

INVESTIGATION OF MUTATIONS INDUCED BY
RADIATION AND RESTRICTION ENDONUCLEASES

Kim E. Haworth

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1995

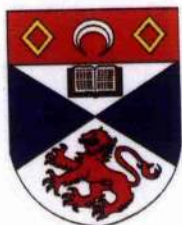
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**INVESTIGATION OF MUTATIONS INDUCED BY
RADIATION AND RESTRICTION ENDONUCLEASES**

KIM E HAWORTH



**THESIS SUBMITTED FOR THE DEGREE OF PH.D. TO THE
DEPARTMENT OF BIOLOGY AND MEDICAL SCIENCES,
UNIVERSITY OF ST. ANDREWS.**

NOVEMBER 1994



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Declaration

I, Kim Elizabeth Haworth, hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification

Signed K.E. Haworth

Date

31-1-95

I was admitted to the faculty of Science of the University of St. Andrews under Ordinance general No. 12 on NOV 91 and as a candidate for the degree of Ph.D on OCT 92

Signed K.E. Haworth

Date

31-1-95

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the degree of Ph.D.

Signature of Supervisor

Dr Peter Bryant

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Acknowledgements

I would like to thank my supervisor Dr P.E. Bryant for all his help, advice, understanding and his enormous patience (and for putting up with my bad moods) throughout the duration of this project. His advice concerning this thesis has been greatly appreciated although many of the mistakes or areas of controversy that remain are probably due to my own pigheadedness. I would also like to say a special word of thanks to Mr John Macintyre for his excellent technical support and for continually digging me out of the "holes" that I have continually dug myself into! I would like to thank my colleagues in the lab, Shoshana, Nan, Peter, Gary, Jean, Tina, Lynne and Angus for all their help, advice and strong black coffee throughout my three year stint at St. Andrews. I feel that it is necessary to express my gratitude to Ian and Chris upstairs for their invaluable help, advice and sarcasm and for Ian for the race to the finish (and the forthcoming meal at the Vine Leaf!!), to Professor A. T Natarajan at Leiden, Holland and Dr Judy Fantes at the Western General Hospital, Edinburgh for helping me with the FISH work. I would like to say a big thanks to Dr Richard Ireland for his help with the statistics and correcting my English (and of course his love, understanding and the use of his car!!!). My parents and family have also helped and encouraged me throughout the project and my undergraduate days. Without their support (both moral and financial) I doubt if I could have ever completed. To them I am deeply grateful. I feel that it is important to mention my flatmates past and present, for putting up with my bouts of grumpyness and frustration as the end of the project approached. I would however, like to say a special thank you to Carol, Tana, Duncan, Nigel and Hylarie, who not only helped me maintain my sanity but, also took such excellent care of me in the spring of 1992 while I was ill and to my parents for continuing the care. Also deserving of a mention are Dr Bob Callow and Dr David Southern at the University of Manchester, who dragged me (kicking and screaming!!) through my undergraduate degree and without whom I would have quit Genetics years ago. Finally, I would like to thank British Nuclear Fuels plc for their financial support and also the many other people who helped me along the way, the list too long to mention, to them I am also very grateful.

Abbreviations

AMP: Adenosine monophosphate

aprt: Adenosine phosphoribosyl transferase gene

5-AZ: 5 Azacytidine

Bam HI: Restriction endonuclease isolated from *Bacillus amyloliquefaciens*,
recognising and cleaving the nucleotide sequence G↑GATCC.

BCIP: 5-Bromo-4-chloro-3-indolylphosphate

bp: Base pairs

BSA: Bovine serum albumen

cDNA: Complementary DNA, a DNA copy of a mRNA molecule

CHO: Chinese hamster ovary cells

DABCO: 1, 4 Diazabicyclo (2, 2, 2) octane

DAPI: 4, 6 Diamido-2-phenylindole

DNase: Deoxyribonuclease

ds: Double stranded

dsb: Double strand DNA break

dNTP: Deoxyribonucleotide triphosphate

E.coli; *Escherichia coli*

Eco R1; Restriction enzyme purified from *E.coli* RY13 which cleaves the
nucleotide sequence G↑AATTC

EDTA: Ethylenediaminetetra-acetic acid

FCS: Foetal calf serum

FISH: Fluorescent *in situ* hybridisation

FITC: Fluorescein isothiocyanate

Gy: Gray, unit of radiation dose absorbed= 1 joule/Kg

GTE: TE solution containing 1% glucose

HAT: Hypoxanthine, Aminopterin and Thymidine media (Sigma)

HBSS: Hanks balanced salt solution

HBSS/BSA: Hanks balanced salt solution with 1% Bovine serum albumen

hprt: Hypoxanthine guanine phosphoribosyl transferase gene

IMP: Inosine monophosphate

Kbp: Kilobase pairs

LET: Linear energy transfer

MEM: Minimal essential medium

MW: Molecular weight

NBT: Nitroblue tetrazolium

NFDM: Non fat dried milk solution

pBR322: Plasmid purified from *E.coli*

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PE: Plating efficiency

Pvu II: The restriction enzyme isolated from *Proteus vulgaris* recognising and cleaving the sequence GAG↑CTG

PI: Propidium iodide

Pst I: Restriction endonuclease isolated from *Providencia stuartii* which cleaves the nucleotide sequence CTGCA↑G

rDNA: Ribosomal DNA

RCF: Relative centrifugal force, calculated by the equation $1.12 \times r \times \frac{\text{RPM}}{1000} \times \frac{\text{RPM}}{1000}$

RE: Restriction endonuclease

RFLP: Restriction fragment length polymorphism

RNase: Ribonuclease A

rpm: Revolutions per minute

SA-AP: Streptavidin-alkaline phosphatase conjugate

SD: Standard deviation

SDS: Sodium dodecyl sulphate

SE: Standard error

SLO: Streptolysin O, a bacterial cytotoxin isolated from *Streptococcus pyogenes*

SSB: Single stranded DNA break

Taq: Thermostable DNA polymerase isolated from the bacteria *Thermus aquaticus*

TBE: Tris borate EDTA electrophoresis buffer

TE: Tris EDTA solution

6TG: 6 thioguanine

tk: Thymidine kinase gene

TFT: Trifluorothymidine

Tris: Tris (hydroxymethyl) methylamine

UV: Ultra violet radiation

V(D)J: Variable, diversity, joining recombination

V79-4: A male lung fibroblast cell line

XR-1: An X ray sensitive mutant of CHO-K1

xrs-5: An X-ray sensitive dsb repair deficient mutant of CHO-K1

Abstract

The effects of gamma radiation and restriction endonuclease (RE) induced DNA double strand breaks (dsb) upon the mutation frequency and the surviving fraction of three Chinese hamster cell lines V79-4, CHO-K1 and an X-ray sensitive dsb repair deficient cell line *xrs-5* were studied.

The X-ray sensitive *xrs-5* cell line was shown to be more sensitive to both the lethal and the mutagenic effects of gamma radiation having a substantially lower surviving fraction and a higher thymidine kinase (*tk*) mutation frequency per unit dose than the parental CHO-K1 cells. The frequency of induced *hprt*⁻ mutations in the V79-4 cell line was comparable to the induced frequency of *tk* mutations in the CHO-K1 cells.

The effect of blunt- and cohesive- ended dsb upon the surviving fraction and the induced mutation frequency was studied by porating different Chinese hamster cell lines (CHO-K1, V79-4 and *xrs-5*) with RE using Streptolysin O (SLO). The surviving fraction of the different cell lines was reduced with increasing concentrations of *Pvu II*. Increases in the concentration of *Pvu II* produced increases in the frequency of hypoxanthine guanine phosphoribosyl transferase (*hprt*) mutations in the V79-4 cells and *tk* mutations in the CHO-K1 and *xrs-5* cells. However, the *xrs-5* cells were shown to be hypomutable to *Pvu II* compared with the parental CHO-K1 cells. *EcoRI* was ineffective at inducing *tk* mutations in the CHO-K1 cells but was as effective as *Pvu II* at inducing *hprt* mutations in the V79-4 cells.

None of the spontaneously induced V79-4 *hprt*⁻ mutant cells were shown to have observable molecular deletions when analysed by PCR deletion screening. One third of the radiation induced *hprt*⁻ mutants were shown to be deletions. However, too few mutant cells were analysed for any non-random distribution of deletions to be observed. Half of the *hprt*⁻ mutants induced by SLO poration alone were shown to be due to deletions of one or more exons. The distribution of the DNA deletions in SLO *hprt*⁻ mutations appeared to be non-random. The PCR amplification products of exons 7&8 were more frequently lost than any of the other exons in the *hprt* gene. It has been suggested that the SLO provided by the manufacturer used to porate the cells was contaminated with small amounts of endonucleases and exonucleases. The *Pvu II* induced deletions also appeared to be non-randomly distributed. The PCR amplification products of exon 2 were more frequently absent than any of the other exon products. Sequence data from the EMBL library indicated that *Pvu II* had a restriction site adjacent to the exon 2 nucleotide sequence of the Chinese hamster *hprt* gene but not in or bordering the other exons. This provides evidence that the blunt-ended dsb plays a role in the

production of mutations.

Mutation studies indicated that there is only one active copy of the autosomally located thymidine kinase gene in the CHO-K1 cells and their daughter cell lines (Singh and Bryant, 1991). However, whether the other homologous copy has been inactivated or deleted is not known. Experiments attempting to locate the thymidine kinase gene(s) were performed using FISH and a mouse tk cDNA probe. However, these attempts were unsuccessful.

Chapter 1

Introduction

Genetics presents a subtle and universal dilemma, the problem of knowledge - The greatest dilemma will be that of being aware of our own fate or of that of our offspring.

-Steven Jones, The Language of the Genes

1.1 Importance of Radiation Genetics

Although radioactivity has only been known to exist for approximately a century, radiation has been present on Earth since its birth. It was present in the primeval world of single celled organisms hundreds of millions of years ago, damaging their genetic material with high energy particles and photons. Some scientists believe, for example Mani (1991), that it was cosmic radiation that limited the primeval world to the oceans for many millions of years. This was until the ozone layer had been built up around the once unprotected atmosphere of the Earth. This was generated by the escape of plant waste products (Oxygen) into the atmosphere to produce a shield from the assaults of cosmic radiation as it was converted to ozone. Life has adapted to the constraints of radiation and can repair much of the damage produced but not with 100% efficiency. In fact, mutation and genetic variation are the essential factors upon which evolution acts.

Man throughout his evolution has been exposed to radiation in the form of cosmic rays travelling to Earth from the Sun and from radioactive isotopes in the soil. The Sun produces electromagnetic radiation and radioactive isotopes emit both particulate and electromagnetic radiation as they decay to form a more stable state. The twentieth century has brought with it the "Radiation Age". New technologies have increased mans' exposure to radiation, for example air travel, television, medical treatments, nuclear power and nuclear weapons. Medical X-rays and radiotherapy have helped to save lives, X-rays being used in the diagnosis

of broken limbs and revealing cancer at an early stage. Radiotherapy has extended the lives of cancer afflicted patients. Nuclear power was developed to replace the diminishing resources of fossil fuels, attempting to produce endless amounts of clean and cheap electricity. Radiation is a powerful tool which must be treated with care and respect. Although there has been a century of biological research, relatively little is known about the causes of the biological changes which are the result of radiation exposure and the consequences of the evolutionary effects of an increasing exposure to radiation. The study of the effects of ionising radiation on the living cell and the prevention of abuse of radiation is essential if the human race is to ensure that it does not blunder into worse dilemmas than it seeks to solve.

