To the Graduate Council:

I am submitting herewith a dissertation written by Gregory James Horesovsky entitled "A Determination of Whether Restriction Enzymes Can Induce Specific Deletions at the HPRT Locus in CHO 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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and Dean of the Graduate School

A Determination of Whether Restriction Enzymes Can Induce Specific Deletions at the HPRT Locus in CHO Cells.

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Gregory James Horesovsky

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DEDICATION

This dissertation is dedicated to my parents, Vaclav and Eva Horesovsky who instilled in me, at an early age, the love of learning.

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ABSTRACT

Restriction enzymes are particularly informative clastogenic agents in that they produce only a single type of lesion, a double-strand break, at specific recognition sites in the DNA. Using a restriction map, particular restriction enzymes can be selected potentially to generate damage at a specific site in any target gene. We have used a variety of restriction enzymes to generate mutations in the hprt gene in Chinese hamster ovary (CHO) cells. The highest induced mutation frequencies have been with the restriction enzymes Sau3A I, which has a calculated cutting frequency in the genome of 1/338 base pairs, and Rsa I, which has a cutting frequency of 1/227 base pairs (Bishop et al., 1983). Both Sau3A I and Rsa I possess restriction sites in hamster hprt exon DNA, and it is likely that there are numerous recognition sequences in the large intervening introns of the gene which comprise nearly 97% of the hamster hprt locus. Mutants were isolated by selecting for resistance to the base analog 6-thioguanine.

We have used the technique of multiplex PCR to amplify simultaneously all exons of the hamster hprt to screen induced mutants for specific deletions. Multiplex PCR consists of a series of oligonucleotide primers designed to amplify each of the exons of hprt using genomic DNA as template. Each amplified exon produces a discrete band when multiplex PCR products are resolved on an acrylamide gel. The absence of a single exon band or group of bands in a multiplex reaction is diagnostic of a deletion in hprt and also begins to define the size and location of the deletion.

Multiplex PCR of restriction enzyme-induced HPRT-deficient mutants shows that both Sau3A I and Rsa I are capable of generating deletions in the hprt gene, but a different spectrum of type of deletions was induced by the two enzymes. RFLP analysis of exon specific bands and asymmetric PCR analysis was employed to determine whether the breakpoints in some of the mutant clones analyzed were at restriction sites. No changes were observed at restriction sites in hprt exons in the clones analyzed, and it was assumed that most breakpoints occured at restriction sites in the large intervening introns.

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INTRODUCTION

DNA damaging agents are ubiquitous in our environment; ultraviolet radiation, x-rays, insecticides, and many other clastogens are commonly encountered every day. There is an ever-increasing awareness in the general public today of the number of potential carcinogens we encounter on a daily basis. Cell death, mutation, and induction of cancer are listed among the consequences of genetic damage. DNA damage resulting from such exposures is the focus of research of many laboratories. By understanding the mechanisms of repair of damage resulting from exposure to environmental clastogens we can better assess how to protect against their harmful effects.

There are several ways in which the damage resulting from exposure to clastogenic agents may be monitored. Analysis of chromosome aberrations, sister chromatid exchange, or induction of mutations at a specific target locus are all indicative of damage to the DNA. The frequency of chromosome alterations has been used as a biological dosimeter for radiation and, to some extent, for chemically induced DNA damage. The production of chromosome aberrations is actually indicative of misrepair of damage in the DNA. The types of chromosome aberrations observed depends on the stage in the cell cycle during which they were induced. The cell cycle, as first described by Howard and Pelc (1952), consists of G1 phase (gap 1), S phase (DNA synthesis or replication), G2 phase (gap 2), and M (mitosis). Figures 0.1 and 0.2 show the cell cycle and which aberrations are induced at the different

Figure 0.1 The cell cycle as described by Howard and Pelc (1952). G1 = gap one, chromosome consists of a single chromatid. G2 = gap two, chromosome cosists of two sister chromatids.

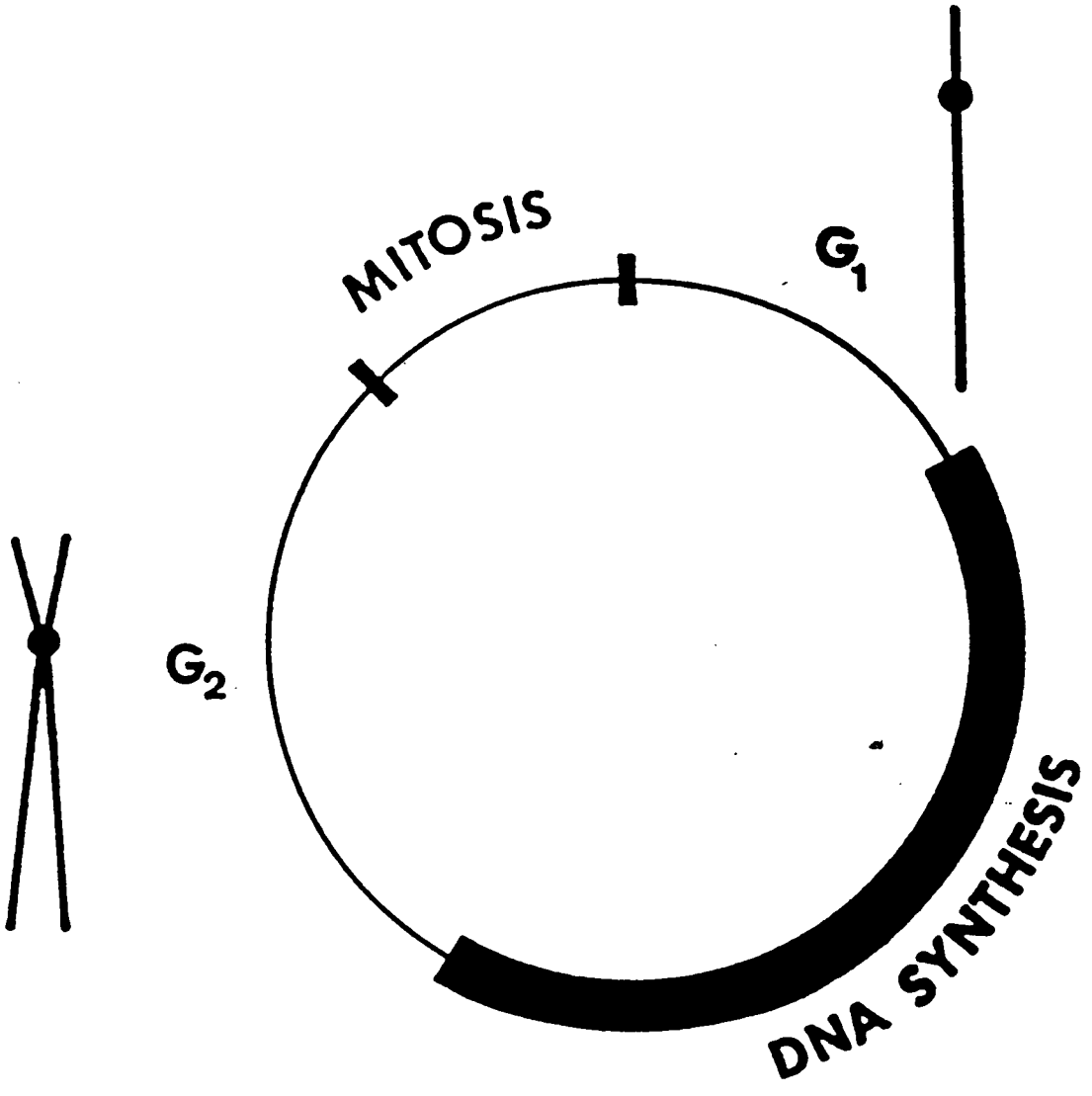


Figure 0.1

Figure 0.2 A schematic representation of the production of chromosome aberrations during G1 and G2 of the cell cycle. During G1 only chromosome-type chromosome aberrations are induced. During G2 only chromatid-type chromosome aberrations are induced.

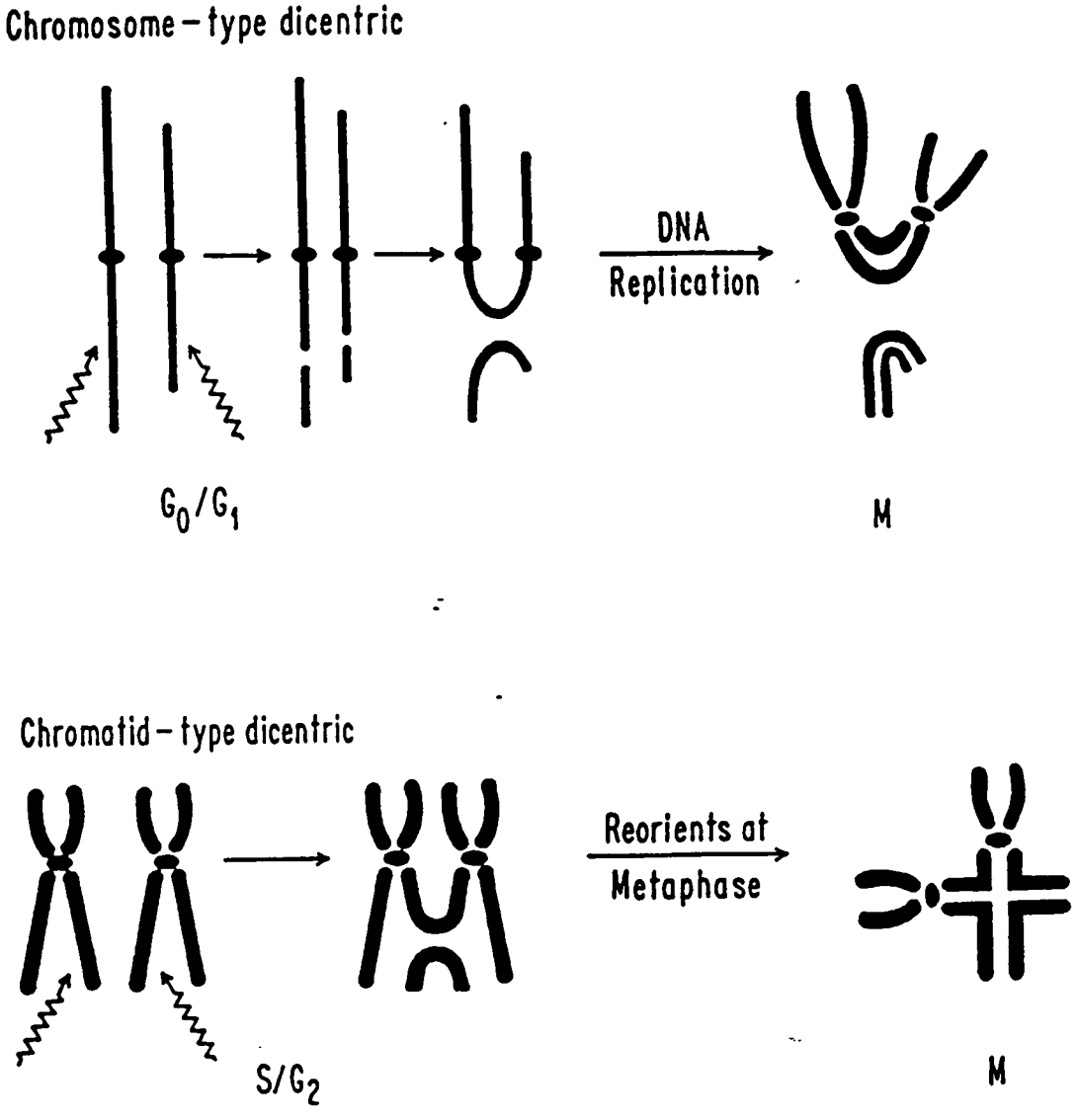

Chromosome-type dicentric
DNA
Replication
G_0/G_1
M
Chromatid-type dicentric
Reorients at
Metaphase
S/G_2
M

Figure 0.2

stages (for review see Savage,1975). Cells that are not actively dividing are considered to be in G_0 of the cell cycle (similar to cells arrested in G1). The observation of chromosome aberrations is performed with the aid of the spindle poison colchicine or its synthetic analog, colcemid. Inhibition of microtubules by colchicine allows the analysis of condensed chromosomes on the mitotic plate by arresting cells in mitosis. During G1 of the cell cycle, the chromosome exists as a single DNA double-stranded helix. Damage induced during this stage of the cell cycle results in chromosome-type aberrations. Since the aberrant chromosome must pass through S phase for observation in mitosis both chromatids are involved in the aberration observed. Damage induced in G2 appears as chromatid-type aberrations when observed in mitosis. Since this damage does not proceed through S phase for observation in mitosis, individual chromatids may be involved in aberrations independent of one another. Damage induced during S phase can appear as either chromosome or chromatid-type aberrations depending on whether or not the damage was induced in regions already replicated. Different clastogens are capable of inducing aberrations during different stages of the cell cycle. Some agents, such as X-rays are said to be S-independent because they do not require passage through S-phase for production of aberrations. Presumably this is due to the type of lesion (double-strand breaks, base damage, etc...) induced by X-rays and the rate of repair processes involved in double-strand break repair. S-dependent clastogens "require" passage through the S-phase in order for damage to result in chromosome aberrations. Ultraviolet radiation and most chemical clastogens [with the exception

of radiomimetic compounds such as bleomycin which are capable of inducing physical breaks in the DNA independent of the cell cycle (Crooke and Bradner, 1976)] are S-dependent clastogens . If damage induced by S-dependent agents is not repaired prior to S-phase, replication through the site of damage results in the production of chromosome aberrations. It has been proposed by Preston (1982) that S-dependent clastogens are actually not dependent on passage through S phase, but that these damages are repaired more slowly than S-independent lesions and are more likely to be present at the time of DNA replication.

The production of chromosome aberrations from DNA double-strand breaks has been proposed to occur by two different pathways. The first, proposed by Sax (1938) is known as the breakage-reunion theory. This theory states that there are three possible outcomes for a DNA double-strand break: 1) rejoining of the original strands; 2) rejoining of the strands with other strands to produce exchanges; and 3) failure of the strands to rejoin resulting in the production of deletions. The second model proposed is known as the exchange hypothesis proposed by Revell (1955,1959). This theory states that unstable lesions in the DNA may decay with two different consequences: 1) No effect upon the chromosome; and 2) decay resulting in an exchange between the DNA strands. According to the Revell hypothesis, all chromosome aberrations may be created by an exchange event. In actuality, it is likely that chromosome aberrations are produced by mechanisms described by both of these models (Duncan and Evans, 1983).

Ionizing radiations are capable of producing a variety of damages to the DNA. Different types of radiations are capable of inducing different spectra of DNA damage based on the rate at which they deposit their energy in the medium through which they pass (this rate is known as the linear energy transfer or LET). Radiations may damage the DNA either by directly depositing their energy in the DNA molecule itself, or indirectly by the induction of reactive species, often oxygen radicals produced by ionization of water molecules. Radiation-induced lesions that can potentially be involved in the formation of chromosome aberrations include DNA-protein crosslinks, single- and double-strand breaks, base damage and bulky lesions (for review, Frankenberg-Schwager, 1990). Low LET radiations produce single-strand breaks and double-strand breaks directly, and sugar and base damage indirectly. High LET radiations produce a similar spectrum of damages except that the amount of direct damage, particularly double strand breaks, is greatly increased (Ward, 1988).

The most commonly occurring lesion produced by ionizing radiations is base damage (Wallace, 1983;Teoule, 1987). These lesions include abasic sites (both apurinic and apyrimidinic), urea fragments, and thymine glycol. Base-damages are repaired by excision repair pathways (Figure 0.3) which involve excision of the DNA around the site of base damage, re-synthesis to fill in the excised region, and finally, ligation of the newly synthesized DNA to the original strand. Failure to repair these lesions by the time of replication results in errors occurring during synthesis resulting in mutations or chromosome aberrations (Evans, 1977).

It has been demonstrated that single strand breaks induced from X-ray treatment are repaired rapidly (50% in the first 2 minutes, Van der Schanns et al.,

Figure 0.3 A schematic representation of the excision repair pathway. There are four steps to this repair process: 1) DNA damage induction; 2) DNA incision around the site of damage; 3) excision of the damaged region and resynthesis; 4) ligation of the newly synthesized DNA.

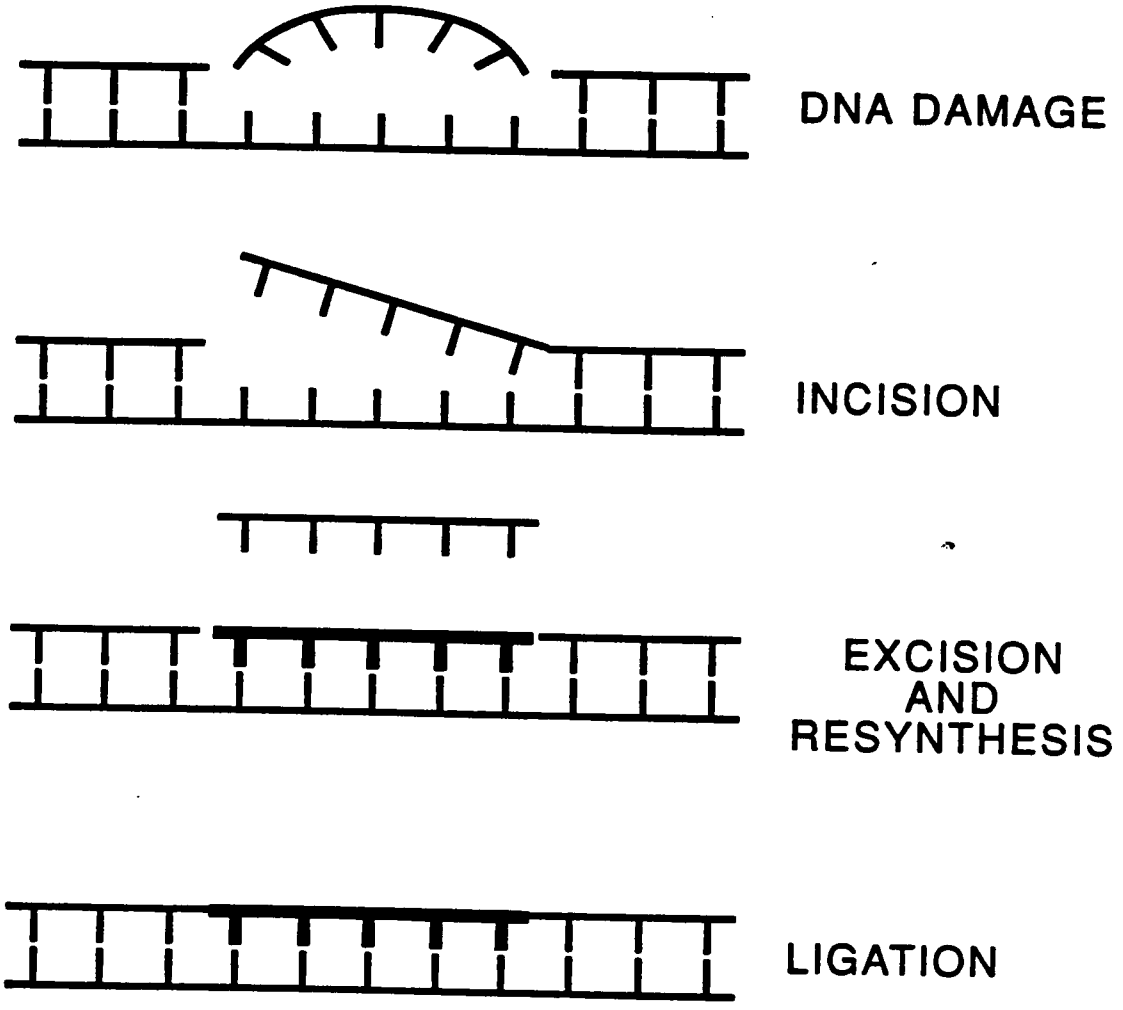


Figure 0.3

1983) and do not contribute significantly to the production of chromosome aberrations, even when repair is delayed (Morgan et al., 1985). Double-strand breaks, on the other hand are believed to be the primary lesion responsible for the production of chromosome aberrations (Natarajan et al., 1986).

An alternative mechanism of repair of DNA double-strand breaks is by recombination. Both non-homologous and homologous recombination has been demonstrated to be greatly enhanced by double-strand breaks in cells derived from a variety of organisms from yeast to higher mammals (Wilson et al., 1982; Vos and Hannawalt, 1989; Cao et al., 1990; Lin et al., 1990). While the mechanisms of non-homologous recombination are presently poorly understood, there exist several different models of homologous recombination repair of DNA double-strand breaks. Resnick (1976) was one of the first to propose a model of DNA double-strand break repair involving recombination. In the Resnick model (Figure 0.4), a DNA double strand break is the initiating event, followed by exonuclease digestion to generate a DNA double-strand gap. Single strand invasion by a homologous segment of DNA into the gapped DNA forming heteroduplex DNA, allows replication repair across the region of damage. Since this first proposal, several different variations of this model have been proposed: the helicase-mediated recombination model (Wake et al., 1985); the discontinuous-heteroduplex model (Ray et al., 1989); the recission-reannealing double-strand break pathway (Ozenberger and Roeder, 1991); the single-strand annealing model (Lin et al., 1984); and the double-strand break repair model (Szostak et al., 1983). There is a significant amount of evidence for each of these models, but

Figure 0.4 The Resnik model of recombination repair. A double strand break is processed to give rise to a double-strand gap followed by single strand invasion into non-gapped homologous DNA, recombination, and repair synthesis.

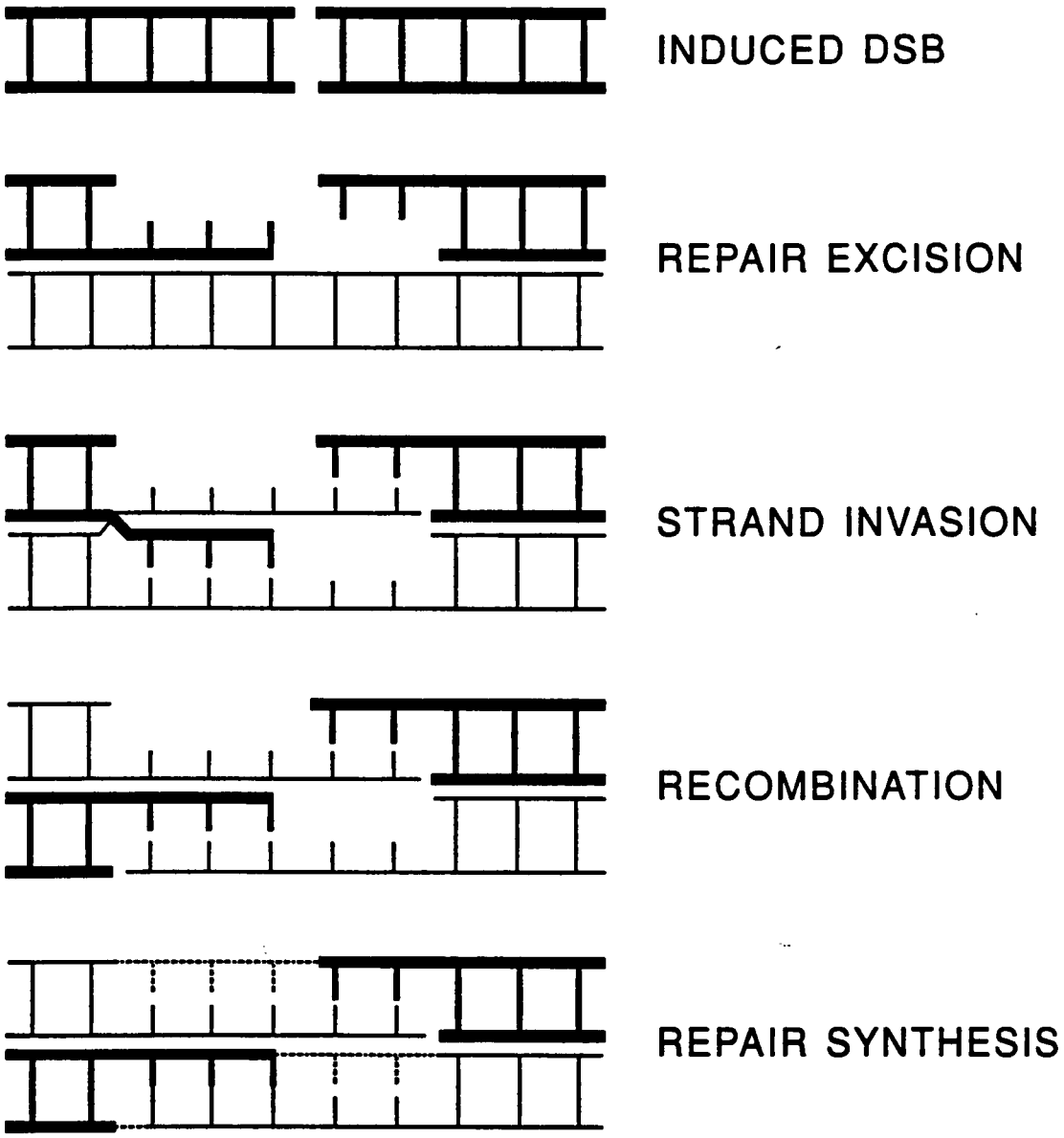

INDUCED DSB
REPAIR EXCISION
STRAND INVASION
RECOMBINATION
REPAIR SYNTHESIS

Figure 0.4

the two models most often used to describe recombination events are the single-strand annealing model (SSA) and the double-strand break repair model (DSBR). The double-strand break repair model (Figure 0.5) proposed by Szostak (1983) involves the production of a double strand break in the recipient DNA. Invasion of a single-strand into a region of homologous double stranded DNA results in the formation of a Holliday junction. Migration of the Holliday junction away from the original site of DNA damage is followed by resolution of this intermediate. Resolution may result in several recombination products, for example, crossover events, gene conversion, and non-crossover events depending on the strands involved in the exchange. The DSBR model predicts that the DNA strand containing the double-strand break is also the recipient of information in gene conversion. This has been shown in a number of laboratories (Nicolas et al., 1989; Voelkel-Meiman and Roeder, 1990)

The other model, single-strand annealing (Figure 0.6) proposed by Lin and colleagues (1984), dictates that both substrates must be gapped and exonuclease digestion of the 5' strand at the double-strand break [or localized unwinding of the homologous double-strand DNA by the enzyme helicase (Wake et al., 1985)] reveals 3' single-stranded regions. These single-strand regions may anneal in regions of homology. Continued degradation of the original 5' strand results in complete annealing of the homologous sequences and a subsequent non-conservative recombination event. Although there is significant evidence for the repair of DNA double-strand breaks according to the DSBR model of Szostak, it appears that the most frequently occurring mechanism of recombination repair is the non-conservative

Figure 0.5 The Szostak double-strand break repair (DSBR) model. A double-strand break is enlarged to a double strand gap, followed by single strand invasion and the formation of two Holliday intermediates which migrate away from the original site of the double strand break. Resolution of the Holliday structures occurs at sites distal to the site of the original double strand break.

