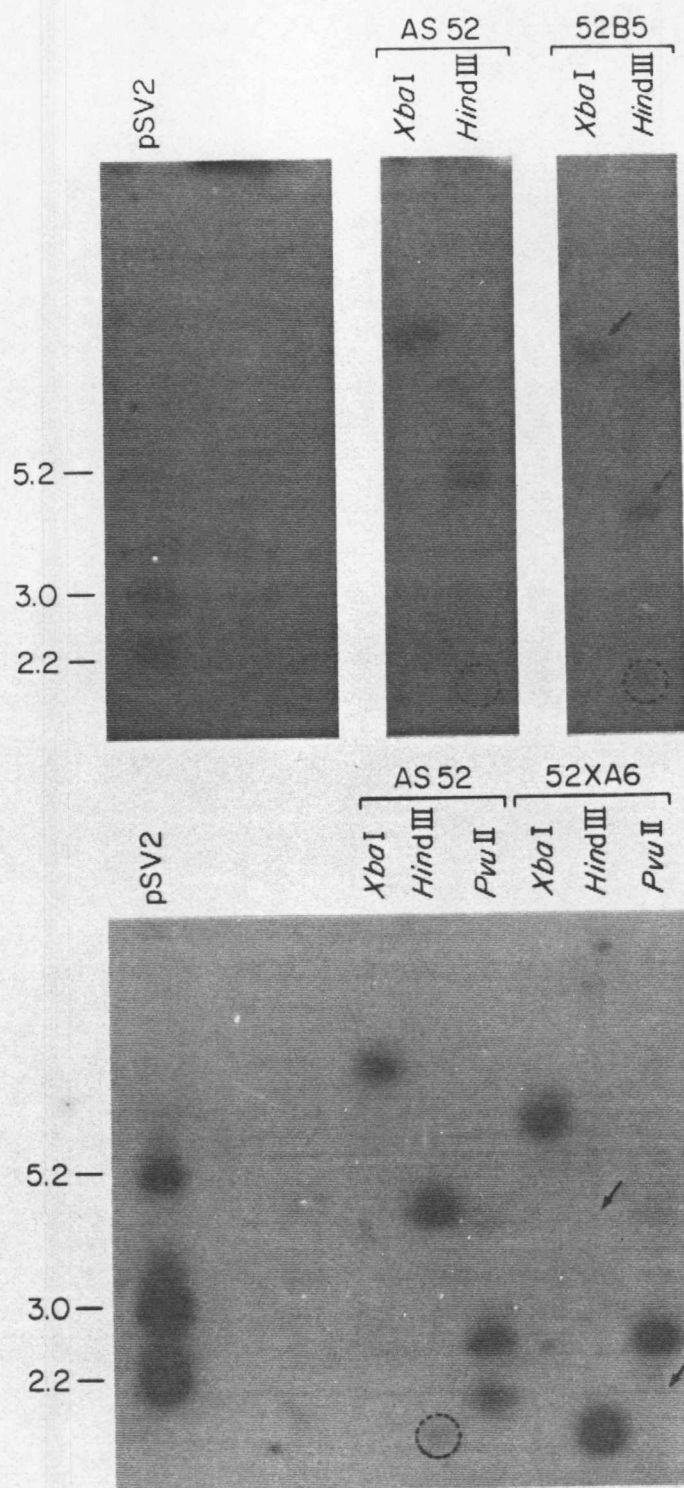


Fig. 3.6

is retained, the 1.0 kb deletion between the HindIII and BamHI sites must include a minimum of approximately 100 bp of the cloned gpt gene region.

Similarly, the deletion in the 52XA6 line is estimated to be 3.1 kb. Again, the HindIII site separating the SV40 early promoter and the gpt structural gene is retained, since the 1.9 kb HindIII fragment is retained in the 52XA6 line (Figure 3.3, p. 55 and Figure 3.6). However, the sequence deletion must occur fairly close to the HindIII site and extend 3.1 kb through the gpt structural gene and on into the genomic DNA, otherwise, an additional HindIII and PvuII hybridization band would be observed. Figure 3.4, p. 58 shows loss of the 4.7 kb HindIII and the 2.2 kb PvuII fragments in the 52XA6 line. We conclude that deletions in the 52B5 and the 52XA6 lines, which apparently include gpt structural gene sequences, provide a molecular basis for the observed TG^r phenotype and lack of XGPRT activity (Figure 3.5) associated with these cell lines.