1.2 What is a mutation ?

A mutation is a heritable change in the nucleotide sequence of a DNA molecule (for example, Lewin, 1990; Adams *et al.*, 1986; Watson, 1973). The protein coding segments of the DNA molecule are split up into codons. A codon is a triplet of adjacent nucleotides which code for a specific amino acid. Some of the mutations that occur in the DNA molecule are synonymous (producing no change in the amino acid product). This is due to the degeneracy of the genetic code. These changes are undetectable in the phenotype of the organism. Some mutations are, however of the non synonymous type, producing changes in the amino acid sequence and often the protein structure. A mutational change is either the result of DNA damage which has been misrepaired by the cell or error in DNA replication.

1.3 The *de novo* biosynthesis of nucleic acids and the purine and pyrimidine salvage pathway mutation systems

Purines and pyrimidines are the two basic building blocks of nucleic acids. Without a ready supply of purines and pyrimidines, DNA replication and cell division would cease. Cells are capable of manufacturing purines from very basic constituents. This pathway is called *de novo*. But they are also capable of recycling purines and pyrimidines from spent nucleic acids. This is called salvage.

a) Purines

i) Biosynthesis of purines

The first step in the synthesis of purines is the synthesis of the purine ring and the second step is the conversion of the purine ring into deoxyribonucleotides. Purines can be synthesised from a scaffolding of ribose-5-phosphate. The synthesis involves a wide variety of other chemical substrates and a large amount of energy, which is supplied in the chemical form of ATP. Figure 1.3.1 shows the source of each of the atoms in the purine ring. The origin of the atoms in the

ring was studied by feeding radioactive nucleotide precursors to pigeons and analysing the waste products (Stryer, 1988). The *de novo* pathway of purine synthesis is similar in most organisms from *E.coli* to pigeons to man.

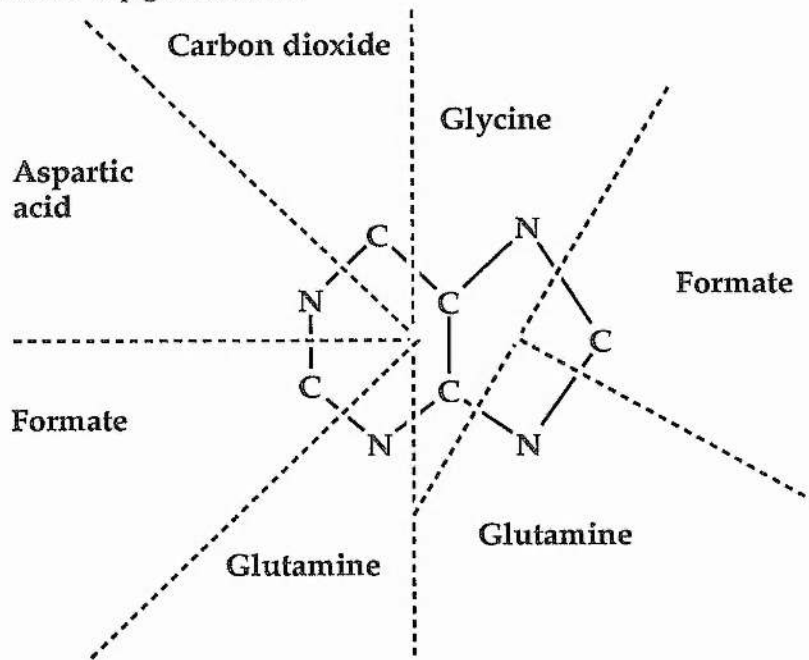


Figure 1.3.1: The origin of the atoms in the *de novo* biosynthesis of purines. There are 11 complex biochemical reactions starting with the basic ribose-5-phosphate and ending with the purine building block inosine monophosphate (from Adams *et al.*, 1986).

The completed ribonucleotide is inosine monophosphate (IMP). IMP is the common precursor of both adenosine and guanosine triphosphate. The manufacture of purines by the *de novo* pathway requires much energy. However, the cell is capable of recycling purines from spent nucleic acids which is far more efficient in terms of energy than the *de novo* pathway. The salvage metabolic pathway is summarised in Figure 1.3.2.

ii) The purine salvage pathway

DNA and RNA from spent nucleic acids are hydrolysed to produce nucleotides and nucleosides. Hydrolytic cleavage of the glycosidic bond between the sugar and the base results in the production of free ribose sugars, pyrimidines and purines. The enzyme adenosine deaminase converts the purines into hypoxanthine which is then phosphorylated by the enzyme *hypoxanthine guanine phosphoribosyl transferase* (HGPRT) to produce the inosine monophosphate (IMP) which is a precursor of the nucleotides and are used to build DNA.

Nucleotide manufacture is controlled in the cell by a negative feedback mechanism. IMP is a

product in both the *de novo* and the salvage pathway. A build up of IMP inhibits the *de novo* pathway.

iii) *The hypoxanthine guanine phosphoribosyl transferase gene (hprt)*

The *hprt* gene is a non-essential gene for cells in culture, which is found in the genome of eukaryotic organisms. It is responsible (see Figure 1.3.2) for the salvage of purines within the cell and is constitutively expressed. In mammals, this gene is located on the X chromosome. It is highly conserved at the protein level and in the exon regions of the DNA. Surprisingly, this conservation extends even to the malarial parasite *Plasmodium falciparum* (King and Melton, 1987). In humans, mutations in the *hprt* gene (which result in the production of an excess of purines in the blood) are responsible for the severe sex linked genetic disorder Lesch-Nyhan Syndrome. The children afflicted with this disabling disease are mentally retarded and show a tendency for self-mutilation. Less severe mutations are sometimes known to cause familial gout, renal stones and neurological abnormalities (Davidson *et al.*, 1988).

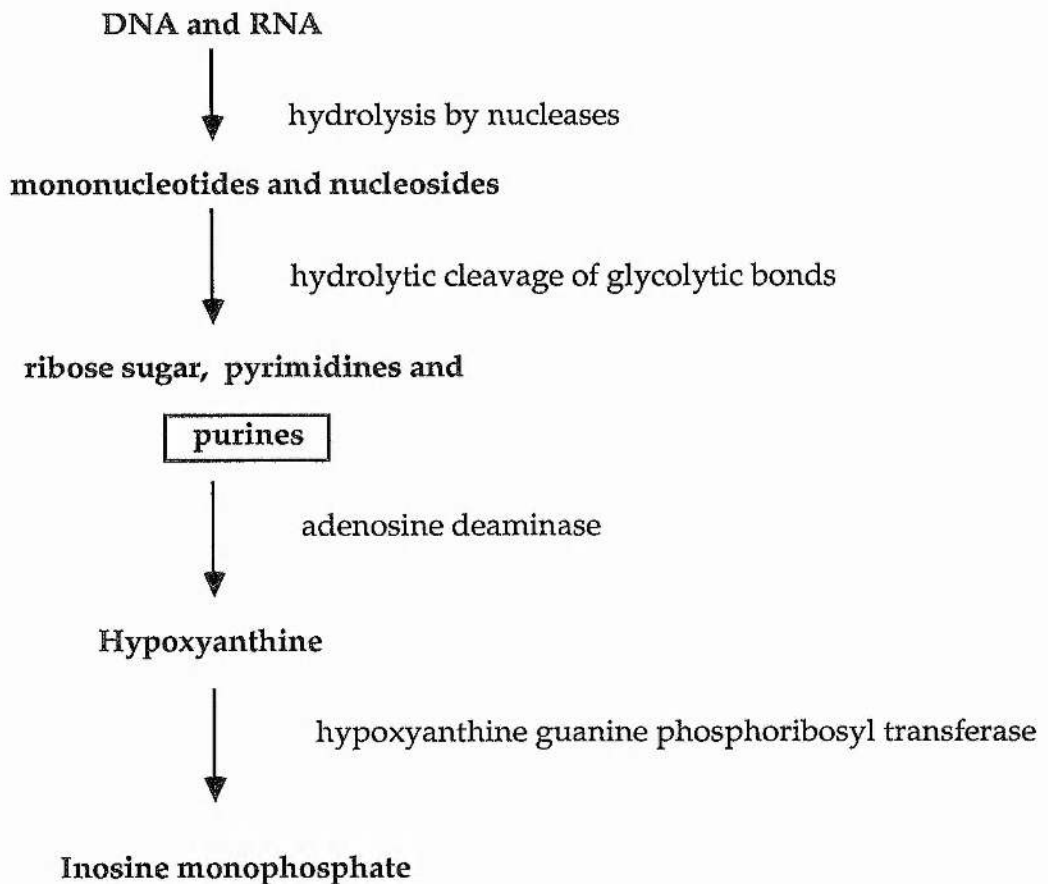


Figure 1.3.2: The purine salvage pathway showing how the nucleotide building block inosine monophosphate is produced from spent nucleic acids.

iv) The hypoxanthine guanine phosphoribosyl transferase (hprt) mutation system

It is possible to select a cell population for the preferential survival of cells which have an inefficient or deficient purine salvage pathway (not lethal to cells in culture). This inefficiency may be due to alterations in the genetic code, producing an aberrant or absent HPRT protein product. Some competitive inhibitors, with varying efficiencies, have been found which select *hprt* deficient mutants (Thacker *et al.*, 1976 and references therein). It has been demonstrated that 6-thioguanine is the most effective inhibitor to date (Thacker *et al.*, 1976 and references therein). The chemical structures of hypoxanthine and 6-thioguanine are shown in Figure 1.3.3.

iii) The Adenine phosphoribosyltransferase (*aprt*) mutation system

The *aprt* gene codes for an enzyme which is involved in the salvage of the purine adenine. The *aprt* gene is autosomally located and is relatively small in size, only 2.5Kbp in the Chinese hamster as compared to the *hprt* gene which in the Chinese hamster is in excess of 40Kbp. The Chinese hamster *aprt* gene has been fully sequenced (Nalbantoglu *et al.*, 1986) and is located on chromosome 3. In the normal diploid Chinese hamster there are two copies of the *aprt* gene present and expressed which consequently makes the selection of *aprt*⁻ mutations impractical (Sankaranarayanan, 1991). However, cell lines have been isolated from the Chinese hamster which are heterozygous and hemizygous at the *aprt* locus. Predominantly the molecular work at the *aprt* locus has been performed on a Chinese hamster ovary cell line which is hemizygous at the *aprt* locus (eg. Meuth *et al.*, 1987; Grovosky *et al.*, 1986).