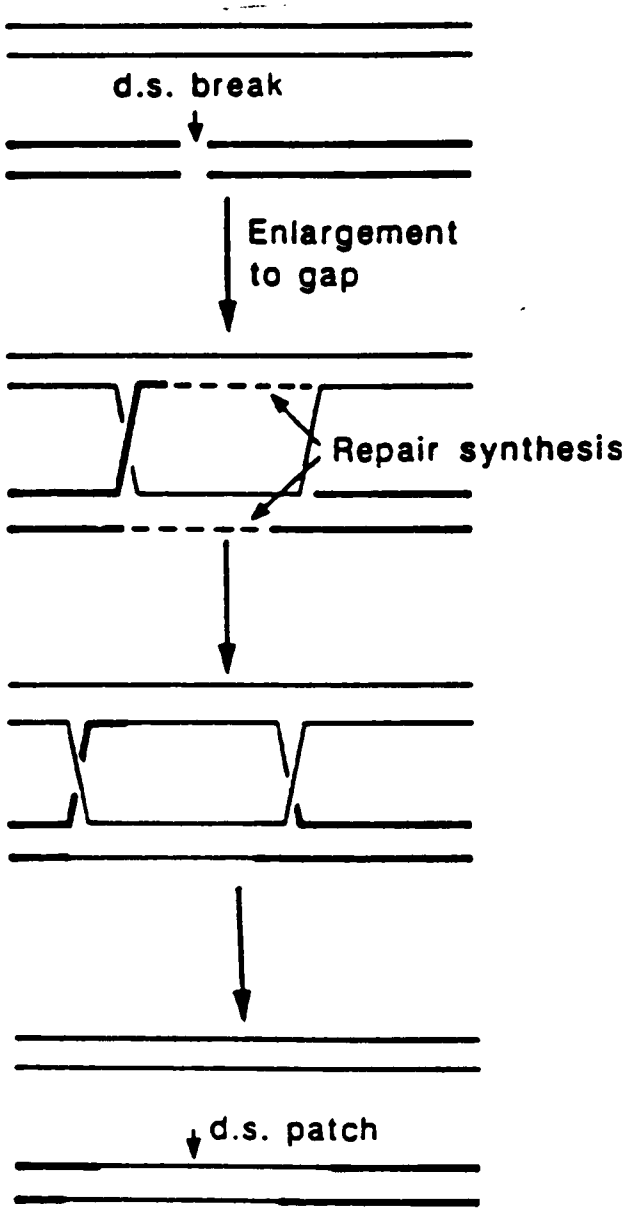


Figure 0.5

Figure 0.6 The Lin single-strand annealing (SSA) model. A double strand break is processed by 5' exonuclease generating long single stranded 3' tails. Eventually, regions of homology become single stranded and may anneal followed by synthesis and ligation.

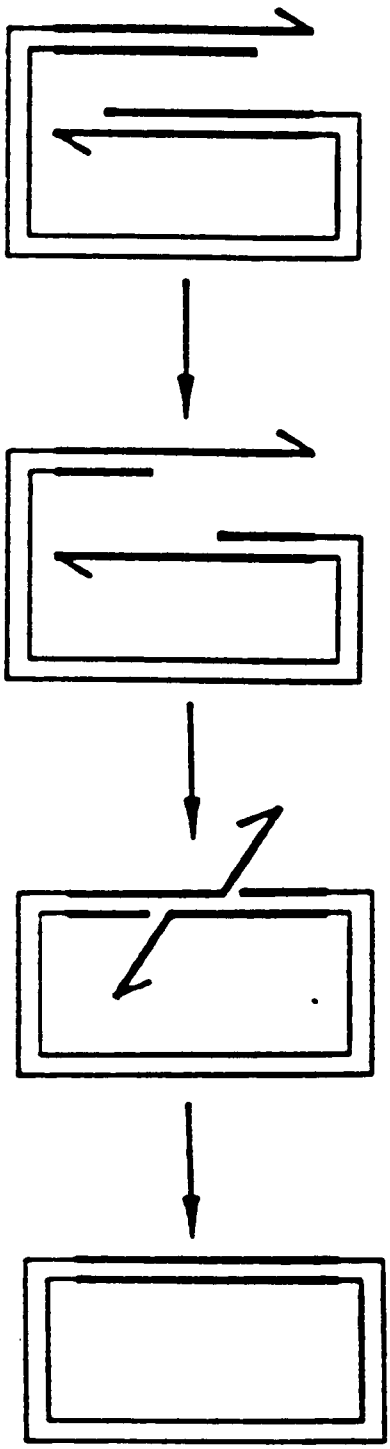


Figure 0.6

SSA model (Folger et al., 1985; Seidman, 1987; Lin et al., 1990a; Lin et al., 1990b; Marryon and Carroll, 1991a; Marryon and Carroll, 1991b). These observations are based on the isolation of proposed intermediates in the SSA recombination. It is very likely that the type of termini present at a particular DNA double-strand break, the local structure of the surrounding DNA, the availability of and physical nature (presence of DNA breaks) of homologous sequences, determine the method of recombination repair.

In order to elucidate the importance of the role of the double-strand break in the production of chromosome aberrations, a clastogen was sought that could produce only double-strand breaks in the DNA. The ability of restriction endonucleases to produce only a single type of break in the DNA, namely a double-strand break, made them ideal for such studies. A number of researchers have used restriction enzymes to induce chromosome aberrations (Bryant, 1984; Natarajan, 1984; Obe and Natarajan, 1985; Zhang and Dong, 1987; Winegar and Preston, 1988) using a number of different techniques to introduce these enzymes into cells. In early studies it rapidly became clear that the method used to introduce restriction endonucleases into the cell was of importance. Many of the cell permeabilization techniques gave poor or inconsistent aberration frequencies. The most successful and consistent method to be used was the technique of osmolytic shock (Okada and Reichsteiner, 1982; Winegar and Preston, 1987). Observations from these studies indicated that restriction enzymes were capable of inducing significant levels of chromosome aberrations in all stages of the cell cycle (Winegar and Preston, 1988).

Additionally, the location of restriction enzyme-induced DNA double-strand breaks is somewhat targeted since they may be induced only at particular recognition sequences. The recognition sequence for different restriction enzymes vary, but usually they consist of a 4 to 8-base palindromic sequence. Breaks in the DNA are usually produced by the enzyme within this recognition sequence and may be of two types: blunt-end or cohesive-end, depending on whether the breaks are oppositely opposed or staggered (Table 0.1). Cohesive-ended termini have single-strand overhangs (the polarity of which may be either 5' or 3' protruding single strands) whereas blunt-ended termini do not possess any single-strand overhangs. The frequency with which any restriction sequence occurs may be predicted based upon the model of Bishop (1983). The Bishop model is based upon a nearest neighbor algorithm that states that certain dinucleotide combinations occur more frequently in the genome than do others. The frequency of induced chromosome aberrations following treatment with any particular enzyme is often a feature of the frequency of the particular restriction sequence in the genome. This indicates that restriction enzymes are capable of recognizing their correct restriction sequence in chromatin inside the cell. The ability of restriction enzymes to digest chromatin with fidelity at the restriction sequence has been further demonstrated by Winegar et al. (1990). It was also observed by Chung et al. (1991) that cytosine arabinoside, a potent inhibitor of DNA polymerase alpha, was able to potentiate restriction enzyme-induced chromosome aberrations. It was concluded from this observation that DNA

TABLE 0.1 Recognition Sequences and End Structure Products of Various Restriction Enzymes

ENZYME	RECOGNITION SITE	END STRUCTURE
Alu I	5' AG CT 3' TC GA	$_{p}$CT $_{HO}$GA
Rsa I	GT AC CA TG	$_{p}$AC $_{HO}$TG
Sau3A I	GATC CTAG	$_{p}$GATCN $_{HO}$N
Eco RI	G AATT C C TTAA G	$_{p}$AATTC $_{HO}$G
Not I	GC GGCC GC CG CCGG CG	$_{p}$GGCCGC $_{HO}$CG

resynthesis was an important component in the repair of restriction enzyme-induced double-strand breaks and that recombination mechanisms might be involved.

The ability of restriction enzymes to induce chromosome aberrations in a manner that indicated digestion of chromatin at restriction sequences opened the door to the idea that they could be used to generate targeted breaks in a specific gene. These breaks could result in the production of a series of well-defined deletion mutations in that target gene. Once the system is optimized, restriction enzymes could potentially be used to study the effects of deleting specific regions of a target gene. The induction of mutations in a target gene should be restricted to certain areas of the target gene based on the frequency of restriction sequence sites for the particular enzyme used. Depending on the structure of the termini resulting from digestion with any particular restriction enzyme, the production of double-strand breaks in a target gene might also lead to increased recombination involving that gene. The outcome of such events would be observed as different induced mutation frequencies. The spectrum of mutations induced by restriction enzymes in a particular target gene might also be expected to differ depending on the type of termini present at the induced double-strand break. This dissertation describes approaches for performing such studies. I will discuss the optimization of treatment conditions, strategies for mutation induction, and subsequent molecular analysis strategies, and techniques used to create mutations with restriction endonucleases in a target gene.

CHAPTER 1

OPTIMIZATION OF PERMEABILIZATION CONDITIONS FOR RESTRICTION ENZYME-INDUCED MUTAGENESIS

SUMMARY

Previous methods of introducing restriction enzymes into living cells, although effective with respect to successfully producing chromosome aberrations in a dose dependent manner, are far from optimal. Osmolytic shock, the most efficient and reliable of these techniques, effectively introduced restriction enzymes into CHO cells, but only a small portion of the treated cell population apparently received an enzyme dose. Subsequently, the distribution of aberrations was overdispersed and followed a Neyman type A distribution rather than the expected Poisson. This had been reported earlier by Winegar (1987). Overdispersion of damage is also evident in cell survival following treatment. Survival following restriction enzyme treatment via osmolytic shock is greater than 95% indicating that a very small portion of the

population received a dose of enzyme. From the level of aberrations induced in this small population it can be assumed that the dose administered would most often be lethal. These conditions are not suitable for mutation induction studies. Ideally a small, sublethal dose should be received by a large segment of the population.

A new approach to permeabilizing cells is cell electroporation. Electroporation of restriction enzymes into cells proved to be more successful than expected. Once parameters for electroporation were optimized, chromosome aberrations were induced at doses 1/10 those used in previous osmolytic shock studies. In addition, overdispersion was not observed with electroporation. At higher doses of enzyme, greater than 90% of the cells exhibited chromosome aberrations and distribution of aberrations throughout the population followed a Poisson distribution. Survival following electroporation with the restriction enzymes Rsa I and Sau3A I was significantly reduced. An intermediate dose where aberration damage and cell survival are in balance should be the most effective at producing mutations in a target gene.

INTRODUCTION

The use of restriction endonucleases as clastogenic agents is now widely accepted. These bacterial enzymes are capable of producing a single type of DNA lesion, namely a double strand break, at a specific restriction sequence in the DNA.

This is in stark contrast to most other clastogenic agents used to date which produce a broad spectrum of damages including base damage and double- and single-strand breaks in the DNA. The damage produced by these other clastogenic agents also has relatively little or no site specificity. For these reasons restriction endonucleases provide the ideal clastogenic agent for studies concerning the formation of chromosome aberrations and the repair of DNA double strand breaks.

Recently, it became clear that the ability of restriction enzymes to produce DNA double- strand breaks could be used to generate mutations in a target gene. An early study showed that following treatment with the restriction enzyme Alu I, there was a small, but significant, increase in mutants at the hypoxanthine phosphoribosyltransferase (hprt) locus in V79 hamster cells (Obe et al., 1986). The low efficiency of mutation induction at this locus may well have been due to the inefficiency of the method used to introduce restriction enzymes into the cell. Several different techniques have been used to introduce restriction enzymes into living cells, but with limited success. These include Sendai virus permeabilization (Bryant, 1984; Natarajan, 1984), trypsinization and pelleting (Obe and Natarajan, 1985) and osmolytic shock (Okada and Reichsteiner, 1982; Winegar and Preston, 1988). Of these, the most consistent and successful results were obtained with the technique of osmolytic shock. Osmolytic shock involves the treatment of cells with a hypertonic medium containing polyethylene glycol and the restriction enzyme of choice. During a brief incubation, the cells form pinocytic vesicles containing the enzyme medium. Another brief incubation, this time in hypotonic medium causes the internalized vesicles to

rupture, thereby releasing the restriction enzymes into the cell. Winegar and Preston (1988) were able to demonstrate, using this technique, that the cutting frequency of the restriction enzyme (based on a nearest neighbor algorithm, Bishop, 1983) is directly proportional to its aberration inducing ability. However, problems with this technique are that the damage inflicted upon the cell population does not follow a Poisson distribution, but rather a Neyman type A distribution, the net effect being an overdispersion of damage as evidenced by the distribution of chromosome aberrations among the cell population. While some cells exhibited a high number of chromosome aberrations, a large number of others showed few or no aberrations. The overdispersion of aberrations is due to a low probability of each cell in the population receiving a dose of enzyme. This probability follows a Poisson distribution. Once in the nucleus, each dose of enzyme is capable of producing multiple aberrations, the distribution of which follows a second Poisson (Winegar, 1987). The problem of overdispersion is not unique to osmolytic shock, but has been observed in all previous techniques and continues to plague new methods of permeabilization.

The problem overdispersion presents to the study of restriction enzyme-induced mutations is a serious one. With only a small population of the treated cells receiving a dose of enzyme, the induction of recoverable mutants within that small sub-population will probably be a rare event. This is further confounded by the fact that in the small population of cells receiving a dose of enzyme, the dose per cell is probably large, resulting in lethality thereby further reducing the probability of isolating the desired mutant cell. Before proceeding with mutation induction studies, the

problem of overdispersion had to be dealt with. Variations in the osmolytic shock technique, as will be discussed in this chapter, initially appeared promising, but eventually gave way to a new approach.

One approach taken to avoid the overdispersion of damage was to introduce a stably integrated plasmid expression vector containing the coding region for the restriction enzyme EcoR I into CHO cells (Morgan et al., 1988). Expression of the gene was controlled by the metallothionein promoter. This approach met with limited ,success for several reasons. Firstly, once expression of the enzyme was induced, although it was capable of producing aberrations, it was difficult to control enzyme levels, making an estimate of dose essentially impossible. Secondly, studying the effects of other enzymes was hampered by the limited availability of cloned sequences for these. However, what did originate from these studies proved to become the most efficient method of permeabilization used to date. Plasmid expression vectors had been introduced via cell electroporation, a technique frequently used to introduce foreign DNAs and other large macromolecules into living cells. Rather than introducing an expression vector coding for a restriction enzyme, the enzymes themselves were introduced into the cells with surprising success. While it is not completely understood how electroporation works, it is believed that delivering a high voltage electrical pulse causes a thinning and localized breakdown of the cell membrane. During this time a very short-lived pore may form in the membrane allowing macromolecules to leak into the cell before the pore closes and the cell continues to grow and proliferate (Winegar et al., 1989). Communications between

our laboratory and Dr. William Morgan's laboratory eventuallyled to the optimization of conditions for electroporation and it is now the technique used exclusively by a number of laboratories. Although some types of aberrations are still overdispersed, on the whole, overall frequency in the cell population is not.

In this chapter a discussion of the evolution of the optimization of cell permeabilization techniques will be presented. Optimal conditions include successful introduction of enzyme into a large segment of the treated cell population at doses that reduce survival to an acceptable level for mutation studies. The significance of the aberration and survival dose response curves will be discussed with respect to successfully performing restriction enzyme induced mutation studies.

MATERIALS AND METHODS

CELL CULTURE

Chinese Hamster Ovary cells (CHO-K1BH4) were maintained in 75cm^2 tissue culture flasks or 100 mm^2 tissue culture plates (Falcon) in Hams F12 medium (Gibco) supplemented with 5% heat-inactivated (56°C, 30 min.) fetal bovine serum (Hyclone), 50 μg/ml penicillin (Squibb) and 50 μg/ml streptomycin (Lilly) in a 37°C incubator humidified 95-100% in an atmosphere of 5% CO_2 in air.

OSMOLYTIC SHOCK

For treatment of attached cells, exponentially growing cells were plated on 35mm^2 tissue culture plates at a density of 4 x 10^5 cells/plate in 2 ml medium and incubated at 37°C for 24 hours. The medium was removed from the plates and the cells were washed once with 2 ml of pre-warmed (37°C) osmolytic buffer(0.5 M sucrose, 5% PEG 6000 in F12, 5% FBS). 100 µl of osmolytic buffer containing either restriction enzyme or enzyme storage buffer in a volume equivalent to the highest dose of enzyme was placed onto the cells. Cells were incubated at 37°C for 2 minutes to allow formation of pinocytic vesicles. Following incubation, the enzyme-containing osmolytic buffer was aspirated and the cells were washed once with 2 ml pre-warmed (37°C) hypotonic F-12 medium (3 parts F12:2 parts sterile distilled H_2O) and then incubated in hypotonic medium for 2 minutes at 37°C. Hypotonic medium was removed by aspiration and 2 ml F12 medium was added. Cells were maintained at 37°C until the time they were to be harvested.

For treatment of unattached cells, exponentially growing cells were removed from three 75cm^2 flasks by trypsinization and pooled together. After pelleting by centrifugation, the cells were resuspended in 9.0 ml osmolytic buffer. Cells were once again pelleted and this time resuspended in 1 ml osmolytic buffer containing the desired number of units of restriction enzyme (New England Bio Labs). Control cells were treated with either osmolytic buffer alone or with osmolytic buffer containing the manufacturer's recommended storage buffer for the particular enzyme. The quantity of storage buffer used corresponded to the volume of restriction enzyme used for that

dose. In experiments where multiple enzyme doses were administered, the highest volume was used. The cells were incubated for 20 minutes at 37°C. Following the incubation period, cells were pelleted and resuspended in 5 ml hypotonic F12 medium and incubated for 2 minutes at room temperature. Cells were pelleted, and resuspended in 10 ml F12 medium. Cell count was determined with a hemocytometer.

ELECTROPORATION

Exponentially growing cells from a 75 cm^2 tissue culture flask were removed by trypsinization and washed once with F12 medium. Cell count was determined using a Coulter counter. A volume containing the total number of cells to be used in an experiment (1 x 10^6 cells/treatment) was transferred to a sterile centrifuge tube, pelleted and washed twice with ice-cold phosphate buffered sucrose (272mM sucrose, 7mM KH_2PO_4 pH 7.4, 1mM $MgCl_2$). Cells were pelleted and then resuspended in a volume equivalent to 0.7 ml/treatment. The desired amount of restriction enzyme was placed at the bottom of a disposable 1 ml electroporation cuvette (Bio-Rad), and 0.7 ml of the cell suspension was added (adding enzyme first ensures even distribution of enzyme in the reaction volume). Cells were electroporated using a Bio-Rad gene pulser electroporator at 300 volts and a capacitance of 250 μFd.

ABERRATION ANALYSIS

OSMOLYTIC SHOCK

The cells not used for the HPRT mutation assay or for survival analysis were plated in 10 ml F12 medium on 100 mm tissue culture plates and incubated at 37°C for 16 hours at which time colchicine was added at a final concentration of 1×10^{-7}M. Cells were prepared for fixation 2 hours later by scraping them from plates with a rubber policeman and pelleting by centrifugation for 2 minutes. Cells were resuspended in hypotonic potassium chloride (0.075M KCl) and incubated at 37° for 13 minutes. Cells were pelleted and fixed in methanol for 5 minutes, pelleted again and resuspended in a second fixative (3 parts methanol to 1 part glacial acetic acid). Cells were dropped onto pre-wetted glass slides and air dried, then stained with 4% Geimsa. One hundred metaphase cells were analyzed for each treatment, and all classes of chromosome aberrations were recorded.

ELECTROPORATION

The fixing protocol was slightly altered for cell treated with restriction enzymes via electroporation. The cell cycle in electroporated cells is delayed by approximately 6 hours and cells are much more fragile. 1×10^{6} treated cells were plated in 10 ml F12 medium on 100mm tissue culture plates and maintained at 37°C for 22 hours at which time colchicine was added to a final concentration of 1×10^{-7}M. Cells were prepared for fixation 2 hours later, at 24 hours post-treatment, by scraping them from plates with a rubber policeman. Cells were pelleted briefly by centrifugation (1 minute) and fixed in hypotonic potassium chloride (0.075M KCl) for 3 minutes. Cells were

pelleted by centrifugation for 45 seconds and resuspended in methanol for 3 minutes. Cells were again pelleted by centrifugation for 45 seconds and then resuspended in a second fixative (3 parts methanol to 1 part glacial acetic acid). Cells were carefully dropped onto pre-wetted glass slides from a height no greater than 2-3 cm. Slides were air dried, stained and scored according to previously described protocol.

CELL SURVIVAL

Immediately following treatment with restriction enzymes, 200- 10,000 cells (depending on dose of enzyme) were plated in triplicate on 60mm tissue culture plates (Falcon) and incubated 7 days at 37°C in 5 ml F12 medium without subculturing. After 7 days, the medium was removed and cells were fixed with 70% ethanol for 2 minutes and then stained with Geimsa (Carolina Biological Supply), 4% by volume in water. Colonies (>50 cells) were counted and recorded as the average from three plates. Percent survival was determined by dividing the average of the number of colonies observed by the number of cells dispensed per plate.

RESULTS

OSMOLYTIC SHOCK

Previous studies of CHO cell permeabilization were performed on cells growing as an attached monolayer in tissue culture plates (Winegar and Preston, 1988.) Aberration data from these experiments gave a clear linear dose response curve indicating that this method was an effective one for introducing restriction enzymes into cells. Table 1.1 and Figure 1.1 show the dose response curves generated when CHO cells growing as attached monolayers were treated with two different restriction enzymes. It can be seen from Figure 1.1 a that treatment with the restriction endonuclease Alu I induced a significant increase in both chromosome-type exchanges and deletions. The sharp increase at 40 units of enzyme is probably an due to poor distribution of the enzyme in the treatment buffer and an effective higher dose administered to some of the cells. Figure 1.1 b shows a similar response for the enzyme Rsa I, but the dose apparently was evenly distributed. In both enzyme treatments, the dose response is linear and proportional to the administered enzyme dose. This increase in aberrations is seen in all stages of the cell cycle following enzyme treatment. While the dose response is clearly significant, it should be noted that the distribution of damage among the cell population does not follow a Poisson, rather it follows a Neyman type A distribution. This type of distribution of aberrations resulting from restriction enzyme treatment via osmolytic shock was first observed by Winegar in our laboratory. The observed effect of the Neyman type A distribution is

TABLE 1.1 The Induction of Chromosome Aberrations in CHO Cells by Alu I and Rsa I Introduced via Osmolytic Shock

Enzyme	Units	No. Cells Analyzed	Chromosome-Type Aberrations/Cell ± SEM			Chromatid-Type Aberrations/Cell ± SEM		
			Dicentrics & Rings	Deletions	Total	Exchanges	Deletions	Total
Alu I	0	200	0.005 ± 0.005	0.005 ± 0.005	0.010 ± 0.007	0.0	0.0	0.0
	10	200	0.065 ± 0.018	0.175 ± 0.029	0.240 ± 0.034	0.040 ± 0.014	0.005 ± 0.005	0.045 ± 0.015
	20	200	0.125 ± 0.025	0.225 ± 0.033	0.350 ± 0.042	0.040 ± 0.014	0.070 ± 0.018	0.110 ± 0.023
	30	200	0.180 ± 0.030	0.225 ± 0.033	0.405 ± 0.045	0.040 ± 0.014	0.035 ± 0.013	0.075 ± 0.019
	40	200	0.635 ± 0.056	0.655 ± 0.057	1.290 ± 0.080	0.175 ± 0.029	0.035 ± 0.013	0.210 ± 0.032
Rsa I	0	100	0.0	0.010 ± 0.010	0.010 ± 0.010	0.0	0.040 ± 0.020	0.040 ± 0.020
	10	100	0.160 ± 0.040	0.030 ± 0.017	0.190 ± 0.043	0.070 ± 0.026	0.0	0.070 ± 0.026
	20	100	0.220 ± 0.047	0.200 ± 0.045	0.420 ± 0.064	0.020 ± 0.014	0.010 ± 0.010	0.030 ± 0.017
	30	100	0.280 ± 0.053	0.340 ± 0.058	0.620 ± 0.078	0.020 ± 0.014	0.0	0.020 ± 0.014
	40	100	0.310 ± 0.056	0.360 ± 0.060	0.670 ± 0.081	0.160 ± 0.040	0.060 ± 0.024	0.220 ± 0.046

Figure 1.1 Aberration induction via osmolytic shock. a) Alu I ○ exchanges, ● deletions. b) Rsa I ▽ exchanges, ▼ deletions.