Discussion

In this study, we report the use of the bacterial gpt gene as a locus for the molecular analysis of mutation in pSV2gpt transformed CHO cells. The gpt gene has been constructed in a SV40-pBR322 recombinant vector to express the bacterial gpt gene product, XGPRT, in mammalian cells (50,52). XGPRT is a purine salvage pathway enzyme with substrate specificity similar enough to mammalian HGPRT that selection protocols used in the isolation of HGPRT deficient CHO mutants (54) are also useful in selecting TG^r mutants of pSV2gpt transformed CHO cells deficient in XGPRT activity. One isolated pSV2gpt transformed CHO line (AS 52)

carries a single copy of the gpt gene integrated into the high molecular weight genomic DNA. Analysis of two selected XGPRT deficient mutants from this line, one spontaneous in origin and the other isolated following X-ray exposure of 400 rads, indicate specific deletions through the gpt structural gene as a molecular basis for the mutant phenotype.

Similar observations of mutation and deletion of significant portions of transforming gene sequences have been previously reported in tk transformed mammalian cells (40,64,75). In these studies, however, the tk mutants described were all spontaneous isolates. The present report is the first to use such a system to analyze mutants following mutation induction in DNA transformed mammalian cells. We report here that mutants at the integrated gpt gene in the AS 52 line can be quantitatively recovered and that partial deletions of transforming sequences can be detected by blot hybridization analysis of recovered TG^r AS 52 mutants.

It is significant that while the cytotoxic effects of X-irradiation in the AS 52 line and in the CHO-K₁-BH₄ line are virtually identical, the recovery of TG^r mutants is much higher in the AS 52 line. A number of TG^r AS 52 colonies were subcloned and assayed for XGPRT activity both in polyacrylamide gels as shown in Figure 3.5, p. 61 and using in vitro enzyme activity assays (data not included). No XGPRT activity was observed in any of the TG^r lines assayed. This observation is consistent with the notion that TG^r colonies arising either spontaneously or following X-irradiation of the AS 52 line are the result of mutation the transformed gpt locus and are selectable as a result of the stringent TG

selection system previously described for the isolation of mutants at the hgp locus in mammalian cells (54). The apparent increase in induced mutation is not unique to X-ray induced mutation. A similar increase is observed in the AS 52 line as compared to the CHO-K₁-BH₄ line following exposure to 254nm UV-light. Again, the cytotoxicity curves are quite similar (KRT, unpublished observations).

The basis of the difference in the slope of the mutation induction curves of X-irradiated AS 52 and CHO-K₁-BH₄ cells has yet to be resolved. There are several possibilities. First, the HGPRT-deficient transformation host, X3/5, was derived following X-irradiation of the parental CHO-K₁-BH₄ line. It is possible that previous X-irradiation may have affected the X3/5 line to make it hypersensitive to mutation induction as a result of effects within a gene involved in a mutation fixation pathway, e.g., a DNA repair gene. Alternatively, the gpt integration event may have occurred within such a gene. While we feel these possibilities are unlikely, they cannot at this time be ruled out.

A second possibility is that the target size of the gpt gene may be sufficiently larger than the effective target size of the mammalian hgp locus to account for the increase in TG^r mutants following X-irradiation of the AS 52 line. That is, assuming mutations in intervening sequences to be silent except for those which occur within sequences necessary for proper splicing, then an estimated 700 bp are required for the HGPRT structural gene (5). Assuming an additional 300 bp for promoter sequences for the hgp gene, a maximum estimate is obtained of approximately 1.0 kb for the hgp gene sequences that would be directly affected