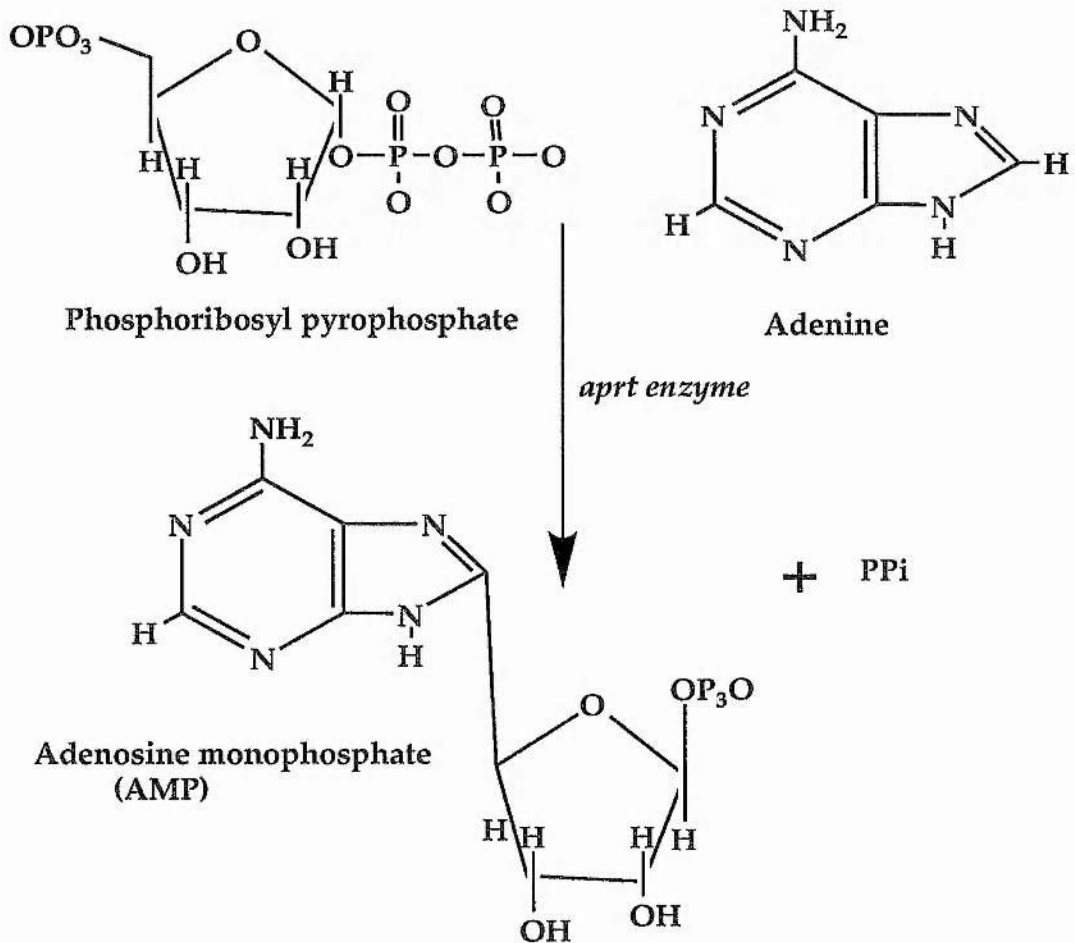


Figure 1.3.4: The synthesis of AMP from the purine adenine and phosphoribosyl pyrophosphate, catalysed by the enzyme APRT (diagram redrawn from Stryer, 1988).

The *aprt* gene product catalyses the reaction between the purine adenine (salvaged from spent nucleic acids and products of digestion) and phosphoribosyl pyrophosphate (Figure 1.3.4) thus producing a mononucleotide in a single enzyme catalysed step.

The enzymatic reaction is controlled by a negative feedback mechanism, where the build up of AMP inhibits the initial reaction which produces it.

Cells which are deficient in the APRT enzyme can be selected for by culturing with the purine analogue 8-aza-adenine. The chemical structures of the nucleotide adenine and the analogue 8-aza-adenine are shown in Figure 1.3.5.

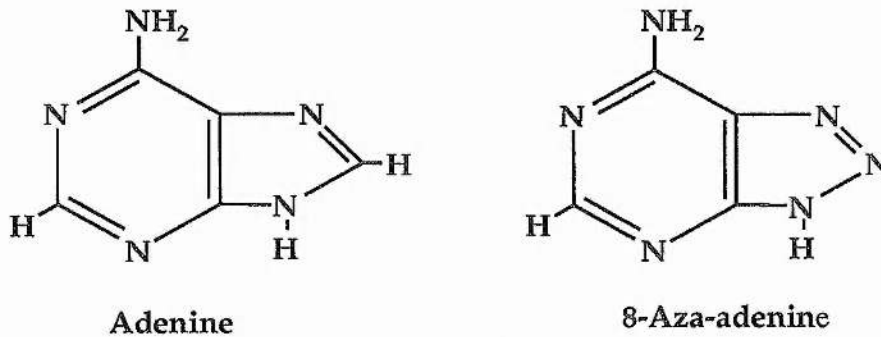


Figure 1.3.5: The chemical structure of the nucleotide adenine and the nucleotide analogue aza-adenine (diagram redrawn from Walker *et al.*, 1979).

b) Pyrimidines

i) Biosynthesis of pyrimidines

The *de novo* biosynthesis of the pyrimidine nucleotide precursor uridine-5-monophosphate from the basic constituents glutamine, aspartic acid and carbon dioxide requires six enzymatic reactions (Rawn, 1983). The origins of the atoms in the pyrimidine ring are shown in Figure 1.3.6.

The *de novo* biosynthesis pathway of the pyrimidine deoxyribonucleotide, thymidine monophosphate from the uridine-5-monophosphate precursor is shown in Figure 1.3.7.

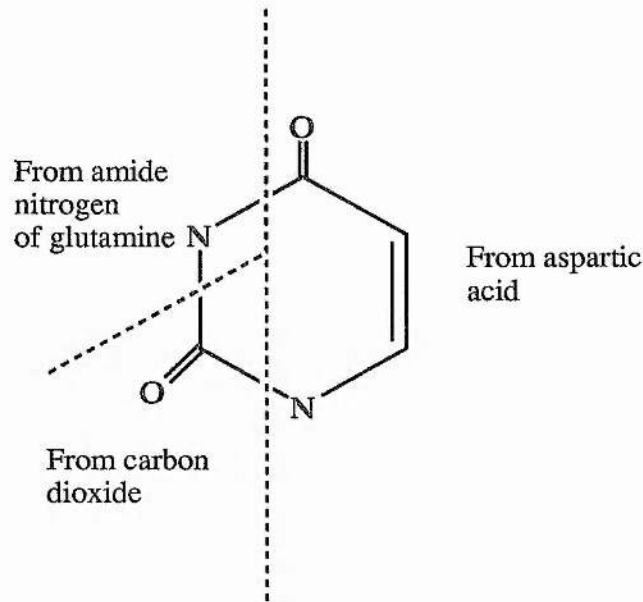


Figure 1.3.6: The origins of the atoms in the pyrimidine ring when manufactured by *de novo* synthesis (redrawn from Rawn, 1983). The reactions used to produce the pyrimidine ring involve the basic ingredients glutamine, aspartic acid and carbon dioxide.

The manufacture of thymidine monophosphate, a substrate of DNA synthesis by the *de novo* pathway is highly energy consuming. Hence, cells are often capable of manufacturing thymidine monophosphate from free cellular thymidine which is the product of the degradation of spent nucleic acids which is far more efficient and is called the pyrimidine salvage pathway. The *de novo* pathway involves the reduction of the ribonucleotide uridine-5-monophosphate by the removal of the oxygen from the hydroxyl of the ribose at carbon position 2 to produce the deoxyribonucleotide, deoxy-uridine monophosphate. This reaction is catalysed by ribonucleotide reductase. From this product, the biosynthesis of thymidine monophosphate is catalysed by the enzyme thymidylate synthetase. This is illustrated in Figure 1.3.7.

ii) The Pyrimidine Salvage Pathway

The process involves the phosphorylation of pre-existing thymidine by the enzyme *thymidine kinase* to produce thymidine monophosphate. The synthesis of thymidine monophosphate by both the *de novo* and the salvage pathways is controlled by a negative feedback mechanism where the build up of the product thymidine monophosphate inhibits the enzymes thymidylate synthetase and *thymidine kinase*.

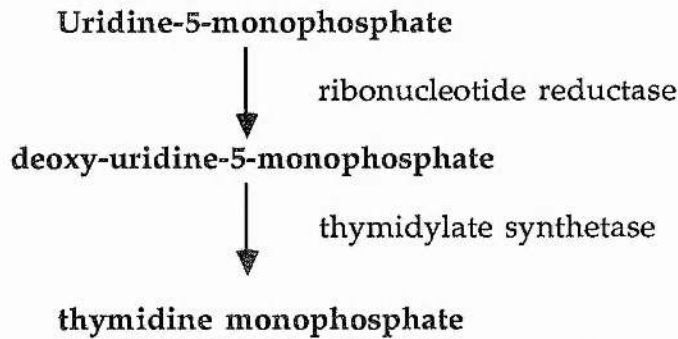


Figure 1.3.7: The manufacture of thymidine-5-monophosphate by the *de novo* pathway.

iii) The thymidine kinase mutation system

The *thymidine kinase* gene (*tk*) is an autosomal gene located on chromosome 17 in humans (Miller *et al.*, 1971), chromosome 11 in the mouse (Kozac and Ruddle, 1977) and chromosome 7 in the Chinese hamster (Stallings and Siciliano, 1981). The *tk* gene is a non-essential gene (a requirement of genes used in mutation assays) in cells maintained in culture. In diploid eukaryotic cells there are two copies of the *thymidine kinase* gene (for example, in the Chinese hamster there will be one copy of the gene on each of the chromosome 7 homologues). Genes controlling enzyme production tend to be recessive in nature. That is two copies of the altered gene need to be present before a change in phenotype is observed, making isolation of *tk*⁻ cells extremely difficult. However, some lines (for example the Chinese hamster ovary cell line, CHO-K1 and the mouse lymphoma line L5178tk⁺) have been shown to be hemizygous or heterozygous for the *tk* gene. These cell lines are therefore ideal for the study of mutation induction at the *tk* locus.

Trifluorothymidine (TFT) is an analogue of the base thymidine. TFT is used to select for cells which are deficient in the enzyme *thymidine kinase*. The chemical structures of the bases thymidine and trifluorothymidine are shown in Figure 1.3.8.

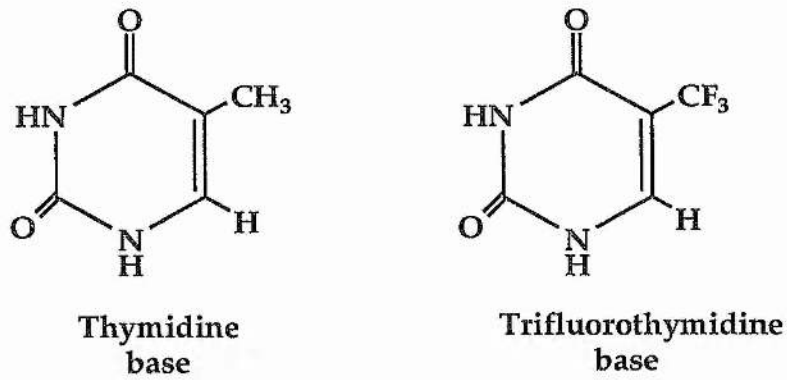


Figure 1.3.8: The structure of the nucleotide base of thymidine and the base analogue trifluorothymidine. The difference in structure is at the carbon (5) position. In TFT the CH_3 group is replaced by a CF_3 group.