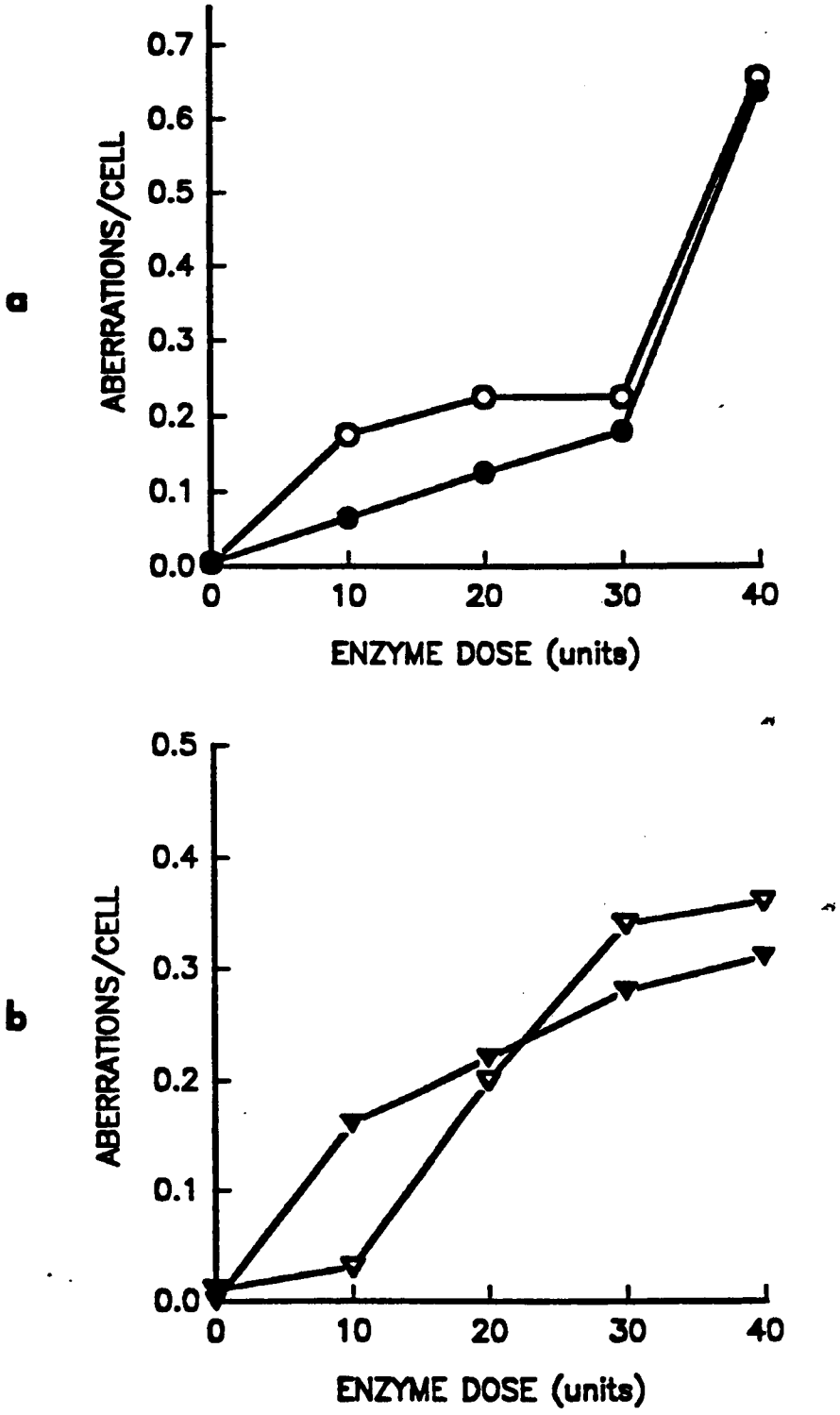

a
ABERRATIONS/CELL
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
ENZYME DOSE (units)
b
ABERRATIONS/CELL
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
ENZYME DOSE (units)

Figure 1.1

one of an overdispersion of aberrations in the cell population. That is, while some cells exhibit a high number of chromosome aberrations, a great many others show few or no aberrations at all. The effect of overdispersion can also be observed in the resulting cell survival following osmolytic shock treatment of attached cultures. In both the enzyme-treated and the non-treated controls, cell survival was greater than 95% (data not shown). If distribution of enzyme dose is overdispersed, then the small population of cells receiving a high dose of enzyme would be killed, while the much larger population of cells receiving little or no enzyme would survive, resulting in a net high survival following treatment. It follows then, that the ideal situation would be one in which all the cells receive an equivalent dose of enzyme and survival in the population would be reduced accordingly. The even distribution of enzyme throughout the cell population is of particular importance in the induction of mutations by restriction enzymes. For these reasons osmolytic shock of attached cultures was abandoned.

In an attempt to avoid the problem of overdispersion, the osmolytic shock technique was modified slightly to treat cells in suspension. Presumably, by treating cells in suspension, more cell surface area would be exposed to the restriction enzyme-containing osmolytic buffer. Cells treated in this manner also exhibited aberrations, but they were still somewhat overdispersed with only 36% of the cells showing chromosomal aberrations. Survival of unattached cells treated with 100 units of the restriction enzyme Sau3A I was decreased to 72% while survivals for untreated control and storage buffer controls were 95% and 70% respectively. It appears that

treating cells in suspension reduces survival of the population somewhat, but it is not clear from these data whether this decrease in survival is due to a more efficient distribution of the enzyme among the cell population, or simply that the amount of treatment buffer entering the cells was increased by this modification. In addition, it has been demonstrated that hypotonic medium can induce chromosome aberrations and reduce cell survival (Nowak, 1987). For these reasons, treatment of cells via osmolytic shock was abandoned altogether.

ELECTROPORATION

An alternative method of introducing restriction enzymes into cells involves the use of electroporation. Electroporation had been used successfully to introduce DNA and other macromolecules into cells and early experiments with this technique with restriction enzymes showed that chromosome aberrations could also be induced. It became clear that several parameters of the treatment had to be taken into account in order to maximize the efficiency of permeabilization. Two parameters of primary importance are the concentration of cells and the resulting time constant (TC) for electroporation. Optimal results were always observed when the time constant (the time required for a particular voltage to be delivered across the cuvette) was around 23 milliseconds. The time constant may be increased by decreasing the volume of the treatment buffer or by decreasing the concentration of cells. Figure 1.2 demonstrates the effect on G1 aberrations induced by 10 units of Rsa I by varying the concentration of cells in an electroporation experiment. The concentration of cells used in the

Figure 1.2 The effect of variable cell number on efficiency of electroporation. All enzyme treatments are with 10 units of Rsa I. Only G1 aberrations were analyzed.

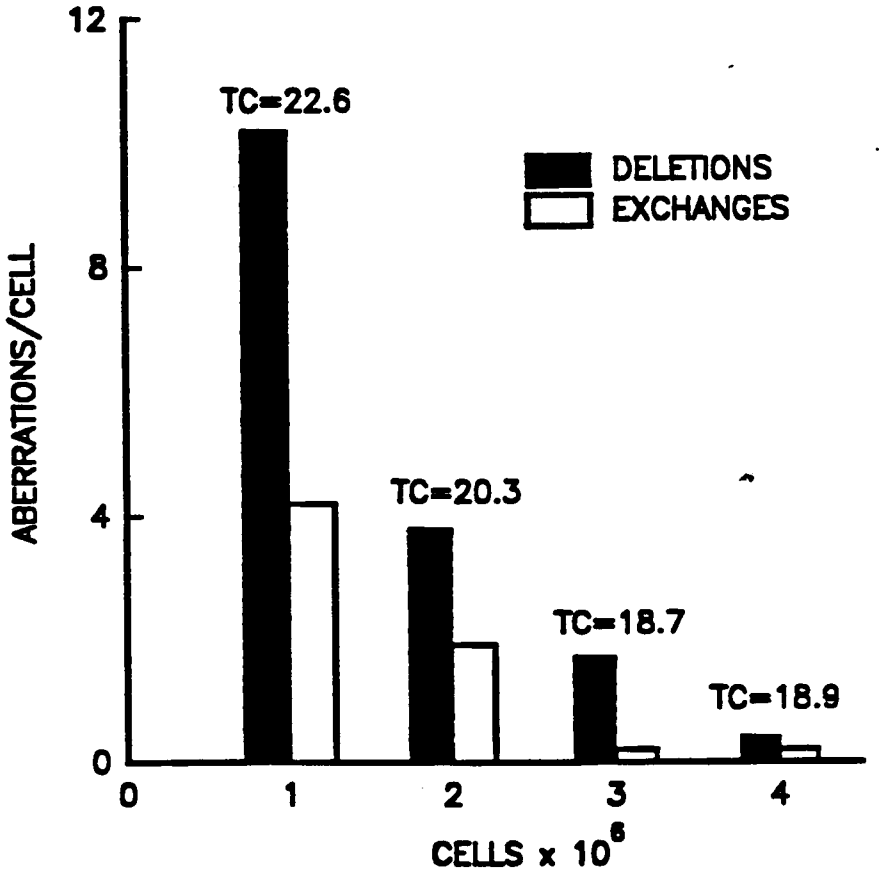

12
8
4
0
ABERRATIONS/CELL
TC=22.6
DELETIONS
EXCHANGES
TC=20.3
TC=18.7
TC=18.9
0
1
2
3
4
CELLS x 10^6

Figure 1.2

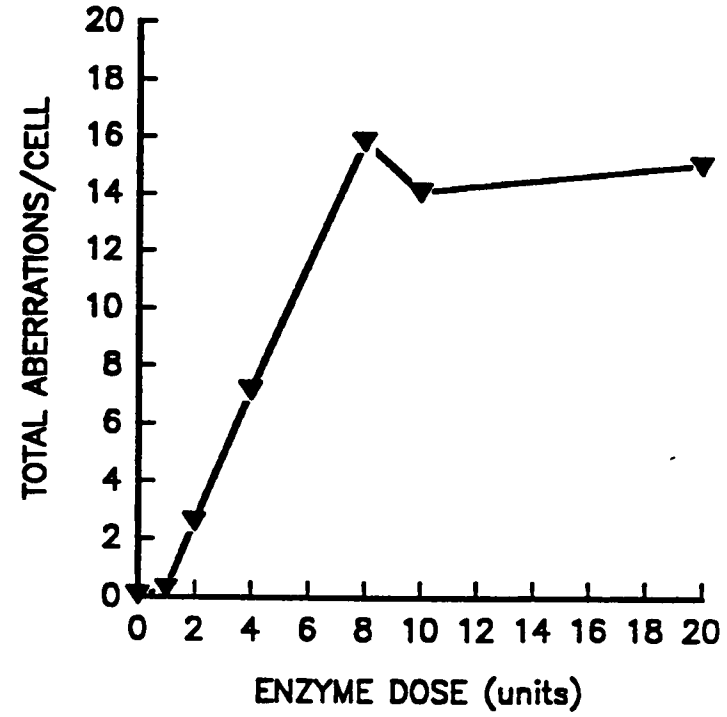
treatment buffer is directly related to the efficiency of aberration induction by restriction enzymes introduced via electroporation. This may be due to an effective "dilution" of the enzyme dose per cell, or due to more efficient permeabilization conditions when cell concentration is reduced. The optimal concentration of cells was found to be $1x10^6$ cells/0.7 ml electroporation buffer.

Once the conditions for electroporation were optimized, aberrations were observed with doses of enzyme much lower than those used in osmolytic shock experiments (Table 1.2 and Figure 1.3). It is of interest to note that the dose spectrum in the electroporation experiment is from 1 to 20 units of Rsa I while the doses used with osmolytic shock span 10 to 40 units of Rsa I. At the highest doses used in electroporation studies, 20 units of Rsa I, there is nearly a forty-fold increase in the level of aberrations compared to the same dose using osmolytic shock. Clearly, electroporation provides a much more efficient means of introducing restriction enzymes into CHO cells. The efficiency of this technique allowed an analysis of restriction enzyme damage utilizing doses 1/10 of those used in osmolytic shock experiments. While the financial implications of this improvement are obvious, the efficiency also indicates that a more accurate dosing of enzyme is achievable with electroporation than with earlier techniques. At doses as low as 1 unit of Rsa I, an increase in G1 aberrations can be observed. This response was also seen with other enzymes and in other stages of the cell cycle (data not shown). Additionally, electroporation allowed the analysis of aberrations induced by rare cutting enzymes that had previously given inconsistent results or had been unsuccessful with the osmolytic shock method. For example, Pvu II a relatively infrequent cutter (1/3386 bp)

TABLE 1.2 The Induction of Chromosome Aberrations in CHO Cells by Rsa I Introduced via Osmolytic Shock or Electroporation

Method of Introduction	Units Rsa I	No. Cells Analyzed	Chromosome-Type Aberrations/Cell ± SEM			Chromatid-Type Aberrations/Cell ± SEM		
			Dicentrics & Rings	Deletions	Total	Exchanges	Deletions	Total
Electroporation	0	100	0.030 ± 0.017	0.030 ± 0.017	0.060 ± 0.025	0.0	0.0	0.0
	1	100	0.170 ± 0.041	0.120 ± 0.034	0.290 ± 0.054	0.050 ± 0.022	0.0	0.050 ± 0.022
	2	100	0.770 ± 0.087	1.820 ± 0.135	2.590 ± 0.161	0.030 ± 0.017	0.0	0.030 ± 0.017
	4	100	2.220 ± 0.149	4.940 ± 0.222	7.160 ± 0.268	0.060 ± 0.028	0.0	0.060 ± 0.028
	8	100	3.730 ± 0.193	12.120 ± 0.348	15.850 ± 0.398	0.070 ± 0.028	0.0	0.070 ± 0.026
	10	100	4.900 ± 0.221	9.180 ± 0.302	14.080 ± 0.370	0.140 ± 0.037	0.0	0.140 ± 0.037
	20	100	4.750 ± 0.218	10.250 ± 0.320	15.000 ± 0.387	0.090 ± 0.030	0.0	0.090 ± 0.030
Osmolytic shock	0	100	0.0	0.010 ± 0.010	0.010 ± 0.010	0.0	0.040 ± 0.020	0.040 ± 0.020
	10	100	0.160 ± 0.040	0.030 ± 0.017	0.190 ± 0.043	0.070 ± 0.026	0.0	0.070 ± 0.026
	20	100	0.220 ± 0.047	0.200 ± 0.045	0.420 ± 0.064	0.020 ± 0.014	0.010 ± 0.010	0.030 ± 0.017
	30	100	0.280 ± 0.053	0.340 ± 0.056	0.620 ± 0.078	0.020 ± 0.014	0.0	0.020 ± 0.014
	40	100	0.310 ± 0.056	0.360 ± 0.060	0.670 ± 0.081	0.160 ± 0.040	0.060 ± 0.024	0.220 ± 0.046

Figure 1.3 A comparison of aberration induction by Rsa I introduced by electroporation and osmolytic shock. a) electoporation, b) osmolytic shock.


TOTAL ABERRATIONS/CELL
20
18
16
14
12
10
8
6
4
2
0
0 2 4 6 8 10 12 14 16 18 20
ENZYME DOSE (units)

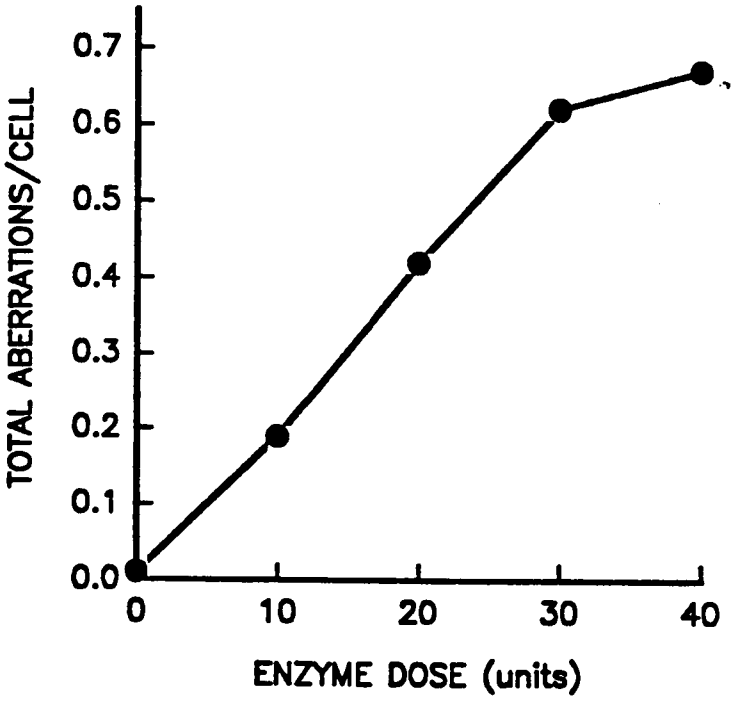

TOTAL ABERRATIONS/CELL
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 10 20 30 40
ENZYME DOSE (units)

Figure 1.3

gave a good dose response curve when introduced via electroporation (Table 1.3 and Figure 1.4).

The distribution of aberrations resulting from restriction enzymes in electroporation experiments followed a Poisson distribution and damage was not overdispersed. This was also observed by other groups using electroporation to introduce restriction enzymes into CHO cells (Winegar and Morgan, 1989). The distribution of damage across the population resulting from the electroporation experiment is graphically represented in Figure 1.3 and shown below:

TREATMENT	% CELLS WITH ABERRATIONS
STORAGE BUFFER CONTROL	6%
1 UNIT Rsa I	20%
2 UNITS Rsa I	44%
4 UNITS Rsa I	74%
8 UNITS Rsa I	86%
10 UNITS Rsa I	97%
20 UNITS Rsa I	91%

At higher doses, nearly every cell received some type of damage and the amount of damage was fairly uniform across that sub-population. Exchange type aberrations follow a Poisson distribution, but deletion aberrations are still overdispersed.

TABLE 1.3 The Induction of Chromosome Aberrations in CHO Cells by Pvu II Introduced via Electroporation

Enzyme	Units	No. Cells Analyzed	Chromosome-Type Aberrations/Cell ± SEM			Chromatid-Type Aberrations/Cell ± SEM		
			Dicentrics & Rings	Deletions	Total	Exchanges	Deletions	Total
Pvu II	0	100	0.0	0.0	0.0	0.020 ± .014	0.060 ± 0.024	0.080 ± 0.028
	10	100	0.520 ± 0.072	0.890 ± 0.094	1.410 ± 0.119	0.0	0.030 ± 0.017	0.030 ± 0.017
	20	100	0.500 ± 0.070	0.870 ± 0.093	1.370 ± 0.117	0.0	0.0	0.0
	40	100	0.870 ± 0.093	1.220 ± 0.110	2.090 ± 0.144	0.0	0.010 ± 0.010	0.010 ± 0.010
	80	100	0.620 ± 0.078	3.820 ± 0.195	4.440 ± 0.211	0.010 ± 0.010	0.010 ± 0.010	0.020 ± 0.014
	100	100	1.460 ± 0.121	2.930 ± 0.171	4.390 ± 0.209	0.020 ± 0.014	0.0	0.020 ± 0.014

Figure 1.4 Aberration induction with Pvu II. ○ deletions, ● exchanges.

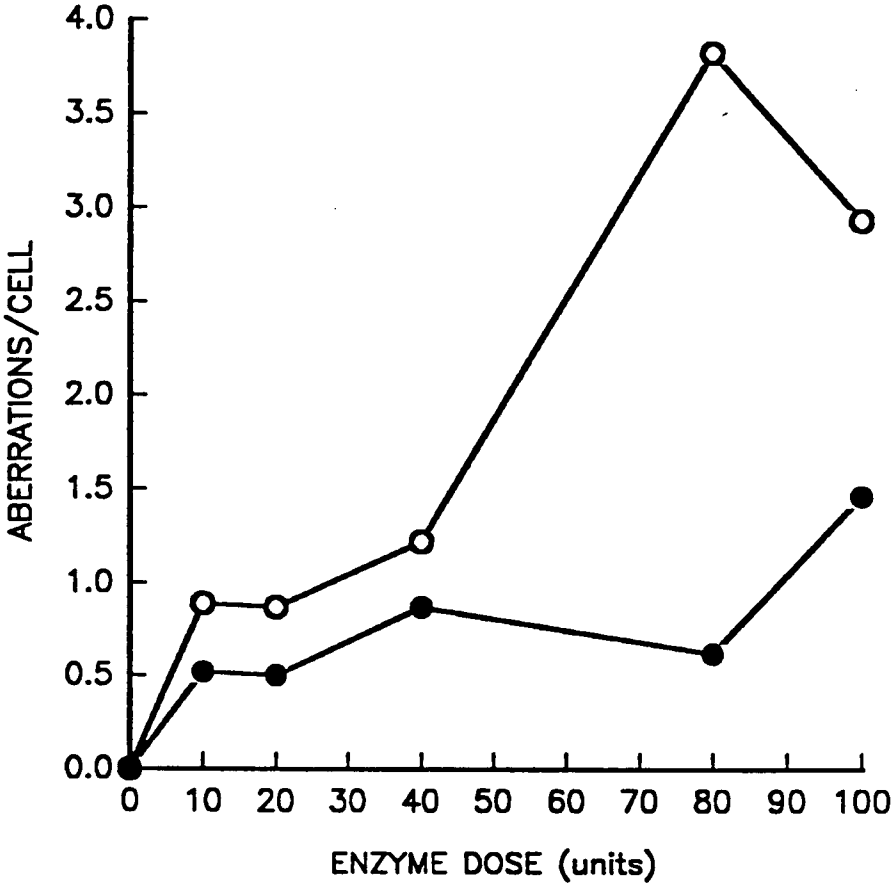

4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ABERRATIONS/CELL
0
10
20
30
40
50
60
70
80
90
100
ENZYME DOSE (units)

Figure 1.4

Overdispersion of deletion aberrations was observed in both osmolytic shock and in electroporation experiments by other investigators (Winegar et al.,1988,1989) using other blunt-end, frequently cutting enzymes. The unusually high number of deletion aberrations with blunt-end cutting enzymes may indicate a difference in the stability of the lesion from that produced by cohesive-end cutters.

The lack of over dispersion of damage was also evident in the survival curves generated with electroporation. Following treatment with the réstriction enzymes Rsa I and Sau3A I, survival is reduced to 27% with doses as low as one unit of enzyme (Figure 1.5). As was expected, once permeabilization techniques could be optimized to deliver an even dose to a large section of the cell population, survival was decreased accordingly. Large doses of enzyme, as might be expected from the amount of aberration damage generated, completely killed the cells, and no survival data is available from these experiments. It might be expected then, that the dose that would be most efficient at inducing mutations would be one at which survival and aberration production were balanced against each other.

Figure 1.5 Cell survival following electroporation with Sau3A I or Rsa I. ○ Sau3A I, ● Rsa I.

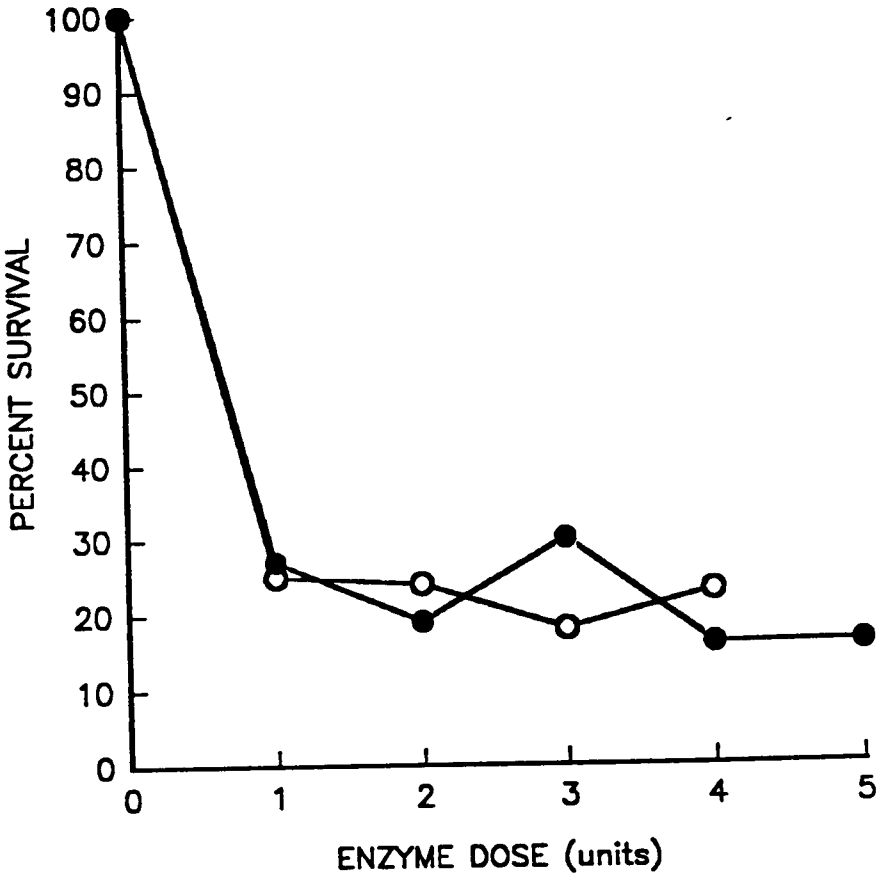

100
90
80
70
60
50
40
30
20
10
0
PERCENT SURVIVAL
0
1
2
3
4
5
ENZYME DOSE (units)

Figure 1.5

DISCUSSION

The results presented show that electroporation is the simplest, most efficient and consistent method used to date to introduce restriction enzymes into CHO cells. The increase in aberrations remained proportional to the administered dose, but showed much higher levels of aberrations than did experiments using osmolytic shock. Transfer across membranes was so efficient in fact that significant increases in aberration induction could be detected at doses as low as 1-2 units of the enzyme Rsa I. In addition to the high efficiency of permeabilization to individual cells, the cell population as a whole was more efficiently permeabilized with greater than 90% of the population demonstrating chromosome aberrations at higher doses of the enzyme. This level of efficiency has been reported by other groups using electroporation to introduce restriction enzymes into living cells (Winegar,et al., 1989; Giaccia et al., 1990).

While some enzymes which met with limited success after osmolytic shock yielded aberration data with electroporation (such as Pvu II), the production of aberrations with some enzymes remained unsuccessful. The inability of an enzyme to produce chromosome aberrations can be due to several factors. The first parameter to be taken into account must be the frequency of restriction sites for that particular enzyme. Enzymes whose restriction sequence occurs very rarely (such as Not I which occurs once every 9×10^6 bp) never induced aberrations in this study [although Winegar (PhD. thesis, 1987) was able to demonstrate some aberration

induction using Not I and osmolytic shock], even at doses as high as 200 units. The actual number of sites available for cutting by a restriction enzyme is probably lower than predicted by the statistical model of Bishop. Mammalian DNA is organized into chromatin and is complexed with various histone and other organizational and regulatory proteins that can effectively mask a restriction site from digestion. In addition, mammalian cells methylate their DNA so that enzymes whose restriction pattern is rich in CpG sequences (again, like Not I: GCGGCCGC), will have fewer sites available for digestion (Winegar et al., 1990).

Other factors contributing to the inability of an enzyme to produce chromosomal aberrations *in vitro* are the salt concentration and temperature requirements for enzyme activity. Taq I for example, isolated from the bacterium *Thermophillus aquaticus* found growing in hot springs and geysers, has an optimal temperature requirement of 65°C - a temperature that would certainly kill CHO cells. The enzyme Sma I on the other hand has a half-life of only 15 minutes at 37°C. Neither of these enzymes would be expected to yield significant levels of chromosome aberrations in these studies. Finally, the age of the enzyme used and variability between enzyme stocks can also contribute to inability or variability of particular enzymes to induce chromosome aberrations.

One point of interest is the marked difference in the abilities of blunt-end frequent cutters to produce a high number of deletion aberrations when compared to cohesive-end cutters with similar restriction site frequencies. Both Alu I and Rsa I produce a high number of deletion aberrations in CHO cells. Winegar (1987)

proposed that Alu I was cutting in the Alu family of repetitive elements found in hamsters. Rsa I, however, another four-base blunt-end cutter should not cut in the Alu repetitive elements. A sharp contrast with another four-base cutter with a similar restriction sequence that produces 4-base cohesive termini, Sau3A I, and Rsa I is readily apparent. Sau3A I is also a very efficient inducer of aberrations, but does not yield the same high numbers of deletion aberrations. Although this may possibly be due to differences in the stability of staggered and oppositely opposing breaks in the DNA, a simple comparison may not be drawn. Dosing of restriction enzymes is difficult even with the most precise measurements. Furthermore, the organization of the genome may be such that Rsa I restriction sequences occur in close proximity to one another giving rise to a high number of deletion mutants following digestion. In addition, Rsa I may simply be a better-cutting restriction enzyme in cells than is Sau3A I. The differences in aberration frequencies resulting from digestion with these two enzymes is likely to be a combination of all these factors.