by potential mutagenic lesions. Comparatively, the XGPRT structural gene is within approximately 1.0 kb insert, with SV40 early promoter sequences accounting for an additional 326 bp and an additional 871 bp which include SV40 splicing functions. Since splicing functions have been demonstrated to increase the efficiency of gene expression in mammalian expression vectors (28,31,51), these sequences have been included in the pSV2gpt vector. Integration of the pSV2gpt plasmid (Figure 3.3, p. 55) occurs very near the BamH1 site within SV40 polyadenylation sequences and therefore loss of these sequences apparently do not significantly effect gpt gene expression. Thus, a target size of approximately 2.2 kb, from the PvuII site to the BamH1 site (Figure 3.3, p. 55) can be estimated for the integrated gpt gene in the AS 52 line. Therefore, sequences sensitive to mutation in AS 52 cells are roughly two-fold greater than sequences sensitive to mutation in the CHO-K₁-BH₄ line. The two-fold difference in potentially sensitive sites, however, does not readily account for the observed 10 to 25-fold difference in TG^r mutants arising following AS 52 vs CHO-K₁-BH₄ X-irradiation. We presume that while target size may have some effect, this cannot totally account for the observed differences in mutant frequencies.

A third possibility for explaining these observations involves the potential effects of imbalances in the pyrimidine and purine pools. Pool fluctuations either as a result of the use of metabolic inhibitors or as a result of defined mutations in pyrimidine pathways have been previously demonstrated to have mutator effects on mammalian cells (44,45,46). AS 52 cells are subcultured in MPA medium (see Materials and Methods), which

blocks de novo purine and pyrimidine biosynthesis and replaces significant concentrations of xanthine, adenine, and thymidine exogenously. It seems plausible that maintenance of the AS 52 line in MPA medium until just prior to mutagen treatment could effect the sensitivity of the AS 52 line to mutation and, therefore, could account for some of the differences in mutant yield.

Finally, an intriguing possibility for the observed differences in TG^r mutant yield in the AS 52 vs the CHO-K₁-BH₄ lines involves selective deletion of gpt sequences as a result of recombinational hot-spots either within the integrated pSV2gpt sequences or within close proximity to the gpt integration site. Albertini et al. (1) have recently shown that deletion mutations in E. coli may arise as the result of slipped mispairing of short homologous regions on either side of deleted sequences during DNA replication, and that these homologous sequences may be as short as a few base-pairs. Similarly, deletions observed in the human β -globin gene family have implicated involvement of repeated sequences in deletion formation in mammalian cells (19). It is interesting to speculate that X-irradiation which is known to increase recombination in CHO cells, i.e. sister chromatid exchange and chromosome aberration induction (56, 57), may promote recombinational activity leading to the deletion of gpt sequences in the AS 52 line. Confirmation of this hypothesis requires sequence analysis of the deletion sites of several mutants derived from the AS 52 line. It is possible that a combination of the above explanations account for the increased mutant yield observed in the AS 52 line.

The small size of the pSV2gpt integration and the well-defined selection protocol for mutant isolation facilitates the future use of the AS 52 line as an easily manipulated system for the molecular analysis of mutation in CHO cells. Specific deletion mutations have been isolated and identified by blot hybridization. Further molecular analyses of mutation with AS 52 and a variety of mutagens are in progress.

CHAPTER IV

SUMMARY AND IMPLICATIONS

This dissertation project has used pSV2gpt transformed CHO cells for the analysis of gene mutation within the transforming gpt gene. The isolation of one transformed cell line (AS 52) carrying a single functional copy of the gpt gene integrated into the AS 52 genome has provided the basis for a system which can be used to analyze mutations at the DNA sequence level in cultured mammalian cells. This system appears to have promise for the future analysis of molecular events in mutagenesis.

While only deletion mutations were detected in these studies, further efforts should provide for the direct analysis of mutant DNA sequences in this system, as well. The gpt gene (1.0 kb) and related plasmid sequences at the insertion site total approximately 4.0 kb which makes the subcloning of mutant DNA sequences from within the AS 52 genome a relatively straightforward task. Once isolated the DNA fragment carrying the mutation can be manipulated for DNA sequence analysis if desired.