Trifluorothymidine (TFT) is taken up into the cell from the surrounding medium and phosphorylated by the salvage pathway enzyme TK to produce TFT- monophosphate. TFT- monophosphate by negative feedback, inhibits the enzymes thymidylate synthetase and TK. Hence, the further production of thymidine-5-monophosphate ceases. The TFT-monophosphate is not a suitable substrate for DNA synthesis and the cell does not use it in the manufacture of DNA. Without a ready supply of thymidine monophosphate (the production of which the TFT-monophosphate has inhibited) DNA replication and cell proliferation ceases. Cells without TK activity will automatically manufacture thymidine-5-monophosphate by the complex *de novo* pathway. Therefore, in these cells, TFT will have no effect on the synthesis of DNA or cell proliferation. However, in cells which have an active *tk* gene, the TFT will be phosphorylated and DNA replication and cell division will be inhibited.

The *tk* gene is autosomally located. Cells which are heterozygous for this gene (having one functional and one non-functional allele) have been cultured. *Tk* mutation work in Chinese hamster cells has been performed on irradiated CHO-K1 lines (Singh and Bryant, 1991; Mussa *et al.*, 1990; Singh, 1991) but *tk*⁻ mutants have not been analysed at the molecular level. From the exceptionally high spontaneous mutation frequency, it is possible to ascertain that there is only one functional *tk* gene in the CHO-K1 genome. However, whether the other copy is present but inactivated or has been totally deleted is not known. There has been very little molecular work performed on the *tk* locus. Some work has been performed on the human B lymphoblast line TK6, which is heterozygous at the *tk* locus, having one functional *tk* copy and one non function *tk* copy. The two alleles of the gene can be distinguished using *Sac* I restriction fragment length polymorphisms (RFLP) and hybridisation of a human cDNA probe to the Southern blot (Yandell, 1986; Sankaranarayanan, 1991). Studies of these heterozygous loci are interesting since they enable the study of autosomal mutations. Also, in autosomal genes

there are more available mechanisms of somatic mutation. There are the usual base substitutions, micro-deletions and complete allele loss which are also found on X linked alleles but there is also the possibility of gene conversion by mitotic recombination which involve the exchange of DNA segments between homologous DNA sequences (Yandell *et al.*, 1986; Sankaranarayanan, 1991). It is believed that homologous recombination may also be as a method of somatic mutation. It has been shown to be a mechanism by which tk^- mutants with two non functional alleles revert back to the tk^+ phenotype (Benjamin *et al.*, 1991; Benjamin and Little, 1992).

1.4 Spontaneous Mutations

When a mutation is said to have occurred spontaneously, one concludes that it has not been deliberately induced, it has occurred at random simply through errors in the DNA replication machinery (Finchman, 1983; Brown, 1989; Lewin, 1990). However, DNA replication is astoundingly accurate, on average only producing approximately 1 error per $10^9 - 10^{10}$ bases copied (Rawn, 1983). Spontaneous mutations are predominantly base pair substitutions. One mechanism by which mutations are thought to arise is nucleotide tautomerism (see later). Other ways in which point mutations could arise are via deamination of the amino bases cytosine and 5-methyl cytosine. Both these models are discussed later. Insertions and deletions may also occur spontaneously. These may be the result of the transposition of transposable genetic elements. Other changes in phenotype may be the result of base modification rather than base substitution.

a) Mechanisms by which spontaneous mutations occur

i) Tautomerism

Each of the nucleotide bases exist in two different forms called tautomeric or structural isomers. Tautomeric isomers have the same chemical formulae but have different three-dimensional configurations (Brown, 1989). The two different forms are interconvertible. This conversion occurs by a change in the position of a double bond and the redistribution of the atoms of hydrogen (Finchman, 1983). The two forms are in equilibrium. The internal environment of the cell produces an equilibrium which greatly favours one tautomeric form. The different tautomeric isomers may have slightly different physical and chemical properties. Some of these rare tautomeric isomers are unable to form hydrogen bonds with the usual complementary nucleotide but are however, capable of forming hydrogen bonds with a "non complementary" base. It is possible for either the base acting as a template or the base which is to be incorporated to undergo a tautomeric shift. It is possible that this could occur on either the leading or the lagging DNA strand. This may result in a point mutation. An example of how this might occur is shown in Figure 1.4.1.

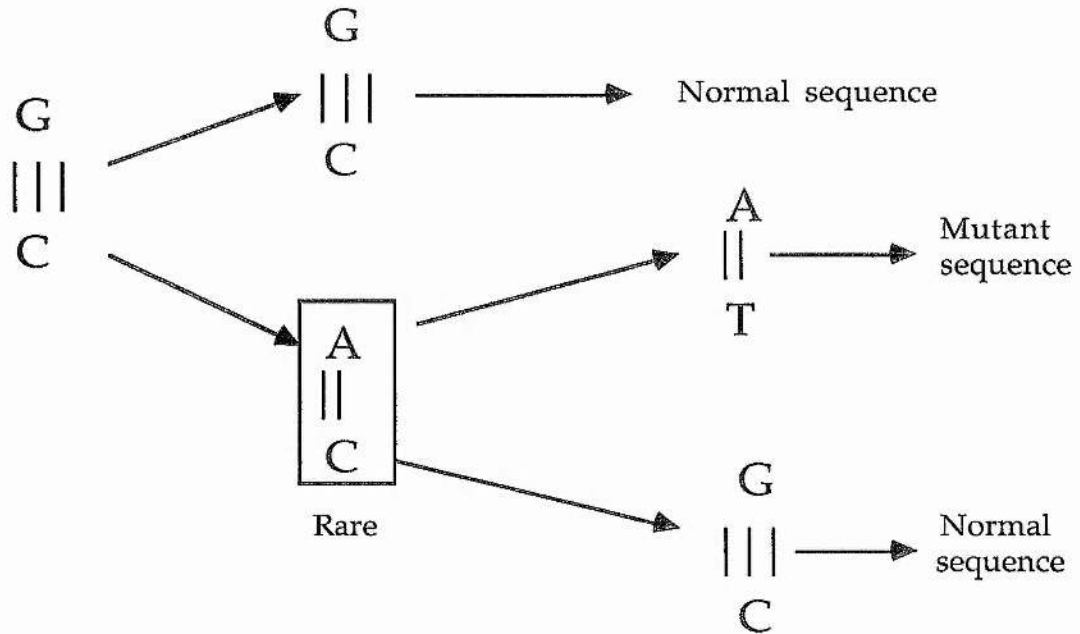


Figure 1.4.1: Tautomeric shift involving cytosine. The cytosine nucleotide normally pairs with guanine. However, when in the rare tautomeric state hydrogen bonds with adenine (behaving like thymine). This, if remaining unrepaired, leads to a transition point mutation (redrawn from Gib de Busk, 1968).

ii) Deamination of bases

In addition to the four bases usually present in DNA, there are also other modified bases. In bacteria there are many, but in eukaryotic cells, base modification is less common. One common modified base in eukaryotes is 5-methyl cytosine. Methylation of cytosine residues is generally found only in specific regions of the genome and is believed to be involved in the regulation of gene expression. Cytosine is sometimes methylated to produce 5-methyl-cytosine. 5-methyl-cytosine suffers from spontaneous deamination which occurs when the amino (NH) group is removed and is replaced by a Keto (O) group (Lewin, 1990). This converts 5-methyl cytosine to thymine yielding a mispaired base. If the generated nucleotide change evades the proof reading mechanism of the cell, a point mutation will occur following DNA replication in the form of a GC to AT transition.

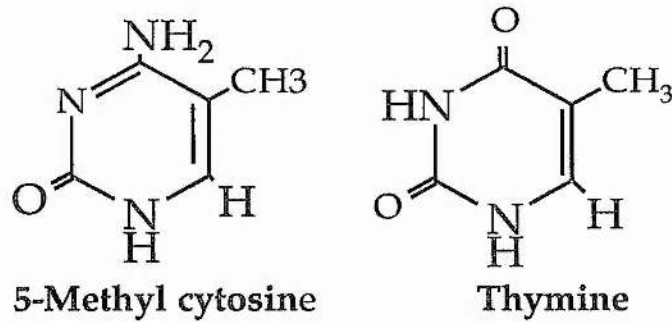


Figure 1.4.2: The Chemical structure of the base 5-methyl cytosine and the deamination product thymine.

Cytosine also undergoes deamination producing the base uracil which is used instead of thymine in RNA. Uracil hydrogen bonds most efficiently with adenine. Hence, if uracil is not removed and replaced by the correct base prior to replication there will be GC to AT transition in the newly produced daughter DNA strand.

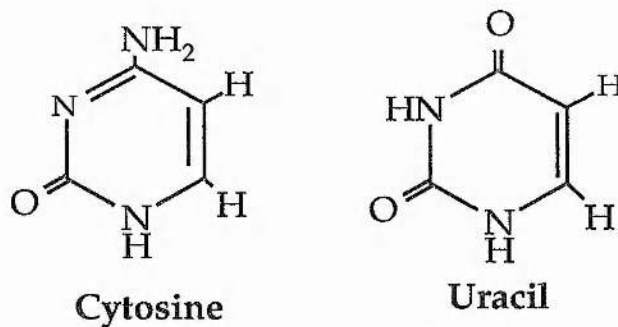


Figure 1.4.3: The chemical structure of the base cytosine and its deamination product uracil, produced by the replacement of the amino group with a keto group.

Spontaneous mutations do exist but at a low frequency. This frequency is much less than one would predict from the rates of tautomeric isomer formation and spontaneous deamination (Rawn, 1983). This can be partially explained by the ability of certain enzymes to proof read the replicated DNA and to repair any anomalies which occur in the DNA sequence. In bacteria, the DNA polymerases have a dual function. They are both able to replicate and proof read the DNA. In eukaryotic cells, the situation is more complicated. There are separate enzymes to perform all the different tasks. Incorrect bases or damaged bases may be removed from the DNA of eukaryotic organisms by excision repair. This mechanism is demonstrated in Figure 1.4.4. Mismatched uracil bases may also be removed by excision repair. However, in the case of uracil prior to excision, the base is removed from the sugar by the hydrolysis of the glycosidic bond by the enzyme uracil glycosylase. The remaining nucleoside is then removed by

exonucleases and replaced by the DNA polymerase.