Another advantage of the electroporation technique over those previously used is that it has built into it an indicator of whether a group of cells has been successfully permeabilized. With previous techniques, there was no such indicator and only the final outcome of the experiment indicated the efficiency of permeabilization. The time constant in electroporation experiments is an excellent indicator of the success of permeabilization. A time constant of 23 milliseconds or greater indicates the optimal conditions for electroporation and consistently gave clear dose responses. A time constant of 23 milliseconds may represent the minimum time for a pore to be held

open to allow restriction enzymes to leak into the cell. Enzyme doses that required high volumes of enzymes in storage buffer to be added to the electroporation buffer had decreased time constants, sometimes as low as 14 milliseconds. This could be yet another factor affecting the inability of a particular enzyme to induce chromosome aberrations. However, super-concentrated enzyme stocks are available from the manufacturer, and using these for higher enzyme doses usually yielded optimal time constants.

With respect to optimizing conditions of permeabilization for restriction enzyme-induced mutagenesis, electroporation seems to have met most of the requirements. Previous attempts to analyze the mutational spectra induced by restriction enzymes met with very limited success. Obe et al. (1986) were able to observe a slight increase in mutants at the hprt locus with Alu I. Although aberrations were induced, the number of aberrations was low and survival was not greatly decreased, indicating that damage may have been overdispersed (a definite possibility since trypsin-pelleting was the method used to introduce restriction enzymes into cells). It is of utmost importance when performing mutation induction studies to treat cells with a chronic rather than an acute dose of restriction enzyme. Overdispersion of restriction enzyme damage, as evidenced by the high survival of cells following treatment with osmolytic shock, results in a very small percentage of the surviving cell population receiving a dose of enzyme. The probability of producing a mutation in a target gene in the cells comprising this small population is relatively low. Similarly, if a large percentage of the treated cell population receives a dose of enzyme, but that

dose is so high that damage to other loci causes lethality to a large portion of the treated cells, then the chances of successfully selecting mutations in the target gene are again low. It is essential that a moderate dose be delivered to a large portion of the treated cell population in order for the investigator to effectively isolate mutations in a target gene.

Having established these conditions, we felt confident that the permeabilization technique was optimized for treatment of CHO cells with a variety of enzymes and could be used to induce mutations in a target gene.

CHAPTER 2

ISOLATION AND PRELIMINARY CHARACTERIZATION OF RESTRICTION ENZYME-INDUCED 6-THIOGUANINE-RESISTANT CLONES.

SUMMARY

With the use of a restriction map of a particular gene, it is possible to develop a strategy whereby a particular region within this gene can be targeted for mutation induction by restriction enzymes. Ideally, the target gene should be a non-essential gene, that may be easily distinguished from the parental phenotype. The Chinese hamster hprt (hypoxanthine-guanine phosphoribosyl transferase) gene fulfills this criterion. The CHO HPRT mutation assay is well-characterized and is used routinely to screen for potential mutagens. The inability of HPRT-deficient cells to metabolize the base analog 6-thioguanine to a toxic metabolite allows the mutants to grow in medium containing this agent. Parental cells with functional HPRT enzyme are capable of metabolizing 6-thioguanine and consequently die.

The cDNA sequence of the Chinese hamster hprt gene has been available for nearly a decade (Konecki et al., 1982) and was used for the selection of restriction enzymes to be used to produce mutations in this gene. Predictions were made, based on restriction site frequency in both exons and introns, of what types of mutations should be produced by different restriction enzymes. A total of over 200 mutant clones have been isolated for all enzymes used, the highest numbers being for Sau3A I and RsaI.

Characterization of the mutants in HAT medium indicated that HPRT enzyme activity levels were low or possibly non-existent. None of the 6-thioguanine-resistant clones tested were able to grow in HAT medium, which selects against HPRT deficient cells. The inability of the 6-thioguanine-resistant clones to incorporate ^{14}C-hypoxanthine into their DNA further indicated that HPRT enzyme activity levels are either very low or totally absent in these clones.

INTRODUCTION

The unique property of site specificity of damage induced by restriction enzymes makes them an excellent tool for studying the consequences of double-strand break damage to DNA. As discussed in Chapter 1, restriction site frequency and the accessibility of these sites for digestion affects the frequency and type of

chromosome aberrations produced by any particular enzyme. Restriction enzymes can also potentially be used to generate damage at targeted regions in the genome. Since restriction enzymes can only digest DNA at their restriction sequence, damage is limited to these targeted regions. Digestion of chromatin with restriction enzymes *in vitro* could be used to probe for recombination "hot spots" or to produce site-specific damage to a target gene. *In situ* targeted mutagenesis is a particularly interesting application for restriction enzymes. To date very little analysis has been done on restriction enzyme -induced damage beyond chromosome aberration analysis. Only one study has been performed with respect to mutation induction by restriction enzymes (Obe, 1986) and conditions for permeabilization were less than optimal. The site specificity of restriction enzyme-induced damage makes it an interesting choice of "mutagen". Most mutagenic compounds produce damage randomly throughout the genome, but since restriction enzymes produce damage only at their restriction sequence, damage is limited to specific target. If the right enzyme or combination of enzymes were to be used, damage could conceivably be induced at virtually any target sequence in the genome. Oligonucleotide directed site specific mutagenesis is used in a number of laboratories to manipulate single codons and substitute the encoded amino acid residue. This approach examines the importance of single key amino acids in the protein. Restriction enzyme mediated site specific mutagenesis on the other hand could be used to create a series of well-defined deletions in a target gene. Such a series of deletions allows for the examination of important regions in a gene as far as function is concerned. Deletions

involving important parts of exons, entire exons, or the entire gene of interest should be produced if the appropriate restriction enzyme is selected. The specificity of the damage should also reduce damage to flanking gene sequences, another property unique to restriction enzyme mutagenesis

In addition to deletions, restriction enzymes are likely to produce a wide spectrum of rearrangements in the target gene including translocations, inversions, frameshifts and possibly point mutations. All of these lesions are likely to result in mutations in the target gene. From preliminary aberration studies, we hypothesized that the most likely type of lesion to be produced by restriction enzymes would be deletions of all or part of the target gene, depending on the restriction site frequency of the enzyme used. Aberration data for nearly every enzyme tested demonstrated a significantly higher frequency of deletion-type aberrations than exchange type. Enzymes which cut very infrequently would be expected to produce mainly total gene deletions while those enzymes which cut more frequently, but not in the coding region of the gene, would be expected to produce both partial (one or more exons) and total gene deletions. In addition, enzymes with sites in the coding region of the gene would be expected to produce small deletions within an exon, partial gene deletions and total gene deletions.

There are several requirements that must be considered in order for restriction enzyme mutagenesis to be considered feasible. The first, efficient permeabilization of cells, was discussed at length in Chapter 1. Additionally, if enzyme selection is to follow any kind of strategy, the sequence of the target gene must be known.

Generally the sequence of a gene is available in the form of cDNA. The cDNA sequence information is very helpful in targeting regions of interest in the coding regions (exons) of the gene, but since the intron sequences are usually not available, predicting deletion patterns for them is generally not feasible. However, the consequences of partial intron deletion could be expected to generally have little influence on enzyme activity, unless they affect mRNA splicing. It has been demonstrated that some intron sequences can affect gene expression (Beenken et al., 1991). Intron sequences for some genes can be quite large and may contribute significantly to the overall size of the gene. It is entirely likely that for genes which possess large intervening introns there will be substantial restriction enzyme damage in these introns. Knowledge of the intron sequences of the target gene would allow for a more detailed estimate of the types of deletions that could be produced by a series of restriction enzyme treatments. However, because of this lack of known intron sequences, estimates based on the size of the introns and restriction site frequency must be used to make a best estimate of intron damage.

Another requirement for restriction enzyme mutagenesis concerns the selection of mutations once they are produced. Mutations in a target gene may be detected in a number of ways, but some systems are far superior to others. A target gene which is essential for the survival of the cell is not a good candidate target gene since mutations will almost certainly result in lethality. For obvious reasons, recovery of these mutants for further analysis is, at best, difficult. Mutations in a target gene that are detectable only as a morphological change are again difficult to isolate for further

analysis. The ideal gene for targeted mutagenesis is a non-essential gene. Selection of mutants relies on a biochemical alteration in the protein product of the target gene. Several target genes fulfil this requirement, for example, APRT, HPRT, TK. The gene we chose for our studies was the Chinese hamster hprt gene.

The Chinese hamster hprt (hypoxanthine-guanine phosphoribosyl transferase) gene is a well characterized gene that has been used routinely as a target in mutagen screening assays (Hsie et al., 1979; O'Neill et al., 1979a). Because it is located on the X-chromosome, hprt is hemizygous in Chinese hamster ovary (CHO) cells. This means that in order to detect a mutant phenotype, it is only necessary to alter the single gene copy. Selection for loss of HPRT activity is simple. HPRT catalyzes the conversion of hypoxanthine to IMP and guanine to GMP in the salvage pathway of purines. In the presence of functional HPRT enzyme, the base analog 6-thioguanine is metabolized to a toxic intermediate. Build up of this metabolite eventually kills the cells and effectively selects against cells with functioning HPRT enzyme. However, if HPRT enzyme is inactivated, of reduced activity or absent due to a deletion or alteration in the gene, then 6-thioguanine is not metabolized and the cell does not die. Therefore, by growing cells in 6-thioguanine-containing medium following treatment, mutations in the target gene resulting in altered HPRT enzyme activity may easily be isolated as 6-thioguanine resistant colonies.

The Chinese hamster hprt gene is divided into 9 exons each separated by a rather large intron with the exception of exons 7&8 which are separated by less than 200 base pairs. While the size of the gene with introns spans a region greater than

30 kb, the cloned cDNA is only 1352bp in length (Konecki et al., 1982). Figure 2.1 represents a cartoon of the hamster hprt gene but is not drawn to scale. With the use of a restriction map, a strategy was selected for digestion of the hamster hprt gene *in situ*. The enzyme Sau3A I, a frequent-cutting enzyme that has consistently produced reliable aberration data was one of the first enzymes selected. Sau3A I possesses two restriction sites in exon 4 and one restriction site in exon 3. Since the restriction site frequency of Sau3A I is high (1/343 bp) and the size of the intervening introns is large (in most cases 1 kb or greater), it is likely that Sau3A I restriction sites are also in the introns. Digestion with Sau3A I then, could produce a variety of deletions in the hprt gene: deletion of the 3' end of exon 3; deletion of most of exon 4; deletion of the 3' end of exon 3 to the 3'end of exon 4; deletion of any one exon or group of exons; and finally total gene deletion. Digestion with Rsa I would be expected also to produce a range of deletions but involving exons 6, 7 & 8, as well as partial and total gene deletions due to its high frequency of restriction sites (1/227 bp). Enzymes like EcoR I or Pvu II which do not possess restriction sites in the coding region of the gene, and have an intermediate frequency of restriction sites, would be expected to cut in intron sequences as well as flanking DNA. The results of digestion with these enzymes would be the loss of single or groups of exons as well as total gene deletions. Finally, digestion with enzymes with rarely occurring restriction sites, such as Sfi I and Not I which cut approximately every $1/2.96 \times 10^5$bp and $1/9.41 \times 10^6$ bp respectively, would be expected to produce only total gene deletions, since the likelihood of a Not I or Sfi I site occurring anywhere in the 30 kb region of hprt is very small.

Figure 2.1 The Chinese hamster hprt gene. Boxes represent the nine exons. Restriction sites for Sau3A I, Rsa I and Hae III are shown.

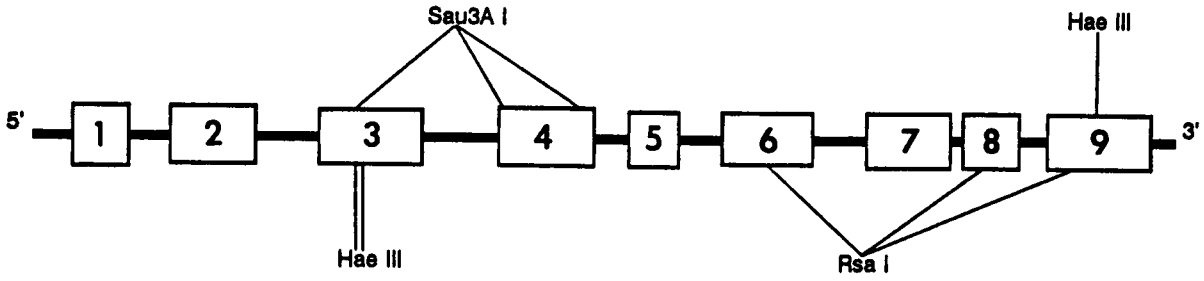


Figure 2.1

Having selected a target gene and a restriction enzyme digest strategy, electroporation experiments were performed to attempt to produce mutations in the hamster hprt gene.

MATERIALS AND METHODS

CELL CULTURE

Chinese Hamster Ovary cells (CHO-K1BH4) were maintained in 75cm^2 tissue culture flasks or 100 mm^2 tissue culture plates (Falcon) in Hams F12 medium (Gibco) supplemented with 5% heat-inactivated (56°C, 30 min.) fetal bovine serum (Hyclone), 50 μg/ml penicillin (Squibb) and 50 μg/ml streptomycin (Lilly) in a 37°C incubator humidified 95-100% in an atmosphere of 5% CO_2 in air.

ELECTROPORATION

Exponentially growing cells from a 75 cm^2 tissue culture flask were removed by trypsinization and washed once with F12 medium. A cell count was determined using a Coulter counter. An aliquot containing the total number of cells to be used in an experiment (1 x 10^6 cells/treatment) was transferred to a sterile centrifuge tube, pelleted and washed twice with ice-cold phosphate buffered sucrose (272mM sucrose, 7mM KH_2PO_4 pH 7.4, 1mM $MgCl_2$). Cells were pelleted and the then

resuspended in a volume equivalent to 0.7 ml/treatment. The desired amount of restriction enzyme was placed at the bottom of a disposable 1 ml electroporation cuvette (Bio-Rad), and 0.7 ml of the cell suspension was added (adding enzyme first ensures even distribution of enzyme in the reaction volume). Cells were electroporated using a Bio-Rad Gene Pulser electroporator at 300 volts and a capacitance of 250 μFd.

ABERRATION ANALYSIS

ELECTROPORATION

The normal fixing protocol was slightly modified for cells treated with restriction enzymes via electroporation. The cell cycle in electroporated cells is delayed by approximately 6 hours and cells are much more fragile. 1×10^6 cells were plated in 10 ml F12 medium on 100mm tissue culture plates and maintained at 37°C for 22 hours at which time colchicine was added to a final concentration of 1×10^{-7}M. Cells were harvested 2 hours later at 24 hours post-treatment by scraping them from plates with a rubber policeman. Cells were pelleted briefly by centrifugation (1 minute) and resuspended in hypotonic potassium chloride (0.075M KCl) for 3 minutes. Cells were pelleted by centrifugation for 45 seconds and resuspended in methanol for 3 minutes. Cells were again pelleted by centrifugation for 45 seconds and then resuspended in a second fixative (3 parts methanol to 1 part glacial acetic acid). Cells were carefully dropped onto pre-wetted glass slides from a height no greater than 2-3 cm. Slides

were air dried, stained and scored according to the previously described protocol (Chapter 1, Materials and Methods).

CELL SURVIVAL

Immediately following treatment with restriction enzymes, 200 cells were plated in triplicate on 60mm tissue culture plates (Falcon) and incubated 7 days at 37°C in 5 ml F12 medium without subculturing. After 7 days, the medium was removed and cells were fixed with 70% ethanol for 2 minutes and then stained with Giemsa (Carolina Biological Supply), 4% by volume in water. Colonies >50 cells were counted and recorded as the average from three plates. Percent survival was determined by dividing the average number of colonies observed per plate by the number of cells dispensed per plate.

HPRT ASSAY

PHENOTYPIC EXPRESSION

Immediately following treatment with restriction enzymes, 1 x 10^6 cells were plated in 10 ml F12 medium on 100mm tissue culture plates. Cultures were maintained at 37°C and were subcultured at a density of 1 x 10^6 cells/plate on days 3,5 and 7 to allow expression of the HPRT$^-$ phenotype (O'Neill and Hsie, 1977, 1979).

MUTANT SELECTION

On day 9, cells were trypsinized and the cell number determined. Cells were plated at a density of 2 x 10^5 cells/100mm tissue culture plate in 10 ml of hypoxanthine-free F12 medium (KC Biological) containing 10 μM 6-thioguanine (6-TG;

Sigma), supplemented with 5% FBS, or 200 cells/60mm tissue culture plate (in triplicate) in hypoxanthine-free F12 medium supplemented with 5% FBS, but in the absence of the selective agent 6-TG. The cultures were maintained at 37°C for 7 days without subculturing.

PLATING EFFICIENCY

After the 7-day incubation period, the 60 mm plates were fixed and stained as described earlier. Plating efficiency was calculated as the average number of colonies/plate divided by the number of cells dispensed per plate. The mutant frequency was calculated by dividing the total number of 6-TG-resistant colonies by the number of cells plated, and correcting for plating efficiency and survival.

MUTANT ISOLATION

After the 7-day incubation, 6-TG-resistant colonies were located, counted, and marked for isolation. Individual colonies were removed by trypsinization in sterile glass cloning rings. 6-TG-resistant clones were maintained in hypoxanthine-free F12 medium supplemented with 10 μM 6-TG and 5% FBS.

CHARACTERIZATION OF 6-TG-RESISTANT CLONES IN SELECTIVE MEDIA

Isolated 6-TG-resistant clones were plated in triplicate on 60mm tissue culture plates at a density of 200 cells/plate in 5 ml F12 medium, hypoxanthine-free F12 medium with 10 μM 6-TG, and HAT medium [F12 medium containing 1 x 10^{-4}M hypoxanthine (Sigma), 1 x 10^{-5}M aminopterin (Sigma), and 4 x 10^{-5}M thymidine (Cal Biochem)]. All media were supplemented with 5% FBS. Cells were maintained at 37°C for 7 days without subculturing. Colonies were fixed and stained as described

in Chapter 1 Materials and Methods. Percent survival was calculated as the average number of colonies observed per plate divided by the number of cells dispensed per plate, relative to growth in F12 medium.

UPTAKE OF ^{14}C-HYPOXANTHINE

HPRT$^-$ clones or K1BH4 positive control cells were plated in triplicate for each of the three time points [one, three and five hours (total of nine plates per clone)] onto 60 mm tissue culture plates and grown to confluence. F-12 medium was removed by aspiration and was replaced with 5 ml pre-warmed F-12 medium containing ^{14}C-hypoxanthine (0.5 μCi/ ml, ICN Radiochemicals) and incubated at 37°C for one, three, or five hours. Following incubation, ^{14}C-hypoxanthine-containing F-12 was removed by aspiration and the cells were washed 4 times with 2 ml phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4, pH~7.3). One ml of 0.2% sodium dodecyl sulfate (SDS, Sigma) was added to each plate and allowed to stand at room temperature for 15 minutes at which time the cells were pipetted up and down several times in order to lyse the cells. 100 μl of the lysate was spotted onto a Wattman 3MM filter paper disc. Filters were dropped into a beaker of ice-cold 10% trichloroacetic acid (TCA; 10 ml TCA per filter) and were stirred occasionally over a twenty-minute period. TCA precipitation was repeated three times. Following TCA precipitation, the filters were dropped into a beaker of ice-cold 95% ethanol and stirred occasionally for 20 minutes. Ethanol precipitation was also repeated three times. Filters were then placed on a 37°C slide warmer and allowed

to air dry completely. Dried filters were placed in individual scintillation vials and 5 ml of scintillation cocktail (Cyto-Scint, ICN Radiochemicals) was added. The amount of ^{14}C incorporated into nucleic acid was determined by normalizing the counts per minute (cpm as determined with a scintillation counter) to mg of protein(as determined by fluorescent spectrophotometry) present in the sample.

RESULTS

ABERRATION INDUCTION

Following restriction enzyme treatment by electroporation, one of the easiest and most reliable ways to determine the success of permeabilization was determined to be the analysis of chromosome aberrations. Generally aberrations from cells treated in G1 were scored due to the relative ease of recognizing and quantitating such chromosome aberrations. Table 2.1 presents the aberration data following electroporation with the enzymes Rsa I and Sau3A I. These data are represented graphically in Figure 2.2. Both enzymes have a high frequency of restriction sites in the hamster genome and produce chromosome aberrations in a manner directly proportional to the dose. The dose response curves generated by these two enzymes indicate that enzyme was successfully introduced into the nucleus. Aberrations were not overdispersed, with the exception of deletions at the higher

TABLE 2.1 The Induction of Chromosome Aberrations in CHO Cells by Rsa I and Sau3A I Introduced via Electroporation

Enzyme	Units	No. Cells Analyzed	Chromosome-Type Aberrations/Cell ± SEM			Chromatid-Type Aberrations/Cell ± SEM		
			Dicentrics & Rings	Deletions	Total	Exchanges	Deletions	Total
Sau3A I	0	100	0.030 ± 0.017	0.010 ± 0.010	0.040 ± 0.020	0.0	0.0	0.0
	10	100	0.230 ± 0.048	0.440 ± 0.066	0.670 ± 0.081	0.0	0.0	0.0
	20	100	0.290 ± 0.054	0.440 ± 0.066	0.730 ± 0.056	0.0	0.0	0.0
	40	100	0.530 ± 0.073	0.830 ± 0.091	1.360 ± 0.116	0.020 ± 0.014	0.010 ± 0.010	0.030 ± 0.017
Rsa I	10	100	0.190 ± 0.043	0.200 ± 0.044	0.390 ± 0.062	0.0	0.0	0.0
	20	100	0.920 ± 0.096	1.980 ± 0.141	2.900 ± 0.170	0.030 ± 0.017	0.010 ± 0.010	0.040 ± 0.020
	40	100	3.450 ± 0.186	10.970 ± 0.331	14.420 ± 0.380	0.060 ± 0.024	0.0	0.060 ± 0.024

Figure 2.2 Aberration induction by Rsa I or Sau3A I. ▽ Rsa I, ○ Sau3A I.

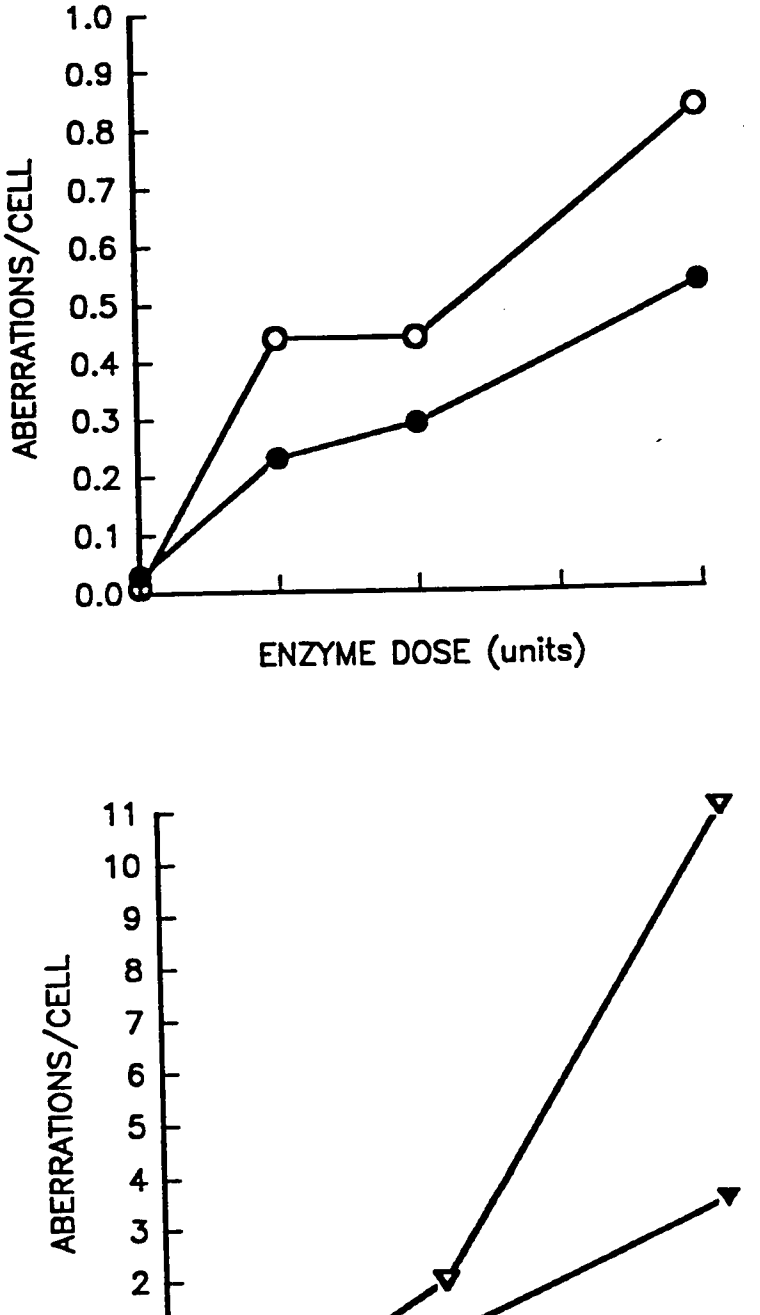

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
ABERRATIONS/CELL
ENZYME DOSE (units)
11
10
9
8
7
6
5
4
3
2
1
0
0
10
20
30
40
ABERRATIONS/CELL
ENZYME DOSE (units)

Figure 2.2

doses of Rsa I. The high efficiency of introduction of enzyme into cells fulfills the initial criteria established in Chapter 1: successful exposure of a large segment of the cell population to restriction enzyme, and the subsequent production of chromosome aberrations.

CELL SURVIVAL

The second criterion that must be met is a significant decrease in cell survival following treatment of cells with restriction enzymes. Figure 2.3 presents the survival curves generated by the same enzyme treatments as those for aberration induction. As might have been expected from the levels of aberrations induced, survival was significantly decreased for both enzymes and was dose-dependent. The survival curve following treatment with Sau3A I appears to be linear. Following an initial steep decline in survival, Rsa I also produces a linear survival curve. The survival of Rsa I treated cells is somewhat lower than Sau3A I treated cells, probably due to the higher frequency of chromosome aberrations produced by this enzyme. Survival and aberration induction following treatment of cells with zero enzyme and zero enzyme plus storage buffer (positive controls) were not significantly different from background.

MUTANT INDUCTION

Since aberration frequency was increased and survival decreased by this treatment regime, cells from this study were plated in hypoxanthine-free F-12 medium containing the base analog 6-thioguanine. In the presence of a functional HPRT

Figure 2.3 Survival following treatment with Rsa I or Sau3A I. ▼ Rsa I, ● Sau3A I.