This kind of approach to mutation studies provides a means by which mutagen-specific changes can be correlated with specific types of DNA base sequence alterations. Some investigators have been successful in analyzing mutagen specificity in vitro in some bacterial systems using this type of correlative approach (18,20,23,70,72,73). Of course, a limitation to these kinds of studies is that types of DNA sequence mutations observed can only be correlated to mutagen activity following analysis of a large number of mutants, since any individual mutation

analyzed may be spontaneous in origin. Nevertheless, the possibility of analyzing point mutations within a selectable locus defined by a fairly small fragment of DNA, as exists in the AS 52 line, is unique among systems currently in use for the molecular analysis of mutation in mammalian cells.

Furthermore, since only a single copy of the E. coli gpt gene is present in the AS 52 genome, the study of mutation at the DNA sequence level is not complicated by the presence of additional copies of the gene in these eukaryotic cells. Analysis of mutant sequences of an autosomal locus such as aprt, for instance, is limited by the presence of two copies of the aprt gene per diploid cell. Since mutant selection at the aprt locus is from aprt^{+/-} to aprt^{-/-}, analysis of induced mutation at the aprt locus must include a means of distinguishing the induced aprt gene mutation from the previously existing aprt gene mutation. Similarly, hgp gene sequences that may be present in the nonexpressing X-chromosome of CHO cells or that may exist as pseudogene sequences observed in hamster, mouse and human cells (24), complicate mutant isolation and DNA sequence analysis of mutation at the hgp locus. Thus, the relatively small size of the gpt gene, the possible presence of the ampicillin resistance gene useful in plasmid rescue and the unique nature of these gpt gene sequences in the mammalian genome all facilitate the direct analysis of gene mutation in these transformed mammalian cells.

While this system provides a means by which the molecular basis of mutation in mammalian cells can be studied, there are shortcomings. The study of mutation in the AS 52 line is the study of mutation by

design. Although the data are consistent with the genomic integration of the gpt gene and mutation induction is observed as expected for a hemizygous locus, the integrated gpt gene does not normally exist within the mammalian genome. Since differences are observed between gpt and hgp^rt gene mutation frequencies in transformed and parental CHO cells, one must consider the possibility of either mutational and/or recombinational hot spots or position effects as a basis for the increased mutant yield in the AS 52 line. Considering recent studies of gene amplification and gene correction (67) which reflect a somewhat fluid nature to the mammalian genome, the relative position of the site of integration and sequences nearby may play a significant role in the ultimate fixation of gene mutation in these transforming sequences. Further studies are in order to define the effects, if any, the integration site has upon induced mutation in the AS 52 line.

Furthermore, the effects cited above may provide an explanation for the spontaneous and induced mutant frequencies observed in the Class III transformed CHO lines. Three independently isolated pSV2gpt transformed lines fit the Class III "phenotype" of an exceedingly high spontaneous mutation frequency at the transforming locus. At least two other laboratories observe similar effects with respect to spontaneous mutant frequencies using different transforming sequences in other mammalian cell lines (18,40,55,57,63). Together these results indicate the non-random nature of gene instability in the transforming locus of DNA-transformed mammalian cells. It would be of interest to isolate integrated pSV2gpt sequences in the Class I, II and III cell lines and compare the pSV2gpt sequences retained as well as study the DNA sequences

at the various sites of integration. Such studies may provide insight into the future utility of DNA-mediated gene transfer in mammalian cells. Results of this study indicate that transforming sequences may integrate into the mammalian genome but in some instances be relatively unstable in that configuration under nonselective conditions. If a significant portion of recipient cells following gene transfer lose the selectable phenotype, that is, at a frequency of 10^{-4} to 10^{-3} per clonable cell as observed in the Class III lines, then DNA-mediated gene transfer in gene correction therapy or in genetic alterations of plant systems will be of limited use. Only further studies with a large number of selectable transforming genes and a wide range of recipient cell types will elucidate the molecular basis of this apparent genetic instability in DNA transforming sequences in mammalian cells.

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LITERATURE CITED

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VITA

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