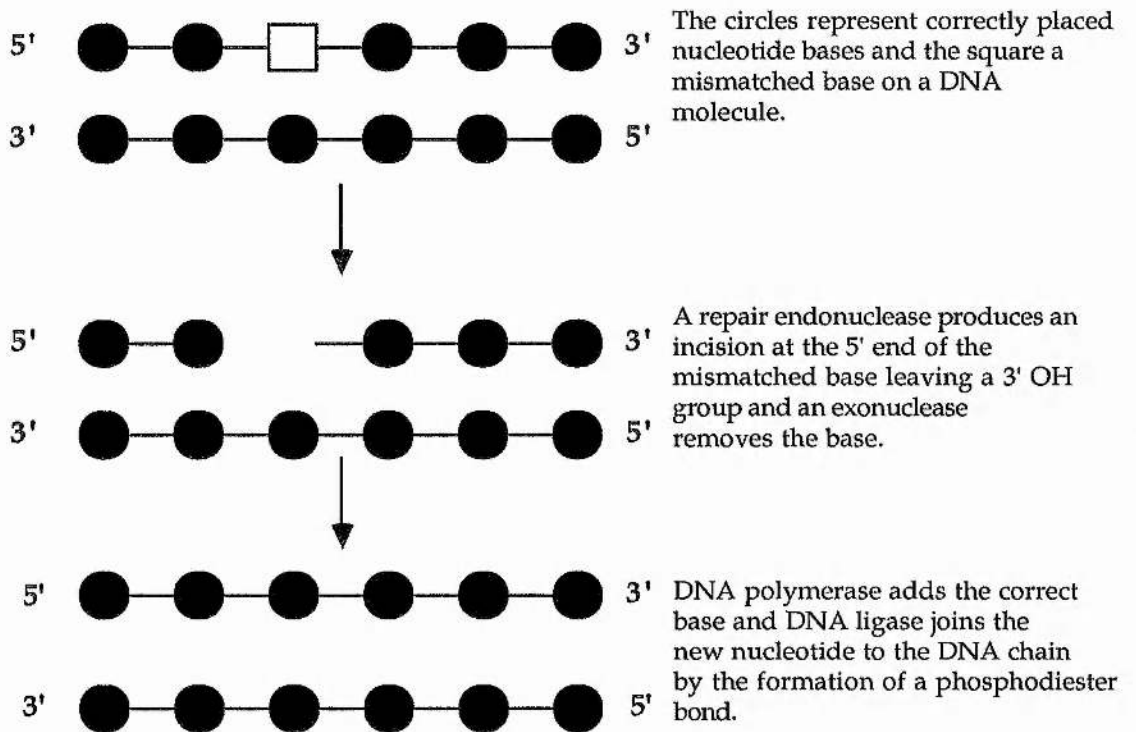


Figure 1.4.4: The mechanism of excision repair which is used to remove and replace mismatched nucleotide bases from the DNA strand.

iv) Transposable genetic elements

Another possible source of mutations are transposable genetic elements or "jumping genes". These are segments of DNA (bound by specific terminal sequences) which are able to duplicate themselves, retain one copy of itself in the original location and transfer the other copy to a target site. They were first discovered by Barbara McClintock in maize (McClintock, 1984 and references therein) where insertion of these elements caused a mutation and an unstable phenotype. They have since been found in a range of other organisms for example, bacteria (Jordan *et al.*, 1968; Shapiro, 1969) and *Drosophila* (Adams *et al.*, 1986 and references therein). These elements produce mutations at the site at which they insert. These locations will determine whether or not a phenotypic change occurs. Transposable genetic elements (or transposons) can cause DNA deletion mutations. A mechanism by which two adjacent transposable genetic elements (with the same orientation) can cause a deletion of the intervening genomic DNA is illustrated in Figure 1.4.5.

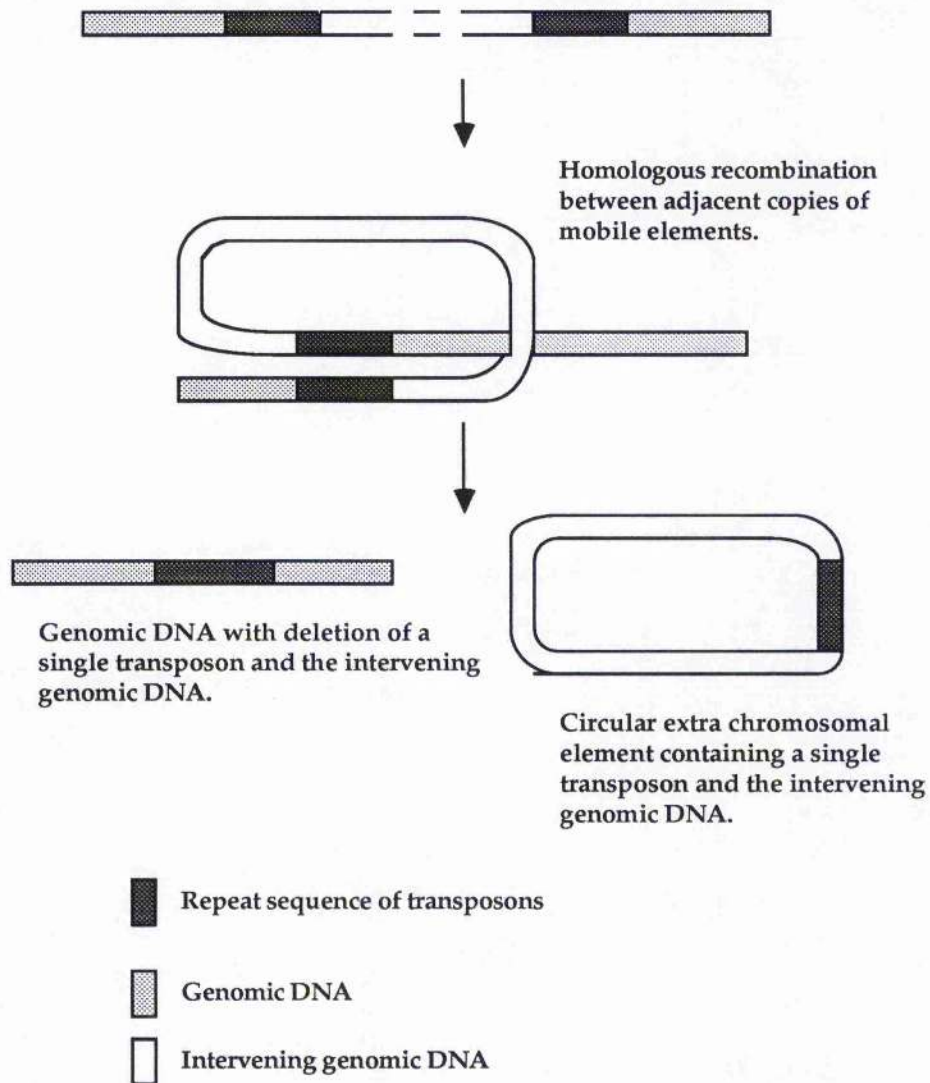


Figure 1.4.5: Diagram shows how two adjacent mobile transposable genetic elements can cause a genetic deletion in genomic DNA by the mechanism of homologous recombination (diagram redrawn from Adams *et al.*, 1986).

vi) Epigenetic changes

Changes in the DNA nucleotide sequence sometimes result in a change in phenotype. However, not all changes in the phenotype are the result of changes in the nucleotide sequence within the DNA molecule. A small proportion are the result of epigenetic changes. These changes are the result of alterations in the patterns of DNA modification (for example, the methylation of the base cytosine to produce 5-methyl cytosine). Gene control via DNA methylation does not occur at random locations. It occurs at specific regions within the genome. For example, the cytosine residues which are located adjacent to guanine residues are preferentially methylated. DNA Methylation is also believed to be tissue specific, stable and clonally inherited (John and Miklos, 1988). Methylation is common in developmentally

regulated genes and accounts for the differential activity in genes which control hormone production (Razin and Riggs, 1980). Methylation at specific regions of the gene is believed to result in gene inactivation. However, this observation is not universal since there is no evidence to suggest that gene control via methylation occurs in invertebrates and a few genes such as the alpha 2 (1) Collagen gene are transcribed despite full methylation (Mckee *et al.*, 1982). Undermethylation of cytosine residues in vertebrate cells is however, associated with active gene expression (John and Miklos, 1988).

b) Molecular analysis of spontaneous salvage pathway mutations

Most changes at the *hprt* locus which occur spontaneously are the result of small genetic changes. This has been found *in vivo* in Lesch Nyhan patients where only 15% of DNA changes are visualised by Southern blot analysis. Similar results have been found *in vivo* in normal healthy humans (Nicklas *et al.*, 1987).

Yandell *et al.*, (1986) isolated spontaneous *tk*⁻ mutations from the heterozygous human TK6 cell line. It was observed that there were two distinct classes of mutations with different growth rates and colony sizes. These subtypes were imaginatively called 'Normal' and 'Slow Growth' mutants. The active *tk* gene and the inactive *tk* gene in the TK6 genome could be distinguished by the differing RFLP patterns produced with *Sac I* digestion and Southern blot analysis with a cDNA probe. In both slow growth and the normal *tk*⁻ mutations, a large proportion of the mutants showed DNA sequence loss. 20 out of the 29 "Normal Growth" mutants showed complete loss of the restriction fragment length polymorphism (RFLP) pattern of one of the alleles and 2 showed a change in the RFLP pattern. The remaining 7 showed no change. In the "Slow Growth" mutation, 22 out of the 24 showed loss of the RFLP of one of the *tk* alleles with the remainder showing no change in pattern. Moore *et al.*, (1985a) and Moore *et al.*, (1985b) noticed a correlation between slow growth and gross chromosomal damage in mouse lymphoma cells. Of the 7 spontaneously induced mutations studied 4 had normal growth and 3 had slow growth. It has been suggested that a proportion of the slow growth mutants in mouse culture cells are the result of non disjunction of chromosomes in mitosis which resulted in the loss of chromosome 11, the chromosome where the *tk* gene is located in mouse cells (Moore *et al.*, 1985a; 1985b).

80 - 90% of mutations occurring spontaneously at the *aprt* locus studied in a Chinese hamster ovary cell line which is hemizygous at the *aprt* locus did not lead to large scale genetic changes (Grosvosky *et al.*, 1986; Sankaranarayanan, 1991). There appeared to be no hotspots for base substitutions. However it was found that deletions, although extending upstream in the 5' direction did not extend beyond the end of the *aprt* gene in the 3' direction (Sankaranarayanan, 1991 and references therein). One hypothesis to explain the above observation is that there is an

essential locus adjacent to the 3' end of the *aprt* gene. Such deletions would therefore be lethal to the cell. Hence, such mutations would be lost from the population. The small deletions observed, although appearing throughout the *aprt* gene seemed to have their end points clustered within exon 5 (Sankaranarayanan, 1991 and references therein).

1.5 Radiation Induced DNA damage

a) The mechanisms involved in the production of radiation induced DNA damage

In the case of high linear energy transfer (LET) radiation (such as alpha and beta particles) some of the damage is believed to be due to the direct action of radiation and the remaining due to the indirect action of free radicals (Hall, 1978). Low LET radiation produces a greater proportion of its damage indirectly in the form of free radical damage as compared to high LET radiation (Milligan *et al.*, 1993a; 1993b). Cellular water is believed to play a critical role in this process by the production of highly reactive free radicals and ions. The free radicals react with the DNA molecule altering its structure (by producing for example, single strand and double strand breaks, base damage, sugar damage and DNA protein cross-links).