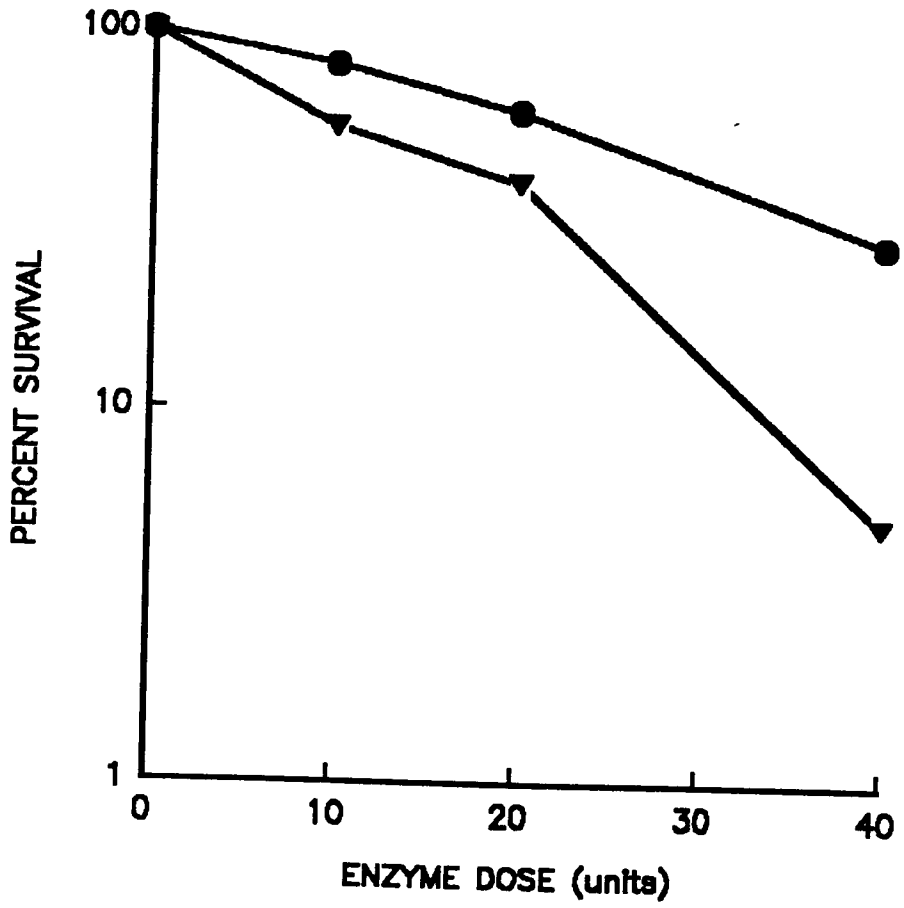


Figure 2.3

enzyme, 6-thioguanine is metabolized to a toxic metabolite resulting in cell killing. The selection medium does not contain hypoxanthine since it is also a substrate for the HPRT enzyme. Thus removing hypoxanthine from the selection medium removes the possibility of competition with 6-thioguanine for HPRT enzyme, and increases the stringency of selection. Cells lacking HPRT activity cannot metabolize 6-thioguanine and, therefore, are capable of growing in the presence of 6-thioguanine. Figure 2.4 presents the mutation induction curves resulting from treatment with Rsa I and Sau3A I. It is clear that both enzymes are capable of significantly increasing the number of 6-thioguanine-resistant cells above background. It is of some interest to note that between 10 and 20 units of Sau3A I, both the aberration induction curve (Figure 2.2) and the mutation induction curve (Figure 2.4) plateau. This plateau is not observed in the survival curve (Figure 2.3) however. Another point of interest is the decline in the number of mutants/10^6 cells at the highest dose of Rsa I while the aberration curve continues to increase and the survival curve continues to decrease at this dose. Presumably,this is due to an effective "saturation" of damage at the hprt locus, i.e. all available restriction sites within the hprt gene have been cut, and only cutting in flanking DNA, or the rest of the genome takes place. This additional damage is sufficiently excessive that "selection against" the survival of hprt damaged cells takes place, resulting in a net decrease of mutants isolated at this dose. A total of 148 mutants were isolated from two treatments with the two enzymes as shown in Table 2.2. Control treatments (zero enzyme and zero enzyme plus storage buffer) did not give rise to any 6-thioguanine resistant colonies.

Figure 2.4 Mutant frequencies following treatment with Rsa I or Sau3A I. ▼ Rsa I, ● Sau3A I.

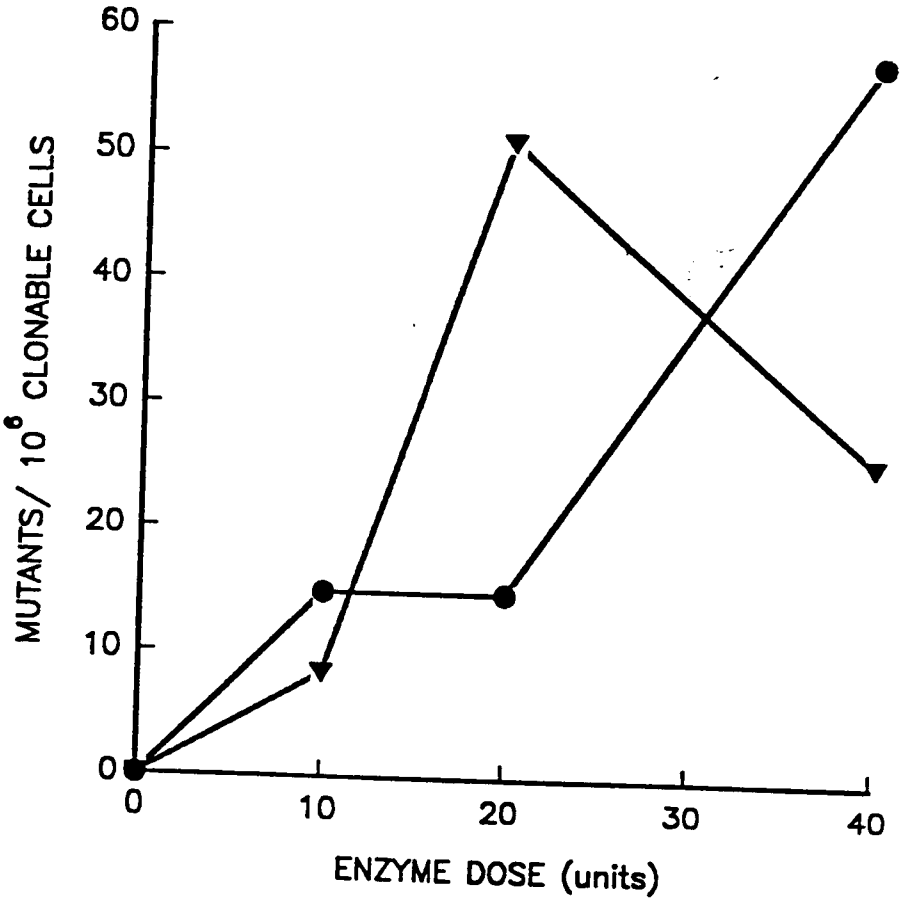

60
50
40
30
20
10
0
MUTANTS/ 10^6 CLONABLE CELLS
0
10
20
30
40
ENZYME DOSE (units)

Figure 2.4

TABLE 2.2 SURVIVAL AND MUTANT FREQUENCIES FOLLOWING TREATMENT WITH THE RESTRICTION ENZYMES Sau3A I AND Rsa I.

ENZYME	DOSE	SURVIVAL	# COLONIES	MUTANT FREQUENCY (mutants/10^6 cells)
CONTROL	0	100%	0	0
Sau3A I	10	82%	21	14.8
	20	62%	23	14.8
	40	28%	49	57.8
Rsa I	10	56%	11	8.4
	20	40%	42	51.2
	40	5%	2	25.7

Other enzymes besides RsaI and Sau3a I were used to attempt to generate mutations at the hprt locus. Table 2.3 lists all the enzymes used in electroporation experiments. The most successful attempts to create mutants at hprt were with frequent cutting enzymes which possess restriction sites in the coding region of hprt. In general, enzymes which are not predicted to cut the hamster genome frequently or which do not possess a restriction site in the hprt coding region, were not efficient inducers of mutations at the hprt locus. An exception to this general observation is Pvu II, which neither cuts frequently nor possesses a restriction site in hprt coding regions. The ability of an enzyme to produce aberrations seems to be of utmost importance in mutation induction studies since those enzymes most efficient at producing chromosome aberrations were also the most effective inducers of mutations at hprt. The inability of some enzymes, like Not I and Sfi I, to produce mutations may stem from the fact that they were not able to cut the DNA. Neither of these enzymes were effective inducers of chromosome aberrations, even when administered doses were very high.

Treatments of cells with Sau3A I via osmolytic shock in preliminary experiments also gave rise to mutant colonies, although the mutant frequency in these experiments was not significantly above background. Nevertheless, since the possibility existed that some of these mutants were induced by damage at a Sau3A I restriction site, these cells were isolated. The total number of 6-thioguanine-resistant clones isolated from all enzyme treatments using both permeabilization techniques is in excess of 200. Since this is a very large number of mutants to analyze at the DNA sequence

TABLE 2.3 RESTRICTION ENZYMES USED TO GENERATE MUTATIONS IN THE CHINESE HAMSTER HPRT GENE

ENZYME	RESTRICTION SEQUENCE	RESTRICTION SITE FREQUENCY		CHROMOSOME ABERRATIONS	6-TG RESISTANT COLONIES
		HAMSTER GENOME	HAMSTER HPRT		
Rsa I	GT⇓AC	1/227 bp	3	YES	YES
Sau3A I	⇓GATC	1/343 bp	3	YES	YES
Hae III	GG⇓CC	1/544 bp	3	YES	YES
Hind III	A⇓AGCTT	1/1927 bp	1	YES	NO
Msp I	CC⇓GG	1/2718 bp	0	YES	NO
EcoR I	G⇓AATTC	1/3248 bp	0	YES	NO
Pvu II	CAG⇓CTG	1/3386 bp	0	YES	NO
Sma I	CCC⇓GGG	1/5.62 x 10^4 bp	0	YES	NO
Sfi I	GGCCNNNN⇓NGGCC	1/2.96 x 10^5 bp	0	NO	NO
Not I	GC⇓GGCCGC	1/9.41 x 10^6 bp	0	NO	NO

level, clones were selected at random from the freezer stock for further analysis and characterization. A bias in the number of clones selected for analysis was imposed in favor of experiments where it was felt that conditions and mutation frequencies were optimal. For this reason, most clones analyzed were induced via electroporation with enzymes that gave mutant frequencies significantly above background.

CHARACTERIZATION IN SELECTIVE MEDIA

After isolation of 6-thioguanine-resistant clones, it was important to determine whether or not the phenotype was "leaky" to the parental phenotype. To select against contamination of clones with parental cells, clones were maintained in selective medium at all times. To determine whether any of the clones were "leaky" to the parental phenotype, clones were plated for survival in selective media (HAT and 6-TG). Growth of a clone in HAT (hypoxanthine, aminopterin, thymidine) medium would signify the presence of a leaky mutation in that clone since HAT medium selects against cells deficient in HPRT enzyme activity. This selection is based on the inhibition of di-hydrofolate reductase by aminopterin and the subsequent blockage of *de novo* synthesis of nucleotides. Cells must rely upon salvage pathways for nucleotide synthesis. A deficiency in a salvage pathway enzyme, like HPRT, hinders the ability of the cell to replicate DNA and eventually the cell dies. Table 2.4 presents the results from the growth of a small fraction of clones isolated in HAT and 6-TG selective media. Survival was always normalized to growth in F-12 which was always considered to be 100%. None of the clones tested in this manner were able to grow

TABLE 2.4 GROWTH OF 6-THIOGUANINE-RESISTANT CLONES IN SELECTIVE MEDIUM

ENZYME	MUTANT ID	F-12	HAT	6-THIOGUANINE
Sau3A I	SI2	100%	0%	87%
	2L1	100%	0%	96%
	SL2	100%	0%	95%
	SO2	100%	0%	95%
Rsa I	RA1	100%	0%	100%
	RA2	100%	0%	105%*
	RB1	100%	0%	102%*
	RC1	100%	0%	98%
Hae III	HA1	100%	0%	107%*
	HB1	100%	0%	106%*
	HC1	100%	0%	95%
POSITIVE CONTROL	K1BH4 (parental)	100%	77%	0%
NEGATIVE CONTROL	RJK88	100%	0%	77%

in HAT medium, but were not inhibited when grown in F-12 or 6-thioguanine media. RJK88, a known complete hprt deletion (Fuscoe et al., 1983) was used as a negative control, and exhibited a similar growth pattern. The parental cell line, CHO-K1BH4, was able to grow in both F-12 and HAT media, but was not able to grow in 6-thioguanine medium. Over 70 mutants were tested in this manner, none of which were able to grow in HAT medium. It was concluded that the clones isolated did not possess any leaky mutations to the wild type phenotype.

HPRT ENZYME ACTIVITY

The inability of mutant clones to grow in HAT medium indicated that HPRT enzyme was either non-functional or not produced in these clones. To test this hypothesis further, the ability of clones to incorporate ^{14}C-hypoxanthine into DNA/RNA was tested. In the presence of even low levels of HPRT enzyme, ^{14}C-hypoxanthine is converted to ^{14}C-IMP and eventually incorporated into nucleic acid. The presence of ^{14}C in TCA-precipitable DNA/RNA indicates the presence of functioning HPRT enzyme. Table 2.5 outlines the results of this experiment. Only the parental cell line CHO-K1BH4, was capable of incorporating ^{14}C into DNA/RNA. While only a small number of clones are listed in the table, a total of 50 were tested, none of which were able to incorporate any ^{14}C into TCA precipitable material. The absence of functional HPRT is probably due to the high stringency of the selective medium. The concentration of 6-thioguanine used (10 μM) is equivalent to ten times the concentration required to completely inhibit the growth of wild type cells (O'Neill and

TABLE 2.5 HPRT ENZYME ACTIVITY ASSAY; INCORPORATION OF ^{14}C-HYPOXANTHINE INTO TCA PRECIPITABLE MATERIAL.

MUTANT ID	INCORPORATION OF ^{14}C-HYPOXANTHINE (nmoles/ mg protein)		
	1 HOUR	3 HOURS	5 HOURS
CHO K1BH4	450.5	1656.7	3347.5
SH3	0.0	0.0	0.0
SM1	0.0	0.0	0.0
S04	0.0	0.0	0.0
SR2	0.0	0.0	0.0
HA1	0.0	0.0	0.0
HB1	0.0	0.0	0.0
HC1	0.0	0.0	0.0
PA1	0.0	0.0	0.0
PB1	0.0	0.0	0.0

Hsie, 1979b). By using such a stringent selection, mutations resulting in partial inactivation of the HPRT enzyme were effectively selected against. This further demonstrates that none of the 6-thioguanine resistant clones tested had any HPRT activity and that they can be thought of as true HPRT deficient cell lines.

DISCUSSION

Upon introduction into CHO cells, restriction enzymes find their way into the nucleus and cut the DNA. The consequences of this damage include a marked increase in chromosome aberrations, a decrease in cell survival among the treated cell population, and following growth in selective medium, an increase in the number of 6-thioguanine-resistant colonies. All of these endpoints are proportional to the administered dose. Clearly, restriction enzymes are capable of inducing mutations at a target gene. This not the first example of mutations arising following restriction enzyme treatment. Obe (1986) demonstrated that following treatment with the restriction enzyme Alu I, there was a small increase in the frequency of mutants at the hprt locus. However when he screened for mutations at the Na^+/K^+ATPase locus, there was no increase above background. Little or no analysis of these mutant clones was performed beyond documenting the increase in mutants above background as another endpoint for indicating restriction enzyme induced effects in

cells. It is not surprising that following treatment with Alu I mutations were induced at the hprt locus since the coding region of the gene possesses 4 Alu I sites, not to mention the number that must be interspersed in the introns. By selecting for ouabain resistance no ouabain-resistant clones were isolated indicating no selectable mutations were induced at the Na^+/K^+ ATPase locus. The cDNA sequence of Na^+/K^+ ATPase α-subunit has been determined for human (Kawakami et al., 1986), pig (Ovchinnikov et al., 1986), sheep (Shull et al., 1985), *Torpedo* (Kawakami et al., 1985), rat (Shull et al., 1988), chicken (Takeyasu et al., 1988), *Drosophila* (Lebovitz et al., 1989) and brine shrimp (Baxter-Lowe et al., 1989). There is little information available in the literature concerning the intron structure of this gene. While the sequence has not been published for the hamster, the sequence is highly conserved from humans to *Drosophila* (Lebovitz et al., 1989) and a reasonable attempt at selecting a restriction enzyme digest strategy could be made. Since Alu I has a very high frequency of restriction sites distributed throughout the hamster genome (1/203 bp), it should at least cut in this gene's introns. Since the Na^+/K^+ ATPase gene is an essential gene, it is likely that the inability to select ouabain-resistant mutants may have been due to a high degree of lethality when Alu I induced changes in the gene. For this reason, the Na^+/K^+ ATPase gene represents a poor choice of target gene. One of the chief criticisms of this experiment is the predicted poor efficiency of introduction of enzymes into the cells. This is due mainly to the method of introduction (trypsinization & pelleting) which, as discussed earlier, has a low efficiency of enzyme introduction. Additionally, the background frequency of mutation at the hprt locus in control cells

was sufficiently high to indicate that a significant proportion of the isolated mutants in the treated population may have been due to spontaneous events.

Perhaps two of the most important factors contributing to the efficiency of an enzyme to induce mutations at a target gene are that enzyme's ability to produce chromosome aberrations and to effectively reduce survival. These two factors were considered carefully when selecting enzymes for mutagenesis. The four-base cutter Sau3A I consistently produces a high number of aberrations, and reduces survival. However, selection of Sau3A I for mutation induction was based on additional criteria Sau3A I possesses three restriction sites at the 5' end of the hprt gene (coding region) and subsequent digestion with this enzyme allows an analysis to be made of damage at the 3' end of the gene. Additionally, two of the sites are on opposite ends of a single exon (exon 4) and digestion with Sau3A I could conceivably target the specific deletion of this exon.

Analysis of the human hprt gene revealed a putative nucleotide binding domain in the first 120 amino acids of the HPRT protein (Argos et al., 1983). This corresponds to the 5' region of the hprt cDNA (in the neighborhood of exons 4 & 5). Strategic selection of restriction enzymes which cut in this region could selectively delete the nucleotide binding domain. HPRT could be very efficiently inactivated following successful digestion of these sites. However, the exon structure of mouse and hamster hprt over this region does not appear to be related to the functional domains proposed for the human protein (Melton et al., 1984). Digestion with Sau3A I would not target this putative active site. Amino acid number 200 has been

implicated as being a key residue in mouse HPRT. Substitution of asparagine for aspartic acid decreases heat stability and kinetic activity of the enzyme (Melton et al., 1984). Since hamster and mouse HPRT enzymes are highly conserved, an enzyme that could induce damage in this region was sought. Fortuitously, there is a Rsa I restriction site 2 bp 5' of the codon for aspartic acid (exon 8). Rsa I also possesses a restriction site in exon 6 and in exon 9. Digestion with Rsa I could produce a series of deletions in the 3' end of the gene.

Both Rsa I and Sau3A I gave mutational spectra. It is interesting to note that Sau3A I gave a higher frequency of 6-thioguanine-resistant mutants than did Rsa I. This may be due in part to the effect of saturation of Rsa I damage as discussed earlier. Since Rsa I produces blunt-end cuts in the DNA, it is more likely to cause deletions at its restriction site and damage outside the target gene is more likely to be lethal. The doses of Rsa administered may have been higher than the optimal dose (25-35 units) for mutation induction. Even so, a fairly large number of 6-thioguanine-resistant mutants were isolated following Rsa I digestion.

Other enzymes used to generate mutations in hprt met with limited success. An attempt to create a small deletion in exon 3 with Hae III resulted in a very low number of 6-thioguanine-resistant colonies being isolated. This may have been due to the general trend of poor aberration inducers being poor mutation inducers. Other enzymes like Msp I, EcoR I and Sma I did not give rise to 6-thioguanine-resistant colonies, nor did they produce very high chromosome aberration frequencies. One reason for the failure of some enzymes to induce recoverable mutants may be simply

that conditions (for example, salt concentration) in the CHO nucleus are not ideal for restriction enzyme-induced DNA cutting. Additionally, enzymes whose restriction sequence is rich in CG sequences may not be able to digest hamster DNA due to the high level of methylation of these sites in the genome. Winegar et al., (1990) demonstrated that methylation of these sites does in fact inhibit digestion. Even after treatment with the de-methylating agent 5-aza-2'-deoxycytidine (AzdC), methylation was not sufficiently reduced to permit digestion with Hpa II (Hpa II cannot digest methylated DNA). Methylation of DNA further reduces the cutting frequency of rare cutting enzymes like Sfi I and Not I since their restriction sequences are rich in CG. This reduction in availability of restriction sites for digestion is the most probable reason for lack of aberration production and subsequent mutation induction by these enzymes.

Although a large number of mutant colonies was isolated following restriction enzyme treatment, the fact that none of them exhibited any HPRT enzyme activity indicates that the stringency of selection may have been too high to allow mutants expressing only reduced levels of HPRT enzyme activity to be isolated. Some of the predicted deletions may not have sufficiently reduced HPRT activity. Even when the concentration of 6-thioguanine was reduced four-fold to 2.5 μM, none of the 6-thioguanine-resistant colonies isolated showed any HPRT activity. By further reducing the stringency of selection, a wider variety in HPRT activity levels may be selected for and this subsequently would also increase the resulting mutant frequency. It must be considered though, that by reducing the stringency of selection, the spontaneous mutant frequency would also increase. This further emphasizes the importance of

removing HPRT mutants from the parental stock prior to mutation induction by growing in HAT medium.

The biochemical characterization of the 6-thioguanine-resistant mutants isolated gives an idea of the levels of hprt enzyme activity present in the isolated clones, but very little can be ascertained about the molecular nature of the mutations involved. The inability of any of the mutants to revert to the parental phenotype indicates that a majority of the mutants could be deletions. Spontaneous mutation rates at the hprt locus have been documented to be 1×10^{-5} (data not shown and Li et al., 1988). Additionally reversion generally would require a second mutation to occur at the site of the initial event, a relatively rare event. Lastly, 6-thioguanine-resistant clones were continuously maintained in selective medium at very high stringency until the time of characterization. A molecular analysis of the nature of the mutations resulting in HPRT inactivation is essential in order to determine the role of restriction enzymes in targeted mutagenesis. Those mutants containing deletions of some or all of the hprt gene must be characterized in order to determine the size and location of the deletion. Point mutations need to be analyzed at the sequence level in order to determine whether damage leading to the mutation could have been produced by the particular restriction enzyme used. In the event of a translocation or inversion, the breakpoints need to be localized with respect to a restriction enzyme site. However, cytogenetic analysis of some of the mutant clones demonstrated no obvious chromosomal rearrangements. Chapter 3 details the molecular approaches taken to analyze some of the mutants created following exposure to restriction enzymes.

CHAPTER 3

MOLECULAR ANALYSIS OF RESTRICTION ENZYME-INDUCED HPRT MUTATIONS

SUMMARY

Multiplex PCR provides an informative method of screening a group of mutant clones for deletions. The technique involves a series of oligonucleotide primers designed to flank each of the individual exons of a gene. Each set of primers generates a discrete amplification product corresponding to that exon. An oligonucleotide cocktail of these primers allows the simultaneous amplification of the exons in a single reaction. PCR amplification products are resolved on an acrylamide gel and the absence of a particular exon band is diagnostic of a deletion of that exon.

Sau3A I-induced hprt mutants showed a series of deletions including some of those expected based on known restriction sites in the coding regions of the gene. A great majority of the deletions appear to have breakpoints at restriction sites in the introns of the gene. This is not surprising since the exons comprise only 1/36 of the 36 kb region containing hamster hprt.

Mutants generated with the enzyme Rsa I, on the other hand, fell into three categories: total gene deletions; deletions of everything but exon 1; and apparent point mutations. The difference in the spectrum of deletions seems to indicate a difference in the repair of blunt-end lesions as opposed to cohesive-end double-strand breaks.

Digestion of exon specific bands from clones exhibiting apparent point mutations was performed in order to asses whether any damage was inflicted at restriction sites in these exons. The enzymes used were the same as those used for mutagenesis, Sau3A I and Rsa I. The inability of these enzymes to digest restriction sites in exon-specific bands would indicate a change at that restriction site. Additionally, exon-specific bands from clones generated with Sau3A I were also digested with the enzyme Cla I. Duplication of a Sau3A I site would generate a new Cla I site. None of these analyses were able to detect changes at restriction sites in hamster hprt exon DNA.

Sequence analysis of exon specific bands was also performed to investigate possible changes at or around restriction sites in particular exons. None of the clones analyzed showed changes in the sequence at or around the restriction site.

While it has been clearly demonstrated that restriction enzymes are mutagenic agents, apparently there are differences in the repair processes used to repair blunt-end double-strand breaks and cohesive-end double-strand breaks occurring in the hamster hprt gene. Our results are somewhat different from the observations reported by Dr. W. Morgan (unpublished results) who showed small changes at the restriction

site for Pvu II- induced mutations at the aprt locus in AS52 cells. Further analysis of restriction enzyme-induced apparent point mutations may demonstrate changes at restriction sites in exon DNA. However, the possibility exists that the selection of the enzyme used for mutagenesis, the target locus selected, and the subsequent repair of different types of double-strand breaks may contribute significantly to the spectrum and types of mutations observed.

INTRODUCTION

The CHO HPRT assay is one of the most widely used systems for the detection of potential mutagens. The ease with which mutant colonies can be selected make it ideal for screening large numbers of potential mutagens. In fact, literally hundreds of compounds have been tested with this assay and speculation of whether or not they are carcinogens has been made in part upon the mutational data generated at the hprt locus.