When an energetic electromagnetic or particulate wave strikes or interacts with an atom, electrons are often excited and are consequently promoted to a higher energy level. Sometimes the energy deposited is so large that the electron is expelled from the atomic shell and ionisation is said to have occurred (Hutchinson, 1985). These electrons lose much of their kinetic energy and are taken up by other atoms or molecules to produce ions. Ions are highly reactive and will react with any chemical species compatible and available, including the DNA molecule.

A free radical carries an unpaired electron in the outer shell. Electrons in orbitals are often described as spinning on their axis in a clockwise direction or an anticlockwise direction. One lone electron in an orbital without a partner in the opposite spin results in chemical instability. Water ions can react to form the free radicals $\text{OH}\cdot$ and $\text{H}\cdot$ (Hall, 1978; Hutchinson, 1985). Free radicals are highly reactive, far more so than ions and have a much shorter half life. Hydroxyl radicals will diffuse quickly within the cell and easily react with other molecules, in some cases inactivating them. In other cases, the free radicals can oxidise the DNA molecule abstracting hydrogen atoms from the base producing a series of chain reactions altering nucleotide structure and stability (Hutchinson, 1985). It is believed (Oleinick *et al.*, 1986; Frankenberg-Schwager, 1989) that the free radical of OH is responsible for the introduction of DNA protein cross links. Base damage, which can also lead to the production of single strand breaks by the action of endonucleases and is one of the most common radiation induced lesion (Cerutti, 1976; Frankenberg-Schwager, 1989). Single strand breaks (SSB) are induced in proportion with

radiation dose (Coquerelle *et al.*, 1976; Frankenberg-Schwager, 1989) as are all DNA lesions *in vivo*. There are many different types of radiation induced DNA damage (see Figure 1.5.1). These primary lesions are believed to be the precursors of genetic mutations. However, there is an incomplete understanding of how a primary DNA lesion leads to a genetic mutation.

i) Single strand break (SSB)

A single break may occur in one of the links of the backbone of the DNA helix. SSB in general occur at the phosphodiester linkage of the DNA chain. The breaks are termed as either "clean" where the break produces a 3' hydroxyl or a 5' phosphate terminus or 'dirty' where the above mentioned termini are not generated. The dirty termini have to be modified prior to ligation. The number of SSB produced is proportional to the radiation dose (Coquerelle *et al.*, 1976; Ahnstrom and Edvardsson, 1974) and approximately 10^3 SSB are produced per cell per Gy in the Chinese hamster (Goodhead, 1994). SSB can be produced by the direct action of X rays, as a result of a local deposition of relatively large amounts of energy at the site of the phosphodiester linkage. However when cells are treated with Low LET radiation it is believed that SSB are caused mainly by the hydroxyl radicals (Milligan *et al.*, 1993b). Single strand breaks can also be produced as a consequence of base and sugar damage. These breaks are transiently caused by endonucleases and are the result of uncompleted DNA repair.

The biological effect of a SSB is more important when it occurs in conjunction with other types of lesions. An example is when a SSB occurs opposite base damage, sugar damage or another SSB. These result in the production of a double strand break which is believed (Bryant, 1984; 1988; 1989) to be the most biologically important radiation induced lesion.

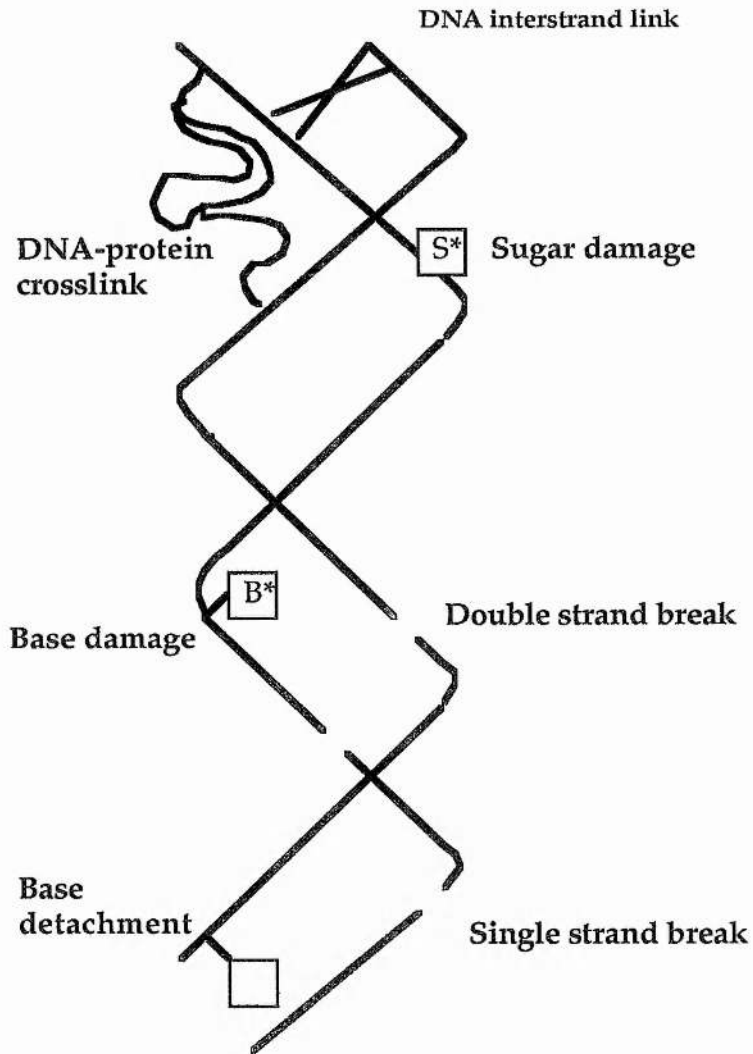


Figure 1.5.1: The above diagram illustrates the spectrum of DNA damage that occurs within the double helix as a result of radiation exposure. Notice that not only are there double and single strand breaks in the phosphodiester bond, but also bases and sugars may be damaged or removed. There are also changes in the relationship between the DNA and associated proteins, as shown by DNA protein cross-links (redrawn from Frankenberg-Schwager, 1989).

ii) DNA protein cross-links

DNA in the cell is covalently linked to proteins in the nuclear matrix (for example, Alberts *et al.*, 1991; Oleinick *et al.*, 1986; Frankenberg-Schwager, 1989; Adams *et al.*, 1986;). DNA in chromosomes associated with nuclear proteins is called chromatin. DNA is associated with basic proteins such as histones, which are responsible for the packaging of the DNA molecule (Adams *et al.*, 1986; Rawn, 1983). The DNA is also associated with other non-histone proteins. Some are involved in replication and transcription (for example, Chiu *et al.*, 1982; Oleinick *et al.*,

1986; Adams *et al.*, 1986; Frankenberg-Schwager, 1989). There are other low molecular weight proteins which play a structural role in the chromatin (Adams *et al.*, 1986). There are approximately 6000 DNA-protein cross-links per normal V79-4 lung fibroblast cell (Frankenberg-Schwager, 1989). Low LET radiation induces a further 160 DNA/protein cross-links per Gy per genome in the V79-4 cell line (Oleinick *et al.*, 1986; Frankenberg-Schwager, 1989). The radiation induced DNA-protein cross-links are removed by the cellular repair mechanisms. In V79-4 monocultures DNA-protein cross-links are preferentially repaired in transcribing DNA. There are two components to the repair process, a fast component with a repair time of $t^{1/2} = 1$ hour followed by a much slower component (Frankenberg-Schwager, 1989).

iii) Base damage

Base damage is one of the major lesions induced by ionising radiation (Cerutti, 1976; Frankenberg-Schwager, 1989). There are numerous different types of base damage, many caused by the action of hydrogen and hydroxyl radicals on the DNA molecule (Hutchinson, 1985). The bases can be partially destroyed or chemically modified by free radicals. This often results in a change in the physical and the chemical properties of the base (Tubiana *et al.*, 1990; von Sonntag, 1987). More is known about thymine and uracil damage than damage to the other bases. The amount of observed damage which occurs at the DNA bases is believed to be related to the structure of the chromatin (Patil *et al.*, 1985; Ljungman, 1990). Evidence for this is provided from the observation that there is more thymine damage within regions of the chromatin where there is actively replication (Cerutti, 1974) and transcription (Patil *et al.*, 1985). Some of the possible chemical modifications which occur at the thymine base following a chemical reaction with hydroxyl radicals are shown in Figure 1.5.2.

Hydroxyl radicals are highly reactive. As they have one unpaired electron in the outer orbital of the oxygen, the hydroxyl radical is highly electrophilic in nature and attacks regions of the base which have a high electron density. In the pyrimidine bases these regions are the Carbon (5) and (6) double bond. Sometimes the OH radical will add to the pyrimidine ring (as seen in Figure 1.5.2.) and on other occasions will remove a hydrogen atom from the available methyl group (for example in thymine). The pyrimidine radicals generated are also highly reactive and will react again, possibly with water molecules or other available ions. In the presence of oxygen, the pyrimidines may be converted into organic peroxy radicals. The initial hydroxyl radical reaction with pyrimidines sets up a chain reaction producing a multitude of different base modifications (von Sonntag, 1987).

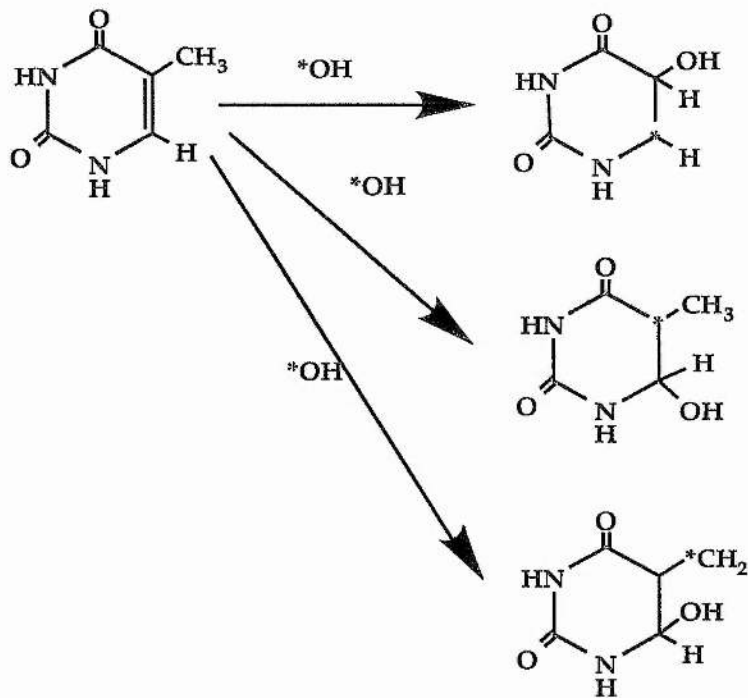


Figure 1.5.2: The reaction of the base thymine with hydroxyl radicals and three possible structures of thymine radical products. The symbol * indicates where the unpaired electron is located in the radical molecule. (redrawn from von Sonntag, 1987).