Although there is a wealth of information on the genotoxicity of many mutagenic agents, it has only been in recent years that a molecular analysis of the types of lesions responsible for the mutational events has been possible. Primarily, the molecular analysis of HPRT mutations in CHO cells became feasible due to the cloning and sequencing of the hamster hprt cDNA (Konecki et al., 1982). The cDNA

sequence of the hamster hprt gene provided the necessary probe for Southern blot analysis of HPRT deficient mutants. With these advances, it was shown that the lesions produced by different clastogenic agents resulting in an inactivation of the hprt gene were very different. Greater than 70% of radiation-induced (X-ray, gamma ray, alpha particle) HPRT deficient mutants arise from large deletions in the gene (Thacker, 1986). X-ray treatment itself results in roughly 50% total gene deletions with a small number of apparent point mutations [no detectable change from wild type on a Southern blot (Xu et al., 1989; Morgan et al., 1990)]. A similar distribution of deletions is observed for γ-ray and α-particle induced 6-thioguanine-resistant mutants. Ultra-violet radiation induced mutations, on the other hand were entirely due to point mutations or very small deletions in the gene (Xu et al., 1989; Vrieling et al., 1989). Alkylating agents, ethyl-methane sulfonate (EMS) and N-ethyl-N-nitrosourea (ENU) also produced only point mutations in hprt (Fuscoe et al., 1983; Thacker & Ganesh, 1989). Mutations arising spontaneously in the hamster hprt gene, as evidenced by Southern blot data, appear mainly to be due to point mutations in the gene (60% point mutations, 10% partial deletions, 30% total gene deletions (Thacker & Ganesh, 1989)). The differences in the spectra of mutational events reflects the differences in the types of damages induced by different clastogens and also the differences in their subsequent repair processes. Ionizing radiations, which induce physical breaks in the DNA, produce almost entirely deletion mutations. Secondary damage induced by radiations (base damage) is reflected by the presence of a small number of point mutations. Alkylating agents such as EMS and ENU, whose damage is characterized

by a chemical modification of the DNA produce only point mutations. The same is true for UV radiation which produces primarily pyrimidine dimers. Since neither of these clastogens is capable of producing a direct break in the DNA it would be expected that the mutational spectra generated by them would be quite different from those for ionizing radiations. These differences in mutational spectra reflect differences in the repair processes involved in correcting damage induced by these agents. It is of interest to see what spectrum of mutational events are induced by restriction enzymes. One might expect that the enzyme's ability to produce only a single type of lesion, a double strand break, might lead to a distribution of mutational events similar to that observed for x-ray-induced mutations. In addition, the radiomimetic agent bleomycin has been shown to produce direct breaks in the DNA by the production of free radicals (Wood and Moses,1989). The mutational spectra following treatment with bleomycin results in approximately 43% of the 6-thioguanine-resistant clones exhibiting large deletions (Köberle and Speit,1991). It might be expected that restriction enzymes would produce a similar spectrum of mutations consisting mainly of large deletions.

Until very recently the only molecular analysis performed on hprt mutations was Southern blot analysis. Digestion of DNA from mutant cells with a variety of enzymes, usually EcoRI, Hind III, and Pst I, gave rise to a series of diagnostic bands on a Southern blot and changes in the presence or mobility of these bands indicated the size of the deletion event (Fuscoe et al., 1983; Gibbs et al., 1987; Carothers et al., 1988; Thacker and Ganesh, 1989). However, although an estimate of the size of the

deletion could be made with Southern analysis, very little could be determined about the exons involved in the deletion or to pinpoint the breakpoint location. It was not known which bands corresponded to which exons, and as such it was difficult to determine if there was any site specificity involved in the generation of hprt deletion mutants. This inherent ambiguity in Southern analysis is particularly enigmatic when looking for specific deletions. Without exon-specific probes, analysis of site-specific deletions is difficult.

With the advent of PCR technology, a way around the ambiguity of Southern analysis was developed. In an attempt to analyze deletions at the Duchenne muscular dystrophy locus, Chamberlin et al., (1988) used a series of oligonucleotide primers to analyze deletion "hot spots" in a target gene. This technique, known as multiplex PCR, has been extended for the analysis of several other genes including human steroid sulfatase STS (Ballabio et al.,1990), human hprt (Gibbs et al., 1990) and most recently, the Chinese hamster hprt gene (Rossiter et al., 1991). Multiplex PCR utilizes a series of oligonucleotide primers that flank each of the individual exons of the gene. Amplification reactions with an oligonucleotide cocktail allow for the simultaneous amplification of all the exons in a single reaction. The discrete exon-specific fragments produced may be resolved on an acrylamide gel. The deletion of an exon or group of exons may be visualized on an ethidium-bromide stained gel by the absence of the corresponding exon band or groups of bands. Multiplex PCR allows not only for an analysis of the size of a deletion, but also the extent and location of the deletion. This is of particular importance when looking for the

production of specific deletions. The exon specific bands generated with multiplex PCR were then used as exon specific probes with which the bands of a Southern blot were assigned to specific exons (Rossiter et al.,1991).

The generation of exon specific fragments lends itself particularly well to the analysis of possible point mutations induced at restriction sites as a variation of restriction fragment length polymorphism (RFLP) analysis. Exon specific bands can be digested with the same enzyme used for mutagenesis. Changes in the resulting restriction enzyme digestion products (banding pattern) as resolved on an ethidium bromide-stained acrylamide gel would be indicative of a loss of a restriction site in that exon. Such a loss of a restriction site would indicate a deletion or alteration at the restriction site. Additionally, the generation of a new restriction site resulting from a duplication of sequence can also be detected. For example, the four base overhang resulting from digestion with Sau3A I (GATC) could be filled in and then re-ligated creating a tandem repeat of the Sau3A I site (GATCGATC) and in the process, a new Cla I site (ATCGAT). The inability of Cla I to digest the exon-specific fragment would indicate that this fill-in/duplication event did not take place. This analysis may be used as a primary screen of point mutations before performing sequence analysis or as an alternative to sequence analysis.

While multiplex PCR allows the analysis of deletion mutants, it is also of interest to look at the distribution of point mutations in hprt. Previously, sequence analysis of the hprt gene would have been a laborious and difficult task. The size of the gene hinders conventional sequence analysis. However another variation of PCR

allows a rapid examination of changes in the coding sequence. Vrieling et al., (1989) used specific oligonucleotide primers that flanked the 3' and 5' regions of hprt cDNA to analyze point mutations in UV-induced HPRT deficient cells. cDNA was synthesized from total RNA isolated from mutant cells and was then used as a template for PCR synthesis. PCR products were ligated into a cloning vector and analyzed for point mutations. However, as Taq polymerase, the DNA polymerase used in PCR synthesis, is error prone, each clone isolated had to be sequenced from two different M13 clones. As was expected, all mutations occurred at di-pyrimidine sites and consisted mainly of transition type point mutations.

The infidelity of the Taq polymerase poses a problem when sequencing PCR products that have been ligated into a sequencing vector. Chen et al. (1991) found that by ligating PCR products into a sequencing vector, one PCR product was being over-represented. To alleviate this problem, more than one ligation product had to be sequenced in order to determine the correct sequence.

Another way of alleviating the error-prone nature of Taq polymerase is to perform asymmetric PCR. Asymmetric PCR involves the synthesis of a specific single-stranded DNA molecule from template DNA by using a 100-fold excess of one of the PCR primers (Rossiter et al., 1991). The single stranded product is ready for sequencing without ligation into a sequencing vector. In this way, no one amplification product is over-represented since the entire "pot" of amplification products is sequenced. If an exon-specific band is used for template in an asymmetric PCR reaction, sequence analysis may be performed on only the region

of interest in a gene. For example, if exon 3 is the only one containing the restriction site of interest, by simply cutting that exon band from a multiplex gel, it may be used as template to generate single stranded sequencing products by asymmetric PCR. With respect to restriction enzyme-induced mutations, this focussing of analysis allows the investigation of changes occurring directly at the restriction sites in the coding DNA.

Unfortunately, only a limited amount of sequence information is available for the hamster hprt gene and only restriction sites in the coding regions may be examined. In order to examine breakpoints involving restriction sites occurring in the intron sequences, sequence information of the directly adjacent DNA needs to be known in order to properly design amplification and sequence primers. The large size of the introns negates the possibility of using multiplex primers to amplify the intron DNA. Sequences greater than 1 kilobase are difficult to amplify, and many of the hprt introns span regions greater than 3 kilobases in length. Until more information becomes available on the DNA sequence of hamster hprt introns, analysis of breakpoints in introns is limited to deletion analysis.

In this chapter, the molecular analysis of 50 restriction enzyme-induced hprt-deficient clones is outlined. Multiplex PCR, asymmetric PCR sequencing and PCR-RFLP analysis is used to characterize the events responsible for mutation at this locus following restriction enzyme treatment.

MATERIALS AND METHODS

CELL CULTURE

CHO cells were maintained in 75 cm^2 tissue culture flasks or 100 mm tissue culture plates in Hams F12 medium supplemented with 5% FBS, 50μg/ml penicillin and 50μg/ml streptomycin. Cells were kept in a 37°C incubator humidified 95-100% under an atmosphere of 5% CO_2 in air. 6-TG-resistant clones were maintained under identical conditions except that they were grown in hypoxanthine-free F12 medium supplemented with 5% FBS and 10μM 6-TG.

DNA EXTRACTION

Genomic DNA was isolated from tissue culture cells by a protocol modified from Current Protocols in Molecular Biology (1989). Cells from 10 confluent 75 cm^2 flasks were removed by scraping with a rubber policeman. Cells were pooled, and centrifuged for 5 minutes at 2000 rpm, then washed twice with ice cold phosphate buffered saline (PBS; 137mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4, pH-7.3). The pellet was then resuspended in digestion buffer (100mM NaCl, 10 mM Tris·Cl pH 8, 25mM EDTA pH 8, 0.5% sodium dodecyl sulfate, 0.1 mg/ml proteinase K (Sigma)) to a final concentration of approximately 1 x 10^8 cells/ml. The mixture was incubated at 55°C for 18 hours. Ribonuclease A (RNase A; Sigma) was added to a final concentration of 1 μg/ml and incubated at 37°C for 2 hours. Samples were extracted four times with a mixture of phenol/chloroform/isoamyl alcohol (PCA;

25 parts phenol: 24 parts chloroform: 1 part isoamyl alcohol) and DNA was initially precipitated at room temperature for 30 minutes by the addition of an equal volume of isopropyl alcohol to the extracted aqueous phase. DNA was pelleted by centrifugation at 50 x g for 10 minutes. The pellet was dissolved in 400μl TE (10mM Tris·Cl, 1mM EDTA). 200 μl of 7.5M ammonium acetate was added, followed by the addition of 1200 μl ice-cold 100% ethanol. DNA was re-precipitated at -70°C for one hour and pelleted by centrifugation at 1400rpm for 15 minutes at 4°C. The pellet was washed twice with 70% ethanol and dried under vacuum in a desiccator. The dried pellet was dissolved in 200-500 μl TE and stored at -20°C. The amount and purity of the DNA sample was measured on a spectrophotometer at 260nm/280nm.

PREPARATION OF OLIGONUCLEOTIDE PRIMERS

Oligonucleotide primers for PCR, Multiplex PCR, and sequencing were synthesized on an Applied Biosystems DNA synthesizer (model 391) PCR-Mate. After synthesis, primers were stripped from the column by four 15-minute incubations with 1.5 ml 30% ammonium hydroxide (VWR). Effluents were pooled and incubated overnight (≈16 hours) at 55°C to remove protecting groups from the oligonucleotides. The oligonucleotides were lyophilized and resuspended in 100-200 μl of 0.5M NH_4OAc, gently vortexed to dissolve and centrifuged briefly to pellet any debris. The supernatant was removed and placed in a clean 1.5 ml Eppendorf tube and 1.0 ml absolute ethanol was added to precipitate the oligonucleotide DNA. Precipitation was performed for 30 minutes at -70°C, and then the sample was centrifuged for 15

minutes at 14,000 rpm at 4°C. Alcohol was removed carefully and the pellet was washed twice with ice-cold 70% ethanol to remove any remaining salt. The pellet was dried under vacuum and resuspended in 200 μl of sterile water. The amount of oligonucleotide was determined by OD_{260}.

MULTIPLEX PCR

Table 3.1 lists the primers and their respective final concentrations used for multiplex PCR amplification of the Chinese hamster hprt gene (Rossiter et al., 1991). *In vitro* amplification of 0.4 μg genomic DNA was carried out in a 40 μl reaction mixture of 6.7 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 5 mM β-mercaptoethanol, 6.8 μM EDTA, 67 mM Tris pH 8.8, 10% dimethylsulfoxide with 1.5 mM each deoxynucleotide, 66.7 nM to 1.67 μM each primer and 2 units Amplitaq DNA polymerase (Perkin-Elmer Cetus). The reaction mixture was covered with 50 μl of mineral oil and subjected to 30 cycles of 94°C/1 minute, 58°C/1 minute, 70°C/7 minutes, preceded by 5 minutes at 94°C and followed by 13 minutes at 70°C. Exon 1 was amplified in a separate reaction under identical conditions except that the temperature cycle parameters were 30 cycles of 94°C/1 minute, 60°C/1 minute, 70°C/45 sec, preceded by 5 minutes at 94°C and followed by 19 minutes at 70°C (Rossiter et al., 1991). Following PCR amplification, samples were stored at 4°C. The reaction products (15 μl) were analyzed by electrophoresis through an 8% polyacrylamide gel, stained with ethidium bromide, and photographed on an ultra violet light transilluminator.

TABLE 3.1 OLIGONUCLEOTIDE PRIMERS USED IN MULTIPLEX PCR OF THE CHINESE HAMSTER HPRT GENE

EXON	LENGTH	OLIGONUCLEOTIDE SEQUENCE	%G/C	CONCENTRATION
1	22 mer	5' *GTA CCT GGC CCC AGG AGC CAC C* 3'	73%	66.7 nM
	22 mer	5' *TCC GCT CTG CTG AAG AGT CCC G* 3'	64%	66.7 nM
2	28 mer	5' *AGC TTA TGC TCT GAT TTG AAA TCA GCT G* 3'	39%	1.50 μM
	29 mer	5' *ATT AAG ATC TTA CTT ACC TGT CCA TAA TC* 3'	31%	1.50 μM
3	29 mer	5' *CCG TGA TTT TAT TTT TGT AGG ACT GAA AG* 3'	31%	1.33 μM
	29 mer	5' *AAT GAA TTA TAC TTA CAC AGT AGC TCT TC* 3'	31%	1.33 μM
4	30 mer	5' *GTG TAT TCA AGA ATA TGC ATG TAA ATG ATG* 3'	30%	1.33 μM
	27 mer	5' *CAA GTG AGT GAT TGA AAG CAC AGT TAC* 3'	41%	1.33 μM
5	30 mer	5' *AAC ATA TGG GTC AAA TAT TCT TTC TAA TAG* 3'	27%	1.33 μM
	27 mer	5' *GGC TTA CCT ATA GTA TAC ACT AAG CTG* 3'	41%	1.33 μM
6	29 mer	5' *TTA CCA CTT ACC ATT AAA TAC CTC TTT TC* 3'	31%	1.33 μM
	28 mer	5' *CTA CTT TAA AAT GGC ATA CAT ACC TTG C* 3'	36%	1.33 μM
7&8	29 mer	5' *GTA ATA TTT TGT AAT TAA CAG CTT GCT GG* 3'	31%	133.0 nM
	25 mer	5' *TCA GTC TGG TCA AAT GAC GAG GTG C* 3'	52%	133.0 nM
9	28 mer	5' *CAA TTC TCT AAT GTT GCT CTT ACC TCT C* 3'	39%	1.67 μM
	27 mer	5' *CAT GCA GAG TTC TAT AAG AGA CAG TCC* 3'	44%	1.67 μM

PCR AMPLIFICATION OF EXON-SPECIFIC BANDS

Exon-specific bands resolved on an 8% polyacrylamide gel were cut from the gel with a scalpel and placed in a sterile, siliconized Eppendorf tube and crushed with a teflon pestle. DNA was eluted from the crushed acrylamide fragment by incubating at 37°for 30 minutes in elution buffer (0.5M ammonium acetate, 1mM EDTA, 5mM magnesium acetate, and 0.1% SDS). Acrylamide fragments were pelleted by centrifugation and the supernatant was collected. DNA fragments were precipitated from the supernatant by adding two volumes of absolute ethanol and precipitating at -70°C for 30 minutes followed by a 15 minute centrifugation at 14,000 rpm at 4°C. Samples were dried under vacuum and resuspended in 20 μl sterile distilled water. PCR amplification was performed on a 5μl aliquot of the fragment in a volume of 100μl [10mM Tris-HCl Ph 8.3, 50mM KCL. 4mM $MgCl_2$, 0.001% gelatin, 200μM each deoxynucleotide, and 2.5 units Amplitaq DNA polymerase (Perkin-Elmer Cetus)] with varying concentrations of primer depending on the primer used. Primers used for amplification were the same as those used in multiplex PCR. Temperature cycle parameters for PCR amplification were: 29 cycles(94°C/1 minute,40°C/1 minute, 70°C/2 minutes) preceded by one cycle 94°C/ 3 minutes, and followed by 72°C for 10 minutes. Following amplification, samples were stored at 4°C.

DIGESTION AND ELECTROPHORESIS OF EXON SPECIFIC FRAGMENTS

Exon-specific PCR products were digested with the appropriate restriction enzymes [Sau3A I, Rsa I, Cla I (New England Biolabs)] at 37°C overnight. 5 μl of

agarose gel loading buffer (0.25% bromphenol blue, 30% glycerol, 0.09M EDTA) were added to the reaction mixture and digestion products were electrophoresed through an 8% polyacrylamide gel and stained with ethidium bromide and photographed for analysis.

ASYMMETRIC PCR

Conditions for asymmetric PCR of exon-specific fragments are the same as described earlier except that one of the primers used was diluted 100 fold. Single stranded asymmetric PCR products were purified away from primers using Centricon 30 spin dialysis columns (Amicon). PCR products were dialyzed against sterile distilled water according to a protocol outlined in *Molecular Cloning: a laboratory manual* (Stambrook et al., 1989). Purified single-stranded PCR products were precipitated by the addition of an equal volume of 7.5M NH_4OAc and two volumes of absolute ethanol, precipitation at -70°C for 30 minutes and centrifugation at 14,000 rpm for 15 minutes at 4°C. After washing three times with 70% ethanol, the pellet was vacuum dried and resuspended in 7μl of sterile distilled water.

DI-DEOXY SEQUENCING OF ASYMMETRIC PCR PRODUCTS

Sequencing was performed according to the outlined protocol in the Sequenase Version 2 kit (United States Biochemical). Asymmetric PCR products were annealed to the appropriate sequencing primer (Table 3.2) in a total volume of 10μl (40mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, 50 mM NaCl) by heating to 65°C for 2 minutes

TABLE 3.2 OLIGONUCLEOTIDE PRIMERS USED FOR ASYMMETRIC PCR SEQUENCING OF EXON-SPECIFIC FRAGMENTS OF THE CHINESE HAMSTER HPRT GENE

EXON	LENGTH	OLIGONUCLEOTIDE SEQUENCE	% G/C	CONCENTRATION
3	20 mer	*5' CTG ACC TGC TGG ATT ACA TT 3'*	45%	10 μM
4	20 mer	*5' AAA TGA TTC TTT GCT TTC CC 3'*	35%	10 μM
6	20 mer	*5' AAG GAC ATA ATT GAC ACT GG 3'*	40%	10μM
8	20 mer	*5' AGT TTG TTG TTG GAT ATG CC 3'*	40%	10 μM

and then cooling slowly over a period of 30 minutes to room temperature, and then placed on ice. Incorporation of radio-labelled nucleotide was performed in a volume of 14.5μl [1.5μM dGTP,dCTP and dTTP, 7mM DTT, 10μM (1000 Ci/mmol) ^{35}S-dATP (Amersham) and Sequenase version 2 DNA polymerase (diluted 1:8)] at 19°C for 2 minutes. Immediately following incorporation, 3.5μl of the mix was aliquotted to each of four tubes containing either dideoxy-ATP,-TTP,-CTP, or -GTP mix (final concentration: 3.2μM ddNTP, 32μM of the other dNTPs, and 20mM NaCl) and incubated for 5 minutes at 37°C. The addition of 4μl stop solution (95% Formamide, 20mM EDTA, 0.05% Bromophenol Blue and 0.05% Xylene Cyanol FF) stopped the reaction. Samples were stored at 4°C. Immediately before loading onto the gel, samples were heated to 75°C for 2 minutes. Samples (10μl) were electrophoresed through a denaturing polyacrylamide gel (7% acrylamide, 8M urea) for 2.5 hours at 70 Watts. Immediately following electrophoresis, the acrylamide gel was blotted onto Wattman 3MM filter paper, covered with mylar, and vacuum dried at 80°C for two hours on a gel drier (Drygel Sr. slab gel dryer model SE1160, Hoefer Scientific). Autoradiographs were exposed to Kodak X-O Mat X-ray film for one day and then developed.

RESULTS

MULTIPLEX PCR

In order to analyze the molecular nature of the mutational events giving rise to 6-thioguanine-resistant colonies following treatment with restriction enzymes, it is important to be able to separate deletion mutants from point mutants. Multiplex PCR provides a relatively easy way of performing this analysis, and the results are more definitive than those determined with Southern blot analysis. Figure 3.1 demonstrates the products from a multiplex PCR reaction resolved on an 8% polyacrylamide gel. Each exon produces a discrete band on the gel, the absence of which is diagnostic of a deletion. Not all exons could be amplified simultaneously due to differences in melting profiles. Exons 1, 9 and sometimes 7/8 had to be amplified in separate reactions. Despite many attempts , exon 9 was never successfully amplified. Tables 3.3 and 3.4 show a deletion map of hprt-deficient clones generated with Sau3A I and Rsa I respectively. A total of 50 clones were analyzed using multiplex PCR. Wild type K1BH4 parental DNA was used as a positive control and RJK88 DNA, a Known complete hprt deletion, was used as a negative control. As expected, a variety of deletions were induced following treatment with Sau3A I. In the 30 mutants tested, there were: 3 total gene deletions; 16 partial gene deletions; and 11 clones whose multiplex bands indicated a small deletion or point mutation. Four of the 16 clones exhibiting partial deletions (SL1, SO1, SP2, SP5) involved a loss of either exon three or four, as was predicted. Clone SO1 was particularly interesting in that it showed a

Figure 3.1 Multiplex PCR of Sau3AI-induced HPRT-deficient clones. Lane: a)SU5 b)ST1 c)ST3 d)ST2 e)SP5 f)SP2 g)SO4 h)SM7 i)SM6 j)SM2 k)SL1 l)SJ1 m)SG1 n)SF2 o)SD2 p)SD1 q)SA1 r)K1BH4 parental control s)ϕX174 size standard.

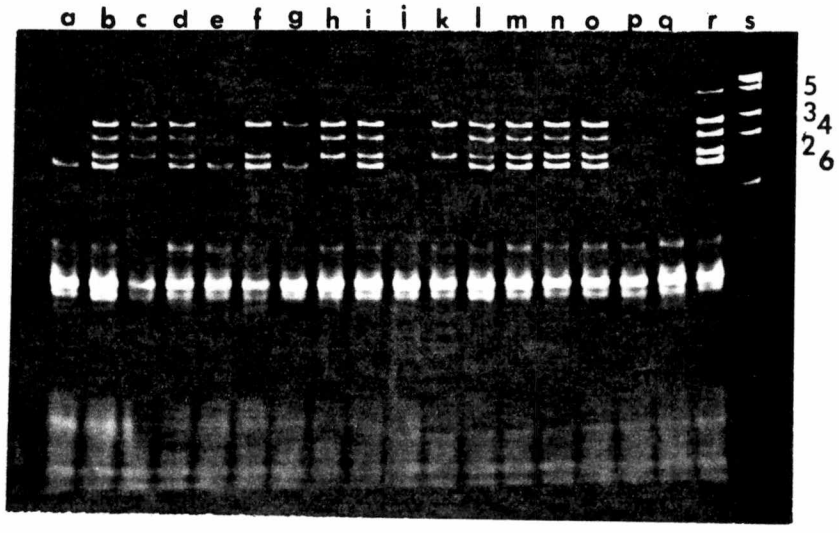


Figure 3.1

TABLE 3.3 MULTIPLEX DELETION ANALYSIS OF Sau3A I INDUCED HPRT MUTANTS

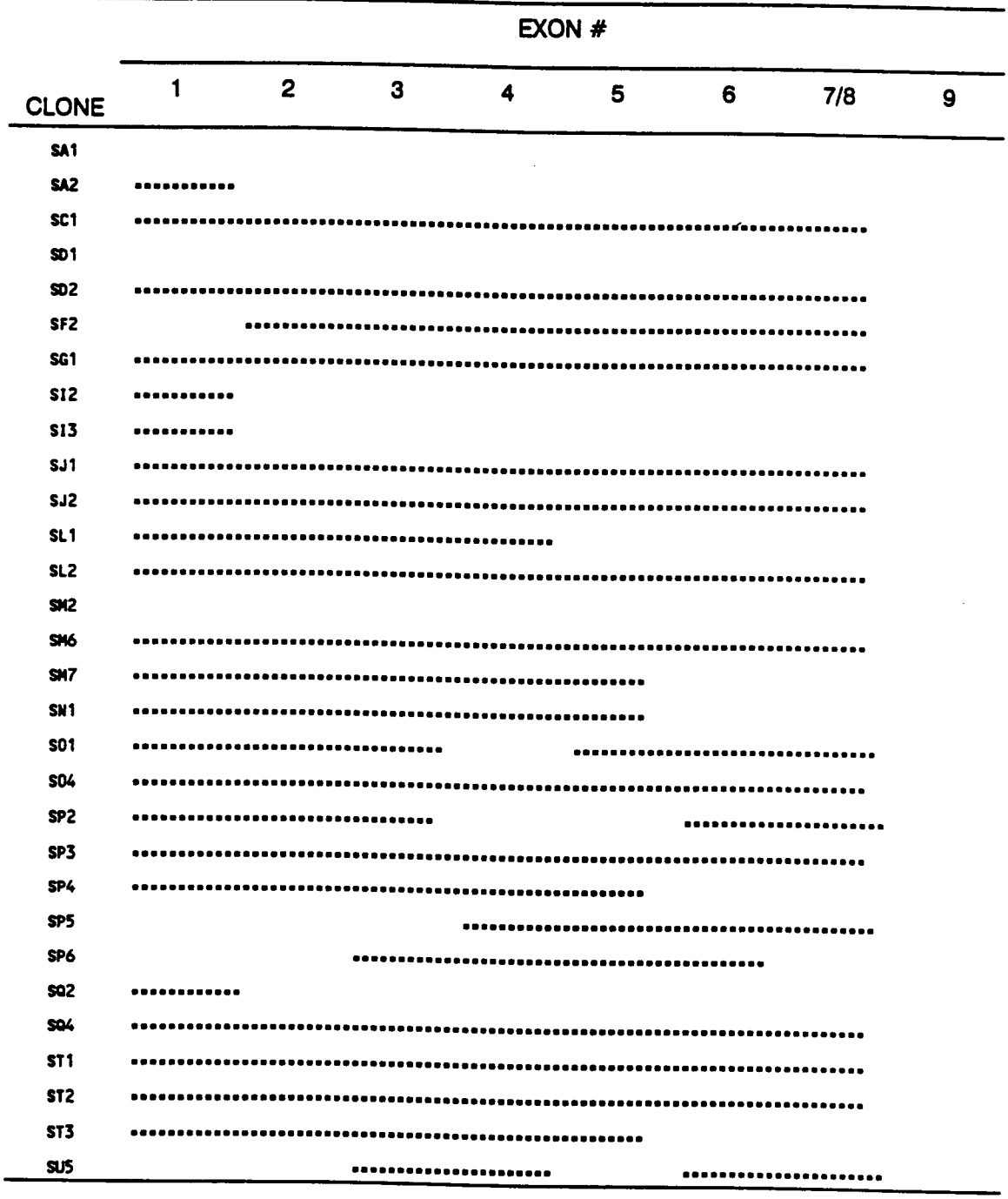


TABLE 3.4 MULTIPLEX DELETION ANALYSIS OF Rsa I INDUCED HPRT MUTANTS

	EXON #							
CLONE	1	2	3	4	5	6	7/8	9
RA2	•	•	•	•	•	•	•	
RA4								
RA7	•	•	•	•	•	•	•	
RB1	•	•						
RC1	•	•	•	•	•	•	•	
RC2	•	•	•	•	•	•	•	
RC3	•	•	•	•	•	•	•	
RD1								
RD2	•	•						
RE1	•	•	•	•	•	•	•	
RE3	•	•	•	•	•	•	•	
RF1								
RF2	•	•	•	•	•	•	•	
RF5	•	•	•	•	•	•	•	
RG2	•	•	•	•	•	•	•	
RH2	•	•	•	•	•	•	•	
RH3	•	•	•	•	•	•	•	
RH5	•	•						
RJ2	•	•						
RP1	•	•	•	•	•	•	•	

loss of only exon 4. The most common types of partial deletions observed were loss of either the 3' or 5' end of the gene, indicating restriction enzyme cutting in hprt introns and the flanking DNA. In one case, clone SP6, both the 3' and 5' ends have been deleted. Clone SU5 represents a deletion of the 5' end of the gene as well as an internal deletion of exon 5. Four of the clones showed a deletion of all of the hprt gene 3' to exon 1.