Less is known about the reaction of free radicals with purines. However, purines are also known to react with the free radicals of water. Examples of possible products following a reaction of hydroxyl radicals with the base adenine are shown in Figure 1.5.3.

However, it must be remembered that water radicals are not the only free radicals generated by the action of radiation on cells. Organic radicals are produced and these will also react with the purine and the pyrimidine bases of nucleotides (Loman and Blok, 1968; Nishimoto *et al.*, 1983; von Sonntag, 1987) changing the nucleotide structure and bonding properties of the DNA molecule. Base damage can be repaired by enzymes in the cell, the main mechanism is excision repair.

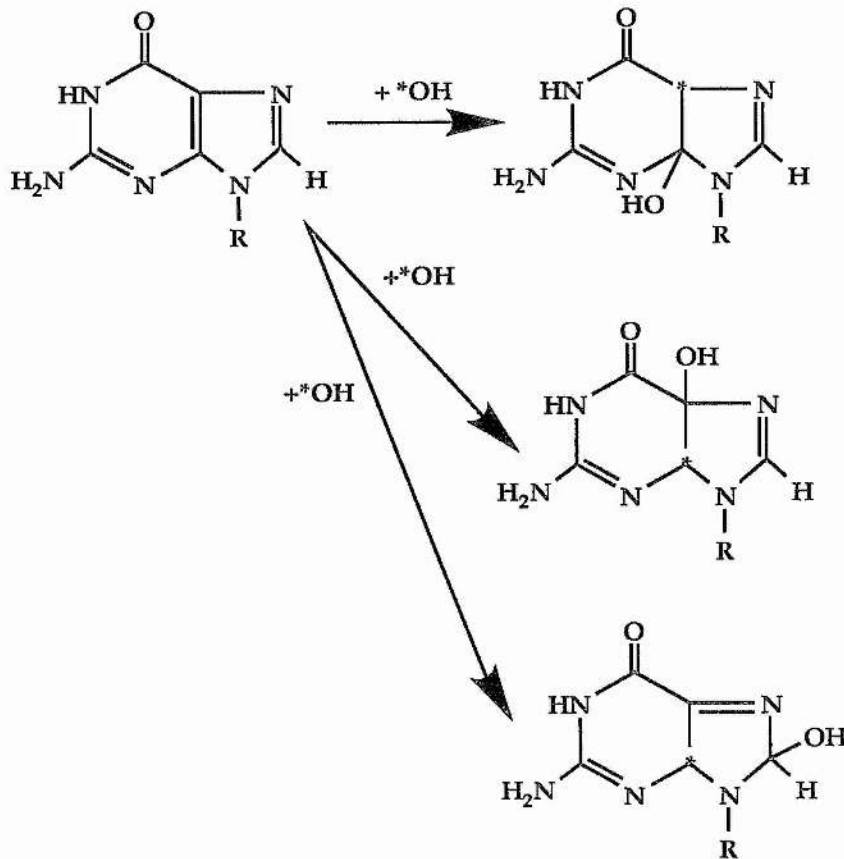
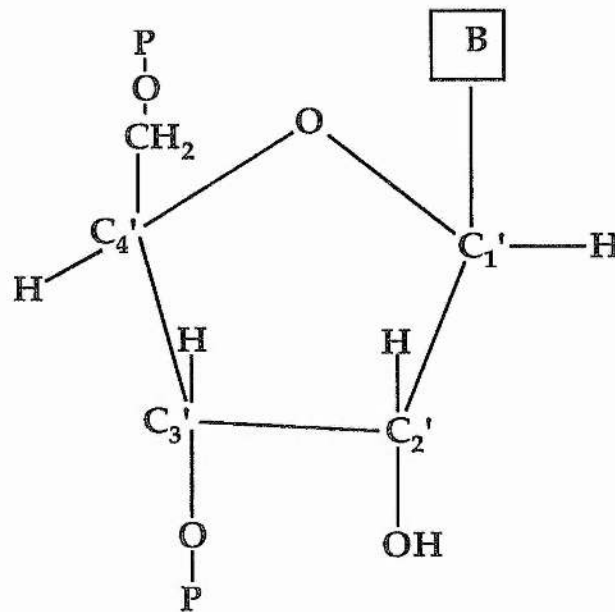


Figure 1.5.3: The reaction of the purine base adenine with hydroxyl radicals showing three possible free radical products. The symbol * indicates the position of the unpaired electron (from von Sonntag, 1987).

iv) Sugar damage

The 2-deoxyribose sugar is the backbone of the DNA molecule, linking itself into the polynucleotide chain by phosphodiester bonds (Figure 1.5.6 shows the structure of the deoxyribose sugar in the deoxyribonucleotide). Sugar damage may be the result of both the direct action of radiation and the indirect action of free radicals. When the DNA is irradiated some of the bases lost are unaltered (Scholes *et al.*, 1949). Evidence suggests that damage to the sugar group may cause base loss and breakage in the DNA strand (Deeble and von Sonntag, 1984; Lamaire *et al.*, 1984). Single breaks in other bonds will result in the weakening of the DNA chain and two breaks in the sugar ring may produce a single strand break.



B= Base
P=Triphosphate

Figure 1.5.4: The chemical structure and the numbering of the carbon atoms on the deoxyribose sugar of a deoxyribonucleotide.

Hydroxyl and hydrogen radicals can react with the sugar by removing a hydrogen atom producing a sugar radical (von Sonntag, 1987; Hutchinson, 1985). If the hydrogen removal occurs at the carbon (1) position it is possible that a series of biochemical reactions will result in the loss of an undamaged base from the nucleotide. A mechanism by which this could occur is shown in Figure 1.5. Attacks on the sugar by free radicals at the Carbon (4) position is believed to initiate a reaction which results in the production of a strand break (von Sonntag, 1987).

v) Double strand break (dsb)

This is the breakage of the two strands of the DNA double helix at points which are less than 3 nucleotides apart (Bryant, 1984; 1988; 1989). There are many different types of dsb and it is easy to categorise them in terms of the types of the termini. As for SSB (see above) the termini of the DNA strands can be described as either "clean" or "dirty". The dsb is believed to be one of the most important DNA induced lesions and responsible for much of the cellular, mutagenic and oncogenic effects of radiation (Bryant, 1989). It is believed that radiation produces both blunt and cohesive dsb termini. However, most of the radiation induced dsb termini are thought to be cohesive and 70% of the termini are thought to be "dirty" requiring "enzymatic cleaning" prior to ligation (Bryant, 1984).

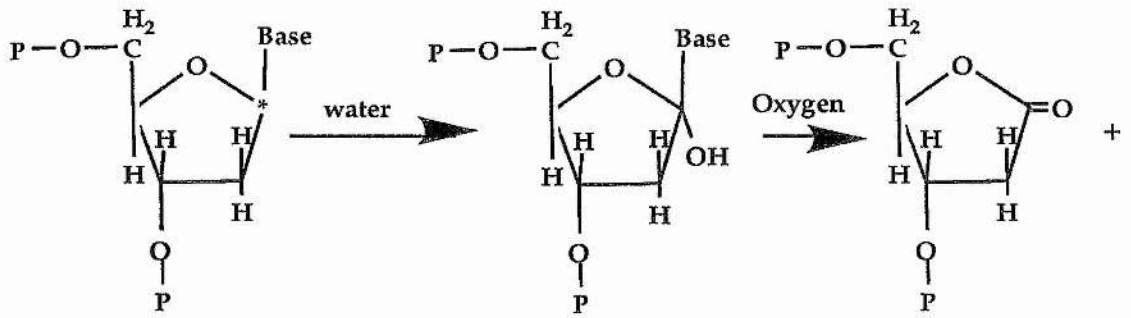


Figure 1.5.5: A possible reaction of a sugar radical which could result in the loss of the base from the nucleotide (redrawn from von Sonntag, 1987). P is an abbreviation for the triphosphate group.

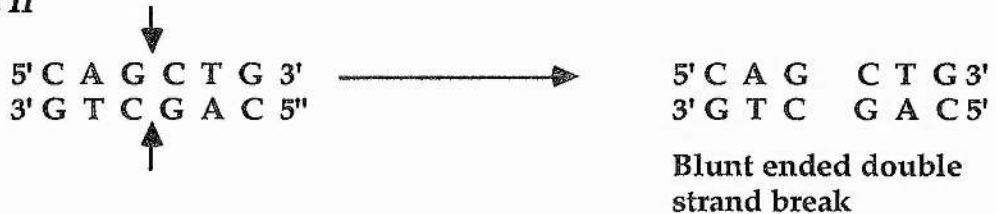
Irradiation of cells has been shown to increase the frequency of chromosome aberrations. Chromosome aberrations are the result of an unrepaired dsb early in interphase prior to the chromosome material being duplicated. During the replication of the DNA, the break is also replicated and these breaks can be observed cytogenetically as chromosome fragments in the following mitosis. Cells lines which have been shown to be defective in dsb repair produce more aberrations per unit dose than the wild type repair proficient parent cell line (Kemp and Jeggo, 1986).

Cell lines defective in dsb repair have also been shown to be more sensitive in clonal assays to radiation than dsb repair proficient wild type cells. This has been demonstrated by Frankenberg-Schwager and Frankenberg (1990) in yeast and in Chinese hamster cells by Jeggo and Holliday, (1986), Zdzienicka *et al.*, (1988), Costa, (1990), Shadley *et al.*, (1991) and Singh and Bryant, (1991).

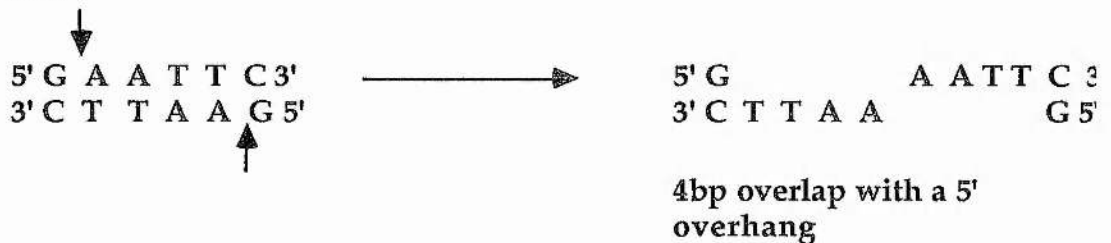
The dsb is also implicated in the induction radiation induced mutations and recent work using restriction enzymes suggests that the blunt end termini dsb are more effective at inducing mutation than dsb with the cohesive termini (Obe *et al.*, 1986; Singh and Bryant, 1991; Singh, 1991). There is also accumulating evidence (Whaley and Little, 1990; Helbig *et al.*, 1994) that the dsb induces mutations by the mechanism of DNA deletion. The chemotherapeutic drug adriamycin, which interacts with topoisomerase II to produce transient dsb, has also been demonstrated to be mutagenic at the *hprt* locus. The mutations generated are predominantly small scale deletion of less than 100bp (Helbig *et al.*, 1994).

Type II restriction enzymes (RE) are derived from bacteria and are capable of cleaving the DNA to produce specific types of DNA dsb. They represent a bacterial "immune system" protecting the cell against the parasitic infection of bacteriophage DNA. Different RE recognise different DNA sequences within the DNA molecule and cleave to produce different types of termini (for example, see Figure 1.5.6). Most RE recognition sequences are 4bp or 6bp long and are palindromic in nature.

i) *Pvu II*



ii) *Eco RI*



iii) *Pst I*

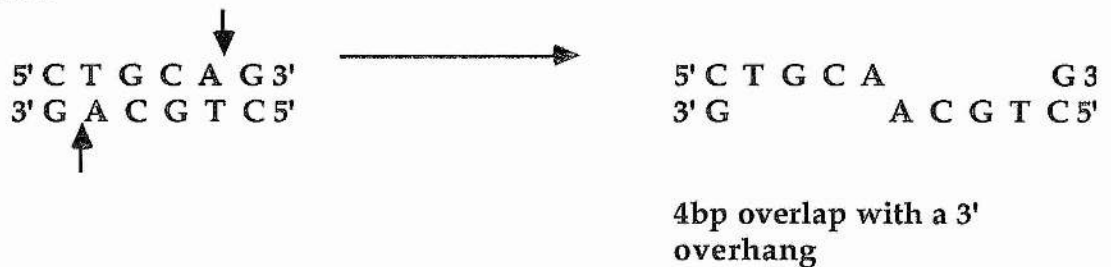


Figure 1.5.6: The restriction sites and the types of breaks which the restriction enzyme *Pvu II*, *EcoRI* and *Pst I* produce.

As radiation produces such a wide spectrum of DNA damage, it is impossible to attribute any particular primary lesion to any particular type of mutation. It is widely believed that RE can mimic the action of radiation to produce specific types of dsb (Bryant, 1984; 1989; Winegar and Preston, 1988). It is important to note that radiation produces a combination of "clean" and "dirty" dsb termini unlike RE which exclusively produce "clean" termini. Restriction enzymes have been used to induce chromosome aberrations (Bryant, 1984; 1989; Natarajan and Obe,

1984; Obe *et al.*, 1985; 1986; Winegar and Preston, 1988; Bryant and Johnston, 1993; Bryant *et al.*, 1993; Liu and Bryant, 1993a; 1993b; 1994) and gene mutations (Singh and Bryant, 1991; Singh, 1991; Kinahi *et al.*, 1992; Tatsumi-Mirajima *et al.*, 1993; Ashraf *et al.*, 1993).

b) Molecular analysis of salvage pathway mutations following irradiation

Analysis of *in vivo* *hprt* mutations of human survivors of the Hiroshima Atom bomb revealed that exposed individuals had a significantly higher proportion of mutant T cells with structural alterations than unexposed controls (Hakada *et al.*, 1989). Duplication and insertion mutations which were extremely rare in spontaneously arising *hprt*⁻ mutants were also observed in radiation induced mutations in hamster cells (Breimer *et al.*, 1986; Thacker *et al.*, 1990). Evidence also suggests that the dose given may also effect the types of structural changes observed (Whaley and Little, 1990). The data obtained by Whaley and Little (1990) indicated that with high linear transfer energy (LET) radiation (for example from ¹²⁵I), the higher the radiation dose the greater the proportion of mutants with large structural changes. Low doses of high LET radiation (for example 0.01mCi/ml of ¹²⁵I) did however produce a similar spectrum of *hprt* mutations to X-rays in human T cells. The molecular changes induced at the *hprt* locus as a result of irradiation do not appear to be distributed randomly along the gene. Evidence suggests (Xu *et al.*, 1989; Thacker *et al.*, 1990) that there are more observed molecular changes at the 3' end of the *hprt* gene than at the 5' end. Whether this is a result of differences in susceptibility to mutation induction at the different ends of the gene, preferential repair of the 5' end of the *hprt* gene or due to differential selection of mutations at the 5' and the 3' ends of the *hprt* gene is uncertain.

Yandell *et al.*, (1986) also isolated 30, 1.5Gy X-ray induced *tk*⁻ mutants of the TK6 cell line. The active and the inactive *tk* gene could be identified from the different RFLP patterns they produced following *Sac* I digestion and Southern blot analysis of the DNA with a cDNA probe. Molecular analysis revealed that 20 had lost the RFLP of one of the alleles and the remainder showed no change in pattern from the wild type TK6 cells. The mechanism of sequence loss in this line is unknown. From the data obtained, it is not possible to tell whether there has been loss of the whole chromosome, a micro-deletion causing the loss of the *tk* allele or a change in the restriction pattern of the functional *tk* allele to that of the non function allele.

1.6 Mutation Hotspots

Mutations are not distributed randomly within the genome. There are sites within the genome where mutations occur with a greater frequency (Watson, 1973; Lewin, 1983), more than one would statistically expect from a random distribution (Lewin, 1983). These sites are called mutation 'hotspots'.

Regions in the genome which are rich in guanine and cytosine are often sites where there is a high frequency of base substitution following DNA replication. These substitutions are predominantly GC to AT transitions. Some *E.coli* strains possess the restriction endonuclease *EcoRII*, which is capable of cleaving the palindromic sequence \uparrow CCAGG in the genome of invading parasitic DNA. To protect its own DNA from self digestion, the cytosine at the second position is methylated. There are three spontaneous hot spots in the *lac I* gene for point mutations and these correspond to the sites where cytosine is subject to DNA methylation (Coulondre *et al.*, 1978; Lieb, 1991). 5-Methyl cytosine spontaneously deaminates to produce thymine which can lead to transitions following DNA replication. Cytosine methylation has also been demonstrated to occur in eukaryotic organisms in regions of high GpC content and these regions are also believed to be sites where the frequency of mutational events are higher than expected. Deletions are the loss of one or more nucleotide bases from the DNA sequence, whereas insertions are the addition of one or more nucleotide bases into the DNA sequence. Radiation produces predominantly deletion type mutations but deletions do occur spontaneously. Evidence suggests that radiation induced deletions do not occur randomly within the genome (Sankaranarayanan, 1991).

1.7 DNA repair and mechanisms of deletion formation

a) dsb repair

X-rays and gamma rays induce strand breaks in the DNA molecule (see earlier). In order for cells to actively proliferate, these breaks must be repaired. The mechanism by which dsb are repaired is still not fully understood. It is possible that there are different mechanisms of rejoining in different types of cells. For example, bacterial, yeast and mammalian cells all possess different genome structure and organisation and therefore different repair systems may be operating.

There have been several mechanisms of dsb repair proposed, for example direct ligation of breaks and the single strand annealing model (Lin *et al.*, 1984; 1987). Evidence suggests that a proportion of the dsb are repaired by recombination. In support of this, radiation has been shown to increase the frequency of recombination in phage and bacteria (Jacob and Wollman, 1955) in yeast (Lopez and Coppey, 1987) and in *Drosophila* (Proust, 1967; Garcia-Bellido and Merriam, 1972). Nüssbaum *et al.* (1992), using a bacterial plasmid system, illustrated that the dsb induces DNA recombination and that the repair of the dsb on the cut plasmid substrate was dependent upon the presence of an intact sequence homologous to that of the damaged substrate. Resnick (1976) proposed a model for the repair of dsb involving DNA recombination. This model requires that there is a duplicate of the genetic material in the form of either sister chromatids or homologous chromosomes at the time of DNA irradiation. There is much

evidence to support mitotic recombination as a mechanism of DNA repair. Haploid cells have been shown to be more sensitive to ionising radiation than diploid cells (Luchnik *et al.*, 1977; Weibezahan *et al.*, 1985) and a radiosensitive yeast mutant cell line which did not repair dsb was also shown to be deficient in recombination (Resnick *et al.*, 1975). Brunborg *et al.* (1980) suggested that the increased ability of G₂ yeast cells to repair dsb as compared to G₁ cells was due to the ability of sister chromatids in the replicated DNA to recombine. Fonck *et al.* (1984) provided evidence for the temporary exchange of homologous DNA strands during the repair of X-ray induced dsb by measuring the density of DNA strands following bromodeoxyuridine uptake and cell irradiation.

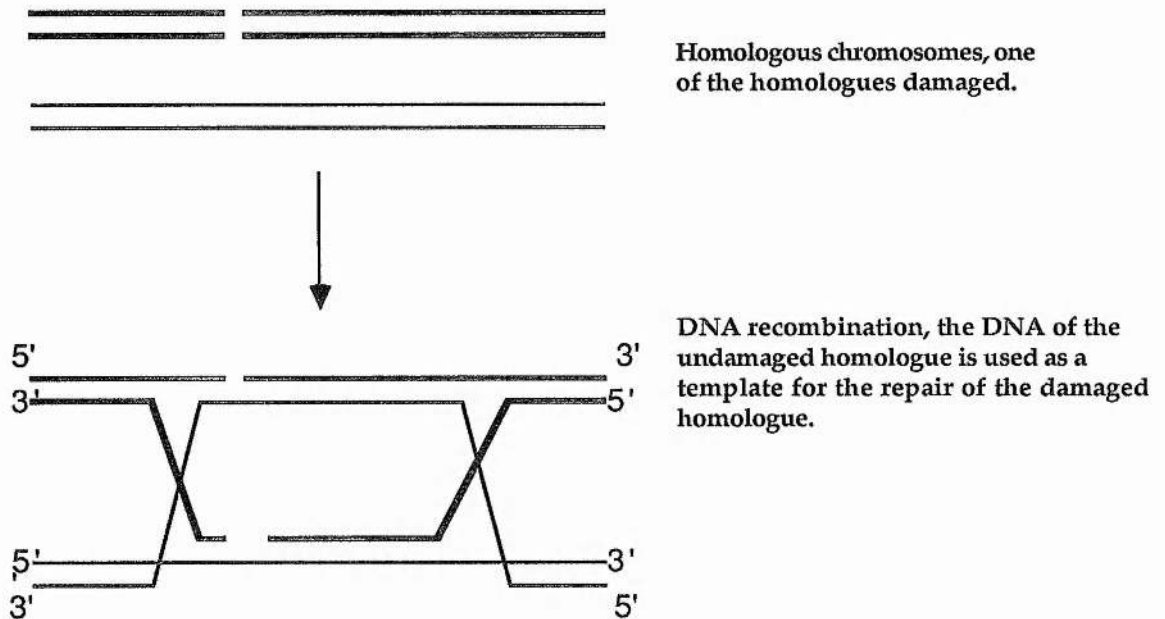


Figure 1.7.1: A possible model of DNA repair by recombination. One of the chromosome homologues has both strands of the DNA damaged at the same place. The other homologue is used as a template for repair. This mechanism has been used to explain gene conversion in yeast cells.

In yeast dsb have been shown to stimulate mitotic recombination and gene conversion (Orr-Weaver *et al.*, 1981; 1988). Evidence suggests that the RAD52 gene product