The multiplex data from clones generated with Rsa I, on the other hand, show a very different distribution of deletions. Thirteen of the 20 analyzed appear to be either very small deletions or point mutations. There are four clones exhibiting a deletion of everything but exon 1, with the remainder being total gene deletions. This is very different from the broad spectrum of deletions generated with Sau3A I. A similar spectrum of deletions was expected to be produced with Rsa I but with deletions involving exons 6 and 8 which both contain an Rsa I restriction site. There is apparently a difference in the ability of Rsa I to digest the hprt gene or a difference in the repair processes utilized to repair damage generated by the two enzymes.

SEQUENCE ANALYSIS

In order to determine if a small change had occurred at the restriction site of the enzyme used to generate mutations in hprt, sequence analysis was performed to examine the DNA sequence directly flanking the restriction site. Asymmetric PCR sequencing of exon specific fragments from exons 3 and 4 in the case of Sau3A I and 6 and 8 in the case of Rsa I was performed on four clones from each enzyme

treatment. Figure 3.2 shows a sequence analysis around the Sau3A I site in exon 3 in three clones. There were no differences in sequence at or around the restriction sites in any of the exons examined.

PCR RFLP ANALYSIS

To determine the nature of the mutational event involved in the clones showing no loss of hprt exons, restriction enzyme digests were performed on PCR amplified exon specific bands. The inability of an enzyme to digest its restriction sequence would indicate a change induced at that restriction site. Digestion of exons 3 and 4 were expected to yield different classes of DNA fragments following digestion with that enzyme. If no changes were induced at the Sau3A I restriction site, two fragments of 157bp and 63bp for exon 3, and three fragments of 104bp, 48bp and 39bp for exon 4 would be expected. Loss of these sites would be expected to generate fragments of the following sizes: Exon 3 loss of Sau3A I site → 220bp fragment; exon 4 loss of site #1 → 87bp & 104bp fragments, loss of site #2 → 48bp & 143bp fragments, loss of both sites → 191bp fragment. Digestion of exons 3 and 4 from Sau3A I generated clones showed no loss of this site in either of these exons (Figure 3.3). Since Sau3A I produces four base cohesive termini with recessed 3' ends, it is possible that a fill-in of the recessed termini followed by a blunt-end ligation could have occurred at the restriction site resulting in a duplication of the Sau3A I site. Digestion with the restriction enzyme Cla I is specific for the duplication of a Sau3A I site. Therefore, digestion of exon 3 or 4 specific bands with Cla I would be indicative of this event.

Figure 3.2 Sequence analysis of the Sau3A I restriction site in hamster hprt exon 3. Lane: a)SF2 b)SM6 c)ST1.

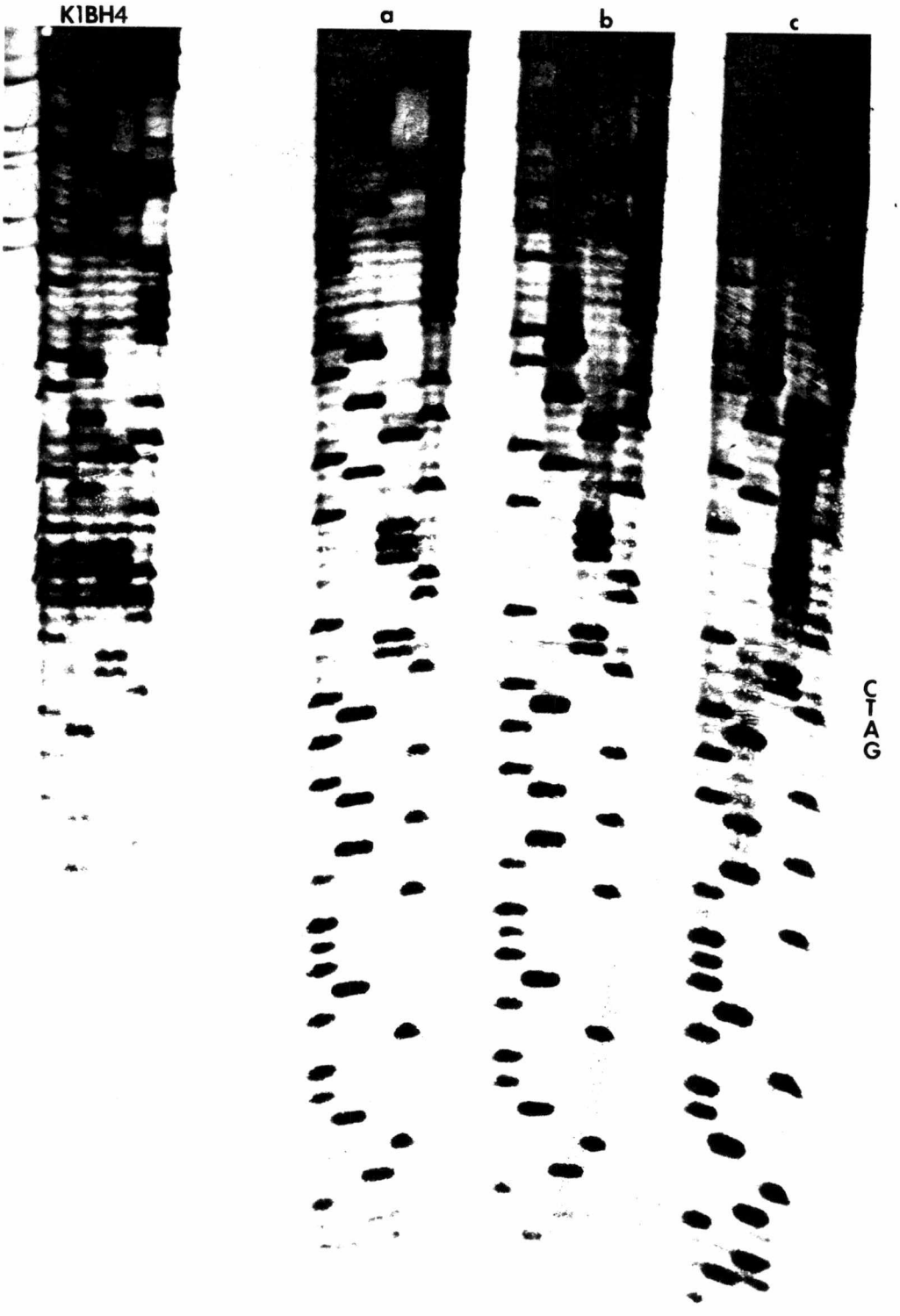


Figure 3.2

Figure 3.3 PCR RFLP analysis of Sau3A I digestion of hamster HPRT exons 3 and 4 from Sau3A I-induced HPRT-deficient mutants. Exon 3, Lane: b) K1BH4 parental control d)SD2 f)SF2 h)SG1 j)SJ1 l)SM6 n)SO4 p)ST2 r)ST1. Exon 4, Lane: a) K1BH4 parental control c)SD2 e)SF2 g)SG1 i)SJ1 k)SM6 m)SO4 o)ST2 q)ST1 s)ΦX174 size standard.

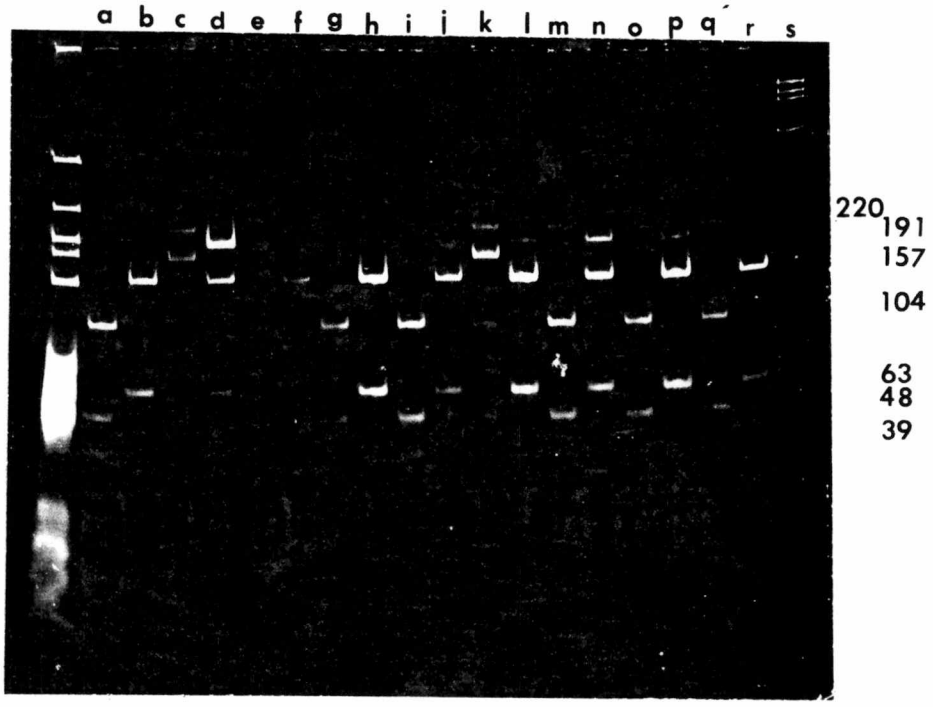


Figure 3.3

Neither exon 3 nor exon 4 specific bands from 9 different clones possessed the new Cla I site.

A similar analysis was performed on exons 6 and 8 from clones generated with the restriction enzyme Rsa I. As with the Sau3A I clones, none of the Rsa clones showed a digestion pattern different from wild type indicating that the Rsa I site remained intact.

DISCUSSION

The hprt mutation assay is one of the most informative systems for screening potential mutagens. As mentioned earlier, what makes the assay so informative is that since the hprt gene is a non-essential gene, it is possible to select for a wide variety of mutations, including deletions and point mutations. Many clastogens have been used to generate mutations in the Chinese hamster hprt gene, and we can now add restriction enzymes to this list. Although there is a wealth of information available on the mutagenicity of compounds at the hprt locus (Li et al., 1988), until recently very little molecular analysis has been performed to determine the nature of these mutations. With the cloning of the Chinese hamster hprt cDNA sequence (Konecki et al., 1982) a probe became available for performing Southern blot analysis of HPRT deficient clones. The analysis of mutations at the hprt locus in Chinese hamster

(Fuscoe et al., 1983, Gibbs et al., 1987, Carothers et al., 1988, Thacker and Ganesh, 1989) as well as at the mouse hprt locus (Vrieling et al., 1988) and the human hprt locus (Gennett and Thilly, 1988) has been performed using the Southern blot technique. Although characteristic banding patterns are known for digestion of hprt with a variety of enzymes, a confounding factor in the analysis of mutants with the Southern blot technique is that often more than one exon is contained in a single band (Rossiter et al., 1991). While it is possible to estimate the size of a deletion with the Southern blot technique it is difficult to determine which exons are involved. This is especially true for the analysis of hamster hprt mutations since a restriction map for the locus has only recently been determined (Rossiter, et al, 1991). In the present study, initial attempts to analyze the molecular nature of restriction enzyme-induced mutations at the hprt locus were performed with Southern blot analysis (data not shown). The inherent ambiguity in the analysis of deletion mutants complicates the analysis of restriction enzyme-induced mutants and the generation of a specific deletion was difficult to detect with Southern blot analysis.

A number of new techniques utilizing PCR technology have been used for the analysis of hprt mutations. Denaturing gradient del electrophoresis (DGGE) has been used to resolve missense mutations in the human hprt locus (Cariello et al., 1988). Competitive oligonucleotide priming has been used to detect single base changes at a specific site (Gibbs et al., 1989). Perhaps most useful is the technique of multiplex PCR for screening deletion mutations that makes analysis much easier and the level of resolution much finer. Multiplex analysis of hprt mutants induced by

restriction enzyme treatment has shown that in addition to some of the expected deletions, a wide variety of deletions can be induced with restriction enzymes. Not only are we able to classify which mutants are due to deletions and which are apparent point mutations, but also we can determine which exons are deleted. Multiplex deletion analysis of hprt-deficient clones generated with the restriction enzymes Sau3A I and Rsa I showed some interesting mutational spectra.

Multiplex deletion analysis of 6-thioguanine-resistant clones did confirm the production of several predicted deletions. Digestion of the hamster genome with the enzyme Sau3A I was expected to result in a series of specific deletions. Digestion of restriction sites within exons 3 and/or 4 was expected to lead to deletion involving these exons. In fact, four of the clones isolated exhibit deletions of exons 3 and 4 (SO1, SP2, SL1, SP5). Each of these clones shows a different type of deletion. Clone SO1 for example has a deletion of only exon 4. This may indicate several different breakpoints at any of several Sau3A I restriction sites. Since the loss of only one multiplex primer sequence is enough to cause the lack of amplification, the failure to amplify a particular exon in a multiplex reaction does not necessarily indicate a loss of that entire exon. Exon 4 contains two Sau3A I sites, one at the 5' end of the exon and one at the 3' end. Digestion of both of these sites causing a loss of most of exon 4 while retaining the primer sites would result in a PCR product of approximately 150 bp. This fragment was not observed on a multiplex gel and therefore, this possibility may reasonably be ruled out. Digestion occurring at one of these two sites and another site in either intron 3 or intron 4, resulting in a loss of one of the multiplex

primer sequences, could also lead to lack of amplification of the exon 4 band. Still, another possibility, digestion of restriction sites in the introns flanking exon four, causing a loss of the exon, would lead to the same result. A similar breakdown of possible breakpoint locations can be performed for the other clone involving both exons 3 and 4. Southern blot analysis using exon specific fragments arising from multiplex amplification could be used here to better define the location of the breakpoint. Other predictions made were that digestion in the large intervening introns would lead to deletions of groups of exons, and digestion in adjacent flanking DNA would result in total gene deletions. As expected, all of these classes were observed with Sau3A I. Many of the clones analyzed showed the deletion of a series of exons indicating a break at a restriction site in an intron toward the center of the gene, and a second break at a restriction site in the flanking 5' or 3' adjacent DNA. These clones are observed on a multiplex gel as having the 5' or 3' end of the gene deleted. The largest number of clones involved a deletion extending toward the 3' end of the gene, considerably more than the deletions extending toward the 5' end. This may indicate the presence of more Sau3A I sites in 3' flanking DNA than in 5' flanking regions. One clone, SP6, has a deletion of both the 5' and 3' exons, while the core of the gene remained intact.

Clone SU5 is of interest because it contains both an internal deletion of exon 5 and a deletion of the 5' exons (exons 1 and 2). The deletion of exon 5 indicates breakpoints occurring at restriction sites in the flanking introns (5 and 6) since exon 5 has no Sau3A I restriction sites while the deletion of exons 1 & 2 indicates

breakpoints in the third intron and in the 5' flanking DNA. The only other cases of internal deletions observed were those involving exons 3 and 4 whose coding sequence is known to possess Sau3A I sites.

One of the most frequently occurring deletions in the hprt-deficient clones analyzed is the deletion of the entire gene except exon I. The first intron in the hamster hprt gene is the largest of the introns, ≈10 kb in size (Rossiter et al., 1991). Considering the restriction site frequency of the enzymes used in this study, it is highly probable that a significant number of restriction sites exist in this intron and that they are readily available for digestion. However, most deletions involving a breakpoint in the first intron extend toward the 3' end of the gene. There was only one deletion observed in the clones analyzed that showed a deletion of only exon 1 (indicating a second breakpoint in the 5' flanking DNA). Rossiter et al., (1991) in attempting to clone the first hamster hprt exon ran into considerable difficulty because the 5' region of this gene is highly GC-rich, a hallmark of ubiquitously-expressed housekeeping genes (Bird, 1986). It is entirely possible that restriction sites do not exist or are not available in the immediate 5' flanking DNA thereby increasing the likelihood of creating deletions toward the 3' end of the locus. Another possibility is that deletions may extend into a gene in the 5' flanking region resulting in a lethal mutation subsequently masking deletions in the 5' end of hprt.

Deletion of the entire gene except for exon 1 was also observed in hprt-deficient clones induced with Rsa I. It is curious though, that with the exception of these deletions, the only other mutations observed were total gene deletions and

apparent point mutations. One of the predictions made concerning the types of mutations expected from treatment with restriction enzymes was that, due to the nature of the lesion induced, double strand breaks, primarily deletions would be observed [similar to results observed with X-rays (Thacker, 1986)]. While this is true for the enzyme Sau3A I where 63% of the clones analyzed exhibited a deletion of all or part of the hprt gene, it is interesting that the same is not true for mutants generated with Rsa I. Of the Rsa I-generated clones analyzed, 65% showed no deletion of any part of the hprt gene. In light of the frequency of aberrations induced with Rsa I, particularly deletions, it is notable that so few of the hprt-deficient mutants are deletion mutants. It was predicted that due to the presence of restriction sites in exons 6 and 8, that deletions involving these exons would be observed. In a manner similar to that for Sau3A I deletions, it was also predicted that based on its rather high restriction site frequency, there would be numerous Rsa I restriction sites in the large intervening introns, resulting in a similar spectrum of exon deletions to those described for Sau3A I. However, none of these predicted deletions was observed in the clones analyzed. It is highly unlikely that there would be no Rsa I sites present or available for digestion in the 36 kb span of the hamster hprt gene. Why then are there no partial deletions similar to those observed with digestion with Sau3A I? The most plausible explanation is that blunt-end termini are repaired differently from cohesive-end termini.

Following digestion of genomic DNA with restriction enzymes, the induced double-strand break may be repaired according to several different mechanisms. Mis-

repair of the enzyme-induced lesion can result in the production of chromosome aberrations and mutations (for review see Bryant, 1988). The simplest of these mechanisms is a rejoining and ligation of the break. This simple model of repair has been used to explain the differences in the efficiency of aberration production between blunt and cohesive-end cutters. Heitman et al., (1989) demonstrated that *in vivo* scissions of the *E. coli* chromosome with the restriction enzyme Eco RI are predominantly repaired by simple ligation of the double-strand break. It has been hypothesized that hydrogen-bonding in the overlapping single-strand protruding termini of a cohesive-end cut stabilizes these lesions, while blunt-ended termini are not stabilized (Bryant, 1984; Natarajan and Obe, 1984). Singh and Bryant (1991) have also reported a significant difference in the ability of cohesive vs. blunt-end cutters to induce mutations at the thymidine kinase locus. A comparison of the efficiency of the restriction enzymes Pvu II (blunt-end cutter) and Eco RI (cohesive-end cutter) demonstrated an 11-fold higher mutation frequency induced by Pvu II than Eco RI at the same extracellular enzyme concentrations (Singh and Bryant, 1991). The difference in mutation induction frequency between Rsa I (blunt-end cutter) and Sau3A I (cohesive-end cutter) in our study also showed a similar trend. The difference in the mutation frequency observed may again be attributed to differences in the stability of the induced lesions. The rejoining of restriction enzyme-induced double-strand breaks has been shown to depend on the number of bases in the overhang, the polarity of the cohesive end (whether the single-strand overhang is 5' or 3'), and on the base composition of the overhang. North et al., (1990) demonstrated that

rejoining of restriction enzyme-induced double-strand break termini "showed a range of efficiencies with 5' 4-base > 3' 4-base overhangs and 4-base > 2-base > no overhang." The differences in the mutational spectra resulting from generation of cohesive and blunt-end termini may be due to the general trend that cohesive-end termini join more stably with other complimentary cohesive termini, either adjacent (resulting in no mutation) or non-adjacent (giving rise to deletions, inversions, and translocation events). The high frequency of deletions observed at the hprt locus following treatment with Sau3A I may indicate that other Sau3A I digestible sites are closely aligned allowing an interaction between non-adjacent complementary cohesive termini. The high stability of joining of cohesive termini due to hydrogen bonding makes it very likely that these termini will remain hydrogen-bonded to one another until they are ligated. Therefore, although the probability of a cohesive-ended terminus recombining with another, non-adjacent cohesive terminus may be low, once joined, the new interaction is likely to persist until ligation can seal the breaks. The low number of deletion mutants isolated at the hprt locus following treatment with Rsa I on the other hand, would indicate that the distribution of Rsa I digestible sites is significantly different so as not to generate the same spectrum of deletion mutations that was observed with Sau3A I. The general instability of the blunt-ended termini would be expected to result in a higher number of partial deletion mutants. However, Rsa I digestible sites may not be distributed in the introns of the hprt locus in such a way as to allow an interaction of termini leading to partial hprt deletions. Because of the instability of a blunt-ended terminus, interactions with other termini are likely to

be far more transient than are those associated with cohesive termini. The nature of blunt ended termini makes them particularly amenable to ligation repair. It was demonstrated by Winegar and Preston (1987) that blunt-ended termini were capable of interacting with other termini (blunt and cohesive) in a synergistic fashion to produce chromosome aberrations. While this indicates that a portion of blunt-ended termini may be repaired by direct ligation other studies by Winegar and Preston indicated that additional mechanisms may be involved in the repair of restriction enzyme-induced double-strand breaks. Winegar and Preston (1987) suggested that DNA re-synthesis was an integral component in the repair of double-strand breaks produced by restriction enzymes based on the observation that the repair inhibitor ara-C is able to potentiate restriction enzyme-induced chromosome aberrations (also demonstrated by: Natarajan and Obe, 1984; Obe and Natarajan, 1985). Inhibition of double-strand break repair with cytosine arabinoside (ara-C) indicated that direct ligation was not sufficient to explain the repair of restriction enzyme-induced double-strand breaks. Ara-C is thought to be a potent inhibitor of DNA re-synthesis either by directly binding to the dCTP binding site of DNA polymerase alpha (Graham and Whitmore, 1970), or by terminating re-synthesis polymerization by direct incorporation into the newly synthesized DNA strand (Major et al., 1982; Heintz and Hamlin, 1983). The inhibition of restriction enzyme induced DNA double-strand breaks indicates that DNA re-synthesis plays an important role in the repair of double strand breaks. The requirement of DNA re-synthesis has been interpreted to indicate the involvement of recombinational processes in the repair of restriction enzyme-induced double-strand

breaks. It is entirely likely that the repair of blunt-ended termini, due to their decreased stability, are more likely to be involved in recombination events and this may account for some of the differences in the mutational spectra observed at the hprt locus for the restriction enzymes used.

In lower eukaryotes homologous and non-homologous recombination both occur frequently. For example, in the unicellular eukaryotic parasite *Trypanosoma bruceii*, the preferred route for insertion of exogenous DNA appears to be via homologous recombination (ten Asbroek et al., 1990). In mammalian systems both homologous and non-homologous recombination mechanisms are used to repair double-strand DNA breaks, but non-homologous recombination of transfected DNA occurs at a frequency 1000 times higher than homologous events (Roth and Wilson, 1985; Lin et al., 1985; Thomas et al., 1986). The differences in the probabilities of non-homologous and homologous recombination between organisms may be due to differences in the complexities of the genomes involved as well as in the repair enzymes themselves. However, the mechanisms of recombination are likely to be conserved between the species, making lower eukaryotic test systems ideal for elucidating the mechanisms of DNA repair resulting in gene conversion and crossover events.

Non-homologous recombination has been shown to be stimulated by damage to the DNA (Vos and Hanawalt, 1989), and in particular, to double-strand DNA breaks (Wilson et al., 1982), so it is entirely likely that a DNA double-strand break induced by restriction enzymes may be the initiating event for non-homologous recombination.

Yorifuji and Mikawa (1990) demonstrated that the co-transfer of restriction enzymes and plasmid DNA into mammalian cells was able to stimulate the integration of linear and circular DNA into the host chromosomal DNA. Non-homologous recombination involving restriction enzyme-cut DNA with other, spontaneously occurring double-strand breaks may account for some of the mutations isolated following restriction enzyme treatment. Illegitimate recombination involving restriction enzyme-induced double-strand break termini and other enzyme-induced or spontaneously occurring breaks may result in a number of mutagenic events including chromosome translocations, oncogene rearrangements, loss of heterozygosity, and mutation at selectable loci such as hprt (for review, Crow and Dove,1987; Meuth, 1987). It has been demonstrated that chromosome fragile sites predispose the genome to deletions and inter-chromosomal recombination (Glover and Stein, 1988). It is likely that fragile sites may provide a break in the DNA capable of interacting with restriction enzyme-induced damage by non-homologous recombination mechanisms although such interactions would be very rare events since most restriction enzyme-induced double strand breaks would be more likely to interact with one another. Nevertheless, such rearrangements involving hprt and fragile sites (a low probability event) provided there was no loss of genetic material would appear as "point mutations" (no change in banding pattern) on a multiplex PCR gel since none of the primer sequences were lost.

While most restriction enzyme-induced breaks may be repaired by a simple re-ligation of the broken ends, it is possible that a modification of these ends may also

occur. The mechanisms of recombination of non-homologous ends has been poorly understood, and until recently very few models have been proposed to explain this type of repair. Modifications involving exonuclease digestion or primed fill-in of cohesive or blunt-ended termini may result in different probabilities of interaction between the termini. It has been shown that non-homologous ends may be joined end-to end in seamless junctions and that joining tends to preserve the original sequence (Wilson et al., 1982). The ability of the cell to repair a DNA double-strand break with cohesive termini consisting of 5' protruding single strand termini (recessed 3' ends serving as primer) may proceed by a "fill-in" reaction followed by blunt end ligation of the break (Pfeiffer and Vielmetter, 1988; Thode et al., 1990). For example, in the case of Sau3A I-induced double-strand breaks, a fill-in reaction involving the 3'-recessed termini generated by the enzyme Sau3A I could result in a tandem duplication of that restriction sequence giving rise to a new Cla I site. However, Cla I digestion of exon 3 & 4 specific bands from HPRT-deficient clones generated with the restriction Sau3A I in our studies did not demonstrate that such a duplication had occurred. The effects of such a duplication of a restriction sequence could cause frameshift mutations at restriction sites occurring in coding regions of a target gene if the new blunt-ended Sau3A I termini were ligated together. Additionally, the new blunt-ended Sau3A I terminus could also now interact with any other available termini resulting in deletion, translocation or inversion of hprt sequence. Pfeiffer and Vielmetter (1988) proposed that cohesive-ended termini may be joined to blunt-ended or cohesive-ended termini with opposite polarity following fill-in of the cohesive termini.

This is particularly interesting when considering the fate of cohesive termini with recessed 5' termini which are unable to serve as primers for fill-in reactions. These termini are filled in with the same efficiency as termini with recessed 3' ends, and are subsequently ligated to other termini (Thode et al., 1990). Presumably, this mechanism is due to an as yet un-identified single-strand binding protein complex that allows another terminus (blunt or cohesive) to serve as a primer for fill-in of the double-strand cohesive-end break. The specificity of the rejoining is assumed to be determined by this unidentified complex. By this mechanism, restriction enzyme-induced breaks could be joined to any other non-homologous double-strand break resulting in deletion, inversion or translocation of genetic information. As mentioned earlier, fragile sites could provide the necessary non-homologous double-strand break for this recombination event to occur, although at a predicted rather low frequency.

Since double-strand breaks induced by the enzyme Rsa I would need no processing to be involved in non-homologous recombination, the rate of their repair by this mechanism might be more rapid than for cohesive-ended termini. However, while double-strand break termini with no homology can be efficiently ligated by the aforementioned mechanisms, the presence of even one base of homology at the terminus can greatly increase efficiency of recombination between these ends (Roth, 1985). Small changes have been observed in the immediate vicinity of the break points including single base additions and deletions (Roth et al., 1985; Landau et al., 1987). It has been observed that the presence of even one base of homology at the terminus of a double-strand break can increase the efficiency of recombination of the

non-homologous ends (Roth and Wilson, 1986; Landau et al., 1987; Pfeiffer and Vielmetter, 1988; Thode et al., 1990). In the case of blunt-ended termini it has been hypothesized that the addition of a single, complementary nucleotide to the end of a blunt-end terminus (presumably by terminal deoxy-nucleotidyl transferase) may aid in non-homologous recombination (Landau et al., 1987). The addition (or deletion by exonuclease digestion) of single nucleotides at the site of a restriction enzyme-induced break could cause frame shift mutations in the target gene. It has been reported by Dr. William Morgan (University of California, San Francisco) that digestion of the APRT gene in AS52 cells (a CHO derivative) with the restriction enzyme Pvu II (a blunt-end cutter), results in predominantly small point mutations at the restriction site for this enzyme (unpublished results and personal communication). These changes may result from a similar mechanism to that described above. As previously stated, in our study, sequence analysis of exons 6 and 8 from four Rsa I-induced hprt-deficient clones showed no changes in the sequence at or around the restriction site. The same is true for sequence analysis (and restriction enzyme RFLP analysis) around Sau3A I restriction sites in exons 3 and 4 in HPRT-deficient clones generated with this enzyme. None of the apparent point mutations analyzed by sequencing showed a change at the specific restriction site. This does not rule out the involvement of non-homologous recombination mechanisms. Many of the apparent point mutations may be large gene rearrangements involving breakage at intronic restriction sequences. Non-homologous recombination of these ends could give rise to these mutations. Such events would not be detectable by multiplex PCR analysis since none of the

primer binding sequences would be lost. The possibility also exists that these mutants may be due to the digestion of restriction enzyme sequences (either intronic or exonic) followed by homologous recombination repair. There is a broad base in the literature that supports the idea that the initiating event in homologous recombination is a double strand break (Ayares et al., 1986; Brenner et al., 1986, Kolodkin et al., 1986; Fraser et al., 1986; Sun et al., 1989; Nickoloff et al., 1989; Ray et al., 1989; Lin et al., 1990; Jessberger and Berg, 1991; Symington, 1991). It is believed that the stimulation of homologous recombination by clastogenic agents such as X-rays may be related to the "cleaning up" of the termini of double-strand breaks resulting from radiation induced damage. Such breaks are thought to need processing before repair can take place. The subsequent exonuclease digestion of these ends results in single-stranded intermediates which have been shown to be important in the initiation of homologous recombination. However, in yeast double-strand breaks created by HO nuclease have also been demonstrated to stimulate recombination (Nickoloff et al., 1989; Rudin et al., 1989). Double-strand breaks induced by this enzyme are restricted to the HO nuclease restriction sequence (MAT locus) and produce a "clean" double-strand break in the DNA (Nickoloff et al., 1986). It follows, then, that the clean ends generated by restriction enzymes should also be capable of stimulating homologous recombination. It remains unknown as to whether these clean ends are processed similarly to breaks induced by X-rays. There is some evidence that restriction enzyme -induced double-strand breaks are subject to exonuclease activity (Marryon and Carroll, 1991).

If homologous recombination initiated by restriction enzyme-induced double-strand breaks is to occur at the hamster hprt locus, what sequences can serve as homologous templates? Since the hprt locus in CHO cells is a hemizygous locus there is no homologous chromosome to be involved in homologous recombination repair. This leaves only three possibilities for recombination involving hprt coding sequences: homologous recombination at the hamster hprt locus may involve sister chromatids, the autosomally-linked pseudogene sequence or repetitive sequences on non-homologous chromosomes. Although the mechanism for the production of sister chromatid exchanges is not clearly understood, it has been demonstrated that restriction enzymes are capable of inducing an increase in SCE formation when cells are treated in the S-phase (Natarajan et al., 1985; Stoilov et al., 1986). This observation may suggest that homologous recombination between the chromatids may be responsible for sister chromatid exchange. However, there are conflicting reports as to whether restriction enzymes are capable of inducing sister chromatid exchanges (Morgan et al., 1989). If restriction enzymes are inefficient at inducing sister chromatid exchanges, this may indicate that double-strand breaks are not good initiators of sister chromatid exchange. This is further supported by the fact that X-rays and other agents that produce DNA strand breaks are also poor inducers of SCE's (Wolff, 1978; Morgan et al., 1985). Homologous recombination may play only a minor role in the production of SCE's. Nevertheless, homologous recombination occurring between sister chromatids is not likely to result in mutations unless mistakes in the re-synthesis of DNA or resolution of homologous recombination intermediates

(such as Holliday structures) occurs. If on the other hand, homologous recombination results in an unequal sister chromatid exchange, duplication of genetic material can occur. This has been evidenced by unequal sister chromatid exchange involving Alu repetitive elements at the Duchenne's muscular dystrophy locus (Hu et al., 1991) and in the LDL receptor gene (Lehrman et al., 1987). The duplication of hprt sequences by unequal sister chromatid exchange resulting from homologous recombination between sister chromatids stimulated by restriction enzyme-induced double-strand breaks would also not be detectable by multiplex PCR deletion analysis, RFLP analysis of exon-specific bands, or by sequence analysis of exon-specific bands.

Homologous recombination involving the autosomal hprt pseudogene sequence on the other hand may indeed give rise to mutations. Since the pseudogene sequence is not expressed, there is no selection against deleterious spontaneous "mutations" occurring at this locus. Furthermore, the pseudogene sequence lacks introns. Homologous recombination involving the pseudogene locus could affect proper splicing of the hprt mRNA by potentially altering splice sites in the hprt locus. There are other candidate sequences that may be involved in homologous recombination repair at the hprt locus. Homologous recombination is not restricted to exon sequences alone. As mentioned earlier, repetitive elements in mammalian genomes are thought to be hot spots for homologous recombination. Homologous recombination between Alu repetitive elements in the human genome are thought to be responsible for the 9;22 translocation (Philadelphia chromosome) in chronic myelogenous leukemia (de Klein et al., 1986) and for XX maleness (Rouyer

et al., 1987) and for tandem duplications in the Duchenne's muscular dystrophy locus (Hu et al., 1991), and for duplications in the LDL receptor gene resulting in familial hypercholesterolemia (Lehrman et al., 1987). It has also been demonstrated that hypervariable minisatellite DNA sequences (utilized for DNA fingerprinting) are hot spots for homologous recombination (Wahls et al., 1990) and that interstitial telomere-like sequences may also be prone to recombination (Hastie and Allshire, 1989). Since it is known that the hamster genome is rich in repetitive elements belonging to the Alu family, these elements may play an important role in homologous recombination. Bearing in mind the large size of the hamster hprt locus, it is likely that intron sequences possess Alu repetitive elements. The generation of DNA double-strand breaks by restriction enzymes at the hprt locus and/or at other sequences in the genome with homology to the hprt gene (or repetitive elements in the intervening introns) could conceivably increase the frequency of homologous recombination involving this locus.

The outcome of homologous recombination is often either a crossover event resulting in a possible translocation of chromosomal DNA, or gene conversion of the recipient DNA sequence. According to the Szostak double-strand break repair model [DSBR (1983)], the strand which receives the DNA double-strand break is also the strand that receives information in homologous recombination-mediated gene conversion. A number of laboratories have demonstrated this to be true (Nicolas et al., 1989; Voelkel-Meiman and Roeder 1990). One of the predictions of both the SSA model (Lin et al., 1984) and the DSBR model of homologous recombination is that

areas of DNA flanking the initial site of damage (double-strand break) are often involved in gene conversion and crossover events either by exonuclease digestion or migration of Holliday structures away from the initial DNA break site (Szostak et al., 1983; Lin et al., 1990). Evidence for gene conversion of regions of DNA flanking the initial site of a double strand break have been performed by inserting recombination hotspots such as HOT1 (Voelkel-Meiman and Roeder, 1990), the HO nuclease site (Nickoloff et al., 1989; Rudin et al., 1989) adjacent to marker genes, or by observing the conversion of markers adjacent to an endogenous recombination hot spot in the promoter region of the ARG4 gene in *Saccharomyces cerevisiae* (Sun et al., 1989; Nicolas et al., 1989; Cao et al., 1990). In all cases, gene conversion was observed at adjacent markers to the initial site of the DNA double-strand break. There is a polarity of gene conversion observed with recombination hotspots that results in high gene conversion frequencies at markers immediately adjacent to the site of the double strand break and decreasing gene conversion frequencies at sites increasingly distal to the break site. However, an exception to this rule was observed by Voelkel-Meiman and Roeder (1990) using the HOT1 recombination hotspot. Sites 50 kbp distal to the breaksite exhibited gene conversion at higher frequencies than markers immediately adjacent to the HOT1 insertion site. It has been hypothesized that HOT1 may specify a *region* of recombination initiation rather than a specific *site* of initiation. The ability for gene conversion events to occur at sites distal to the initial site of damage may explain the absence of changes at restriction sequences in the clones analyzed. A double-strand break occurring at a restriction sequence in an intron or

exon resulting in gene conversion of distal sequences may result in altered mRNA splice junctions, or mutational changes in exons themselves.

Recombination involving repetitive elements most likely would result in translocation-type mutations since resolution of homologous recombination intermediates must be resolved in regions of homology (Parsons and West, 1988). Homologous recombination involving repetitive elements located in non-homologous loci would restrict resolution of the intermediate structures to the repetitive element. The lack of homology beyond the repetitive element would decrease the likelihood of gene conversion of non-homologous coding sequences. On the other hand, homologous recombination between the hprt locus and the autosomally linked pseudogene sequence could result in gene conversion of the hprt locus since there is considerable homology between the coding sequence of hprt and its pseudogene sequence. If homologous recombination repair involving restriction enzyme damage induced at the hprt locus resulted in gene conversion, the hprt locus would be the recipient of new information. Gene conversion using homologous sequences in the autosomal pseudogene sequence as template would almost certainly result in mutation. The sites of mutation would, probably, be distal from the initial site of DNA breakage, a phenomenon frequently observed with gene conversion. RFLP and sequence analysis of exon-specific bands of gene conversion-mutants may not show changes at the restriction site due to the production of gene conversion events at sites distal to the original DNA break. This may explain why no changes were observed at restriction sites in the mutants analyzed. Gene conversion at the hprt

locus would remain undetectable in multiplex PCR analyses, unless "mutagenic" gene conversion of hprt sequences resulted in sequence changes so extensive as to prohibit binding of multiplex oligonucleotides. Amplification of exon-specific bands would be normal.

In addition to gene conversion events, there are several other possibilities that may explain the absence of changes at restriction sites in the clones showing apparent point mutations: 1) The presence of a translocation or inversion event in which the breakpoints are contained in the introns would be undetectable by multiplex PCR, PCR RFLP analysis or sequence analysis of exon-specific fragments. Such large chromosomal rearrangements have been shown to take place following restriction enzyme treatment (Winegar et al., 1990). As discussed, there are many mechanisms by which such large chromosomal re-arrangements may be produced. 2) Damage induced by restriction enzymes in 5' control flanking regions could disrupt normal expression of hprt gene product while molecular analysis of exon material would appear no different from that in parental cells. The inability to transcribe hprt mRNA, because of damage in the promoter region, would confer an HPRT-deficient phenotype while leaving the wild-type gene sequence intact. 3) A very likely assumption is that damage induced by the restriction enzymes Sau3A I and Rsa I at their restriction sites may not have effectively inactivated the hprt gene. Perhaps these sites are not important regions in the gene and would therefore never be selected as mutations that confer resistance to 6-thioguanine. It has been demonstrated that some mutations in genes, primarily single base changes, do not

give rise to a mutant phenotype. These sites have been dubbed silent sites, that is, no single mutation at that site can give rise to a mutant phenotype (Waters et al., 1991; Jaberaboansari et al., 1991). Only damages that led to substantial deletions of pieces of coding DNA of the hprt gene, or that resulted in changes at locations other than a restriction site, could be selected at the stringency of selection used in this study. The studies of Waters et al.,(1991) and Jaberaboansari et al.,(1991) also demonstrated the presence of DSM sites (detectable single mutation), that is sites at which some or all three possible base substitutions are known to give rise to a mutant phenotype. These DSM sites might then appear as mutational hotspots in a gene resulting in a non-random distribution of mutations. Mutagenic events at a DSM site in hprt (due to gene conversion distal to the original DNA break,or some other type of repair error) would almost certainly result in an inactivation of HPRT. 4) The argument that apparent point mutations arose spontaneously can reasonably be ruled out. While the spontaneous mutation rate at the hprt has been shown to give rise to significant background frequencies of so-called "jack-pot" mutations, it is most unlikely that this has occurred in this study. Firstly, one of the ways to avoid the appearance of "jack pot" mutations is, prior to treatment, to grow the parental cells for 1 week in HAT medium. This growth period selects against any pre-existing HPRT-deficient cells. In this study, parental cells were maintained in HAT medium for one week prior to electroporation. Secondly, the lack of any 6-thioguanine-resistant cells in the control cultures indicates that the spontaneous background frequency was very low in this study, especially when compared to the high induced mutation frequencies

observed with restriction enzyme treatment. In light of this, it is very unlikely that spontaneous events could account for such a large number mutations in the restriction enzyme-treated cell populations.

When comparing the differences in the mutational spectra generated with Rsa I and Sau3A I, it becomes clear that different restriction enzymes have different efficiencies in mutation induction. Differences in the location of Rsa I sites and Sau3A I sites may be responsible for their efficiencies in mutant induction at the hprt locus. Similarly, a consideration of these differences may be extended to the efficiency and type of mutations induced at other loci. In contrast to the observations of Dr. William Morgan (personal communication), digestion of the K1BH4 CHO cells with Pvu II in our study did not yield a significant number of mutations at the hprt locus, even though induced aberration frequencies were quite high. This may be due to differences in the distribution of Pvu II restriction sites in the target gene,differences in the organization of the gene locus (intron/exon), or differences in the local DNA environment at the endogenous hprt locus and the insertion site of the aprt locus in AS52 cells. Differences in the local DNA structure at particular restriction enzyme sequences are likely to be important in the ability of that enzyme to digest its target sequence. The packaging of mammalian DNA into chromatin, methylation patterns, complexing with DNA-binding proteins, and sequence-specific geometric structural requirements result in a variety of novel DNA conformations (for review, see Palecek, 1991). Bearing this in mind, it becomes clear that not every restriction sequence in the genome may be available for restriction enzyme digestion.

Further analysis of clones described in this chapter may elucidate the nature of the mutations involved. Additional DNA sequence analysis of these clones may indicate point mutations at restriction sites. Northern analysis of RNA extracts from these clones will show if the hprt gene is transcribed and may shed light on damage occurring in 5' controlling elements. Northern analysis will also aid in identifying those clones possessing translocation events in the hprt gene by changes in or lack of hprt mRNA products. The detection of translocations and inversions is best determined cytogenetically. The technique of *in situ* hybridization would lend itself particularly well to identifying which clones involve translocation events and if specific chromosomes are involved in these rearrangements, particularly with respect to the involvement of known fragile sites.

We have demonstrated that restriction enzymes are indeed capable of inducing mutations at a target gene and have further demonstrated that a large number of the induced mutations are due to deletions of hprt coding material. Further studies using more than one restriction enzyme may improve the specificity of the induced deletions. Additionally, restriction enzyme mutation analysis may now be extended to other non-essential selectable loci. These future directions will be discussed in the next chapter as well as other possibilities for the utilization of restriction enzymes for producing specific genomic alterations.

SUMMARY AND FUTURE DIRECTIONS

The purpose of these studies was to demonstrate the feasibility of using restriction enzymes to produce specific mutations in a target gene. In order to perform this investigation, a more effective and reliable means of introducing restriction enzymes into CHO cells had to be developed. Initial restriction enzyme-mutation studies used osmolytic shock to generate mutations at the hprt locus, but induced mutant frequencies were not significantly above background. Electroporation provided the most effective and consistent means of introducing restriction enzymes into CHO cells. Following treatment with the restriction enzymes Sau3A I and Rsa I, chromosome aberrations were induced in a dose dependent manner, cell survival was reduced, and mutation frequencies at the hprt locus were significantly above background. Clearly, restriction enzymes can be used to generate mutations in CHO cells in a dose dependent manner. While other laboratories have also demonstrated the ability of restriction enzymes to generate mutations at the hprt locus, the increase in mutation frequency above background was not as marked as in the studies presented here. Presumably, this is due to the method used to introduce the enzymes into the cells. Furthermore, in all other previous restriction enzyme mutation studies, no molecular analysis had been performed on the resulting mutants. We have demonstrated that multiplex PCR provides a rapid means of screening restriction enzyme-induced hprt mutants for specific deletions. It is interesting to note that although both of the enzymes that generated mutations at the hprt locus were capable of generating deletions in the target gene, the types of deletions observed

between the two enzyme treatments differs significantly. While the restriction site frequencies of the two enzymes Sau3A I and Rsa I are not significantly different, their cut-end structures are and this may, in part, account for the differences in the spectra of observed deletions. The distribution of Rsa I sites throughout the hprt locus may be such that many Rsa I sites are not available for digestion or may not be in close enough proximity to interact to generate the same spectrum of deletions as observed with Sau3A I. This is not to say that Rsa I is less efficient at inducing deletions at the hprt locus since the frequency of induced deletions is not significantly different between the two enzymes, but that there are differences in the fate of double-strand breaks induced by these two enzymes. As discussed in detail in Chapter 3, the difference may also be due to differences in the stability of the termini at the restriction enzyme-induced double-strand breaks. The difference in stability between the blunt-end terminus produced by Rsa I and the four base cohesive-end terminus induced by Sau3A I may result in different probabilities of their subsequent involvement in alternate repair pathways.

The differences in the repair of double-strand breaks induced by cohesive- and blunt-end cutting enzymes remains a topic of debate. While the calculated restriction site frequencies of the two enzymes used in these studies do not appear to be significantly different with respect to their frequency throughout the genome, on a smaller scale such as the hprt locus the difference is significant enough to result in a different spectrum of induced deletions. Ideally isoschizomers should be used that differ only in their cut-end termini structure. However, until an isoschizomer pair can be found to test this hypothesis, the differences in the stability of cohesive and blunt-

end termini will remain the subject of speculation. Attempts in our laboratory to address this problem with the isoschizomer pair Sma I and Xma I have been inconclusive due to differences in the temperature sensitivities between the two enzymes.

An analysis of the breakpoints in the resulting mutants in this study has yet to be performed. It is very likely that most of the breakpoints occurred at restriction sequences in the introns of the hprt gene since they comprise approximately 97% of the locus. Since the introns of the Chinese hamster hprt gene remain to be sequenced an easy analysis of these breakpoints cannot be performed at this time. Once available, these sequences could be used to generate a restriction map of the entire locus. Candidate restriction sequences in the introns may be examined for their involvement as potential break points by designing oligonucleotide primers that flank the restriction sequence. Several different PCR strategies may then be designed to determine the exact nature and size of the deletions induced by restriction enzymes in this study. The oligonucleotide primers used for multiplex amplification of the Chinese hamster hprt gene could be used to perform such a study, but conditions for amplifying fragments as large as 10 kilobases must first be established.

As mentioned earlier, a number of the hprt-deficient clones identified as apparent point mutants by multiplex PCR may likely possess translocations or inversions involving the hprt gene. Translocations may be detected by in situ hybridization using hprt cDNA fragments as a probe. It will be interesting to observe if any translocation events are observed involving hprt and known fragile sites or recombination hotspots. It was proposed by Winegar and Preston (1987) that

restriction enzymes might be used as a probe for recombination hot spots and fragile sites. A further analysis of some of the mutants generated in these studies may provide some information on that question.

It is evident from these studies that the specificity of the restriction enzymes is important in the spectrum of mutations generated. The enzymes used in this study have relatively high restriction site frequencies and as such demonstrate relatively little specificity in the spectrum of deletions induced. Restriction enzymes with less frequently occurring restriction sites may be able to increase the specificity of deletions in a target gene. Additionally, the use of two or more different restriction enzymes with rarely occurring restriction site frequencies would also increase the specificity of induced-deletions. However, as has been demonstrated here, not all restriction sites may be available for digestion. Additionally, the specificity of induced deletions is limited to the availability of known restriction enzyme sequences and the ability of these enzymes to perform satisfactorily in the nucleus of the cell. Some restriction enzymes are more likely to generate DNA damage than are others based on methylation of restriction sequences, optimal salt concentrations, temperature requirements, and other conditions contributing to the efficiency of digestion.

One of the most logical next steps for this project is to extend restriction enzyme-induced mutagenesis to other selectable loci such as thymidine kinase. It has been demonstrated that the enzyme Pvu II is weakly mutagenic at this locus (Singh and Bryant 1991) in CHO cells. However, no molecular analysis of the induced mutants was performed in this study and enzyme strategies were poorly chosen. Different enzyme strategies will need to be considered for each new target gene

chosen. Additionally, it will be interesting to see what effect using two different enzymes together has on the specificity of production of mutations. Some early attempts at using two enzymes together were made in this study (data not shown) but met with little success. Considerations should also be made concerning the stringency of selection. Changing the stringency of selection may allow the isolation of mutants with partial gene product activity. By decreasing the stringency of selection perhaps a more accurate estimate of the mutagenic activity of restriction enzymes may be made. In these studies only complete inactivation of the target gene was able to be selected at the stringency used. By decreasing the stringency of selection, the effects of a series of well-defined deletions may be determined for the targeted gene.

The ability of restriction enzymes to produce double-strand breaks in the DNA has other implications beyond mutagenesis. Since it has been widely demonstrated that DNA double-strand breaks can increase the frequency of both homologous and non-homologous recombination, restriction enzymes may potentially be used to drive recombination processes. In the same manner that the site-specificity of restriction enzymes can be used to generate specific mutations and sets of deletions, the site specificity may also be used to target homologous recombination of exogenous DNA fragments to specific chromosomal locations. Site specificity would be determined by the restriction sequence frequency, the availability of restriction sequences for digestion, and whether the "geography" of the local DNA environment at the chromosomal restriction site is conducive to recombination. Theoretically, the

greatest limiting factor for targeting such a reaction would be the presence of restriction sites at the area of interest.

The ideal strategy would be to choose enzymes which are capable of cutting only at a specific site in the target locus and nowhere else in the genome. Obviously, such a restriction enzyme is not available, but several strategies for creating enzymes with such specificities have been proposed over the last several years. By linking EDTA.Fe to the amino terminus of a synthetic peptide capable of recognizing the Hin recombination site, it has been demonstrated that site specific-double-strand breaks may be induced within a seven-base region at the Hin recognition sequence (Sluka et al.,1987). Other attempts make use of the specificity of synthetic oligonucleotides. Oligonucleotides may form triple helix structures and when linked to EDTA in the presence of iron may generate a diffusible oxidant capable of breaking the DNA in a localized area usually limited to about 8 bases (Moser and Dervan, 1987). Oligonucleotides have also been linked to Staphylococcal nuclease (Corey and Schultz, 1987; Pei et al., 1990) and to an ellipticine derivative capable of cleaving DNA upon photochemical activation (Perrouault et al., 1990). While all of these systems are capable of breaking the DNA in a site-specific *region* of the DNA, another method allows the production of a double strand break in the DNA at a single specific site. Type II restriction enzymes recognize specific sequences in the DNA and then cleave the DNA at a site removed from the restriction sequence. The type II restriction enzyme Fok I recognizes a specific asymmetric sequence of 5 bases and then produces a 4 base cohesive end-double strand break at a site 9 bases 3' to the restriction sequence (Sugisaki and Kanazawa, 1981). By designing an oligonucleotide

with a hairpin containing the Fok I restriction sequence at the 5'end, and a single-strand sequence complementary to a specific site in the DNA, digestion with Fok I may produce specific double-strand breaks at any particular and unique site in the DNA (Szybalski, 1985; Podhajska and Szybalski, 1985; Koob et al., 1988). To ensure the site specificity of the Fok I mediated site-specific break, it would first be essential to methylate the DNA with Fok I methylase to restrict cutting at endogenous Fok I sites. However methylation of endogenous Fok I sites may prove to result in high cell mortality and preliminary experiments would need to be performed to determine the feasibility of such an approach. The ability to generate a double-strand break at any site would allow the deletion of very specific pieces of DNA from virtually any target gene provided there was a method of selection. Additionally, the production of a double-strand break at a specific site could be used to greatly increase the specificity of both homologous and non-homologous recombination processes at this site. This may prove to be useful in inserting reporter genes next to sequences of interest as well as in targeting insertions in gene therapy and insertional inactivation studies which have traditionally relied on random retroviral insertion processes. We feel that our studies have provided preliminary information on the feasibility of such approaches.

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VITA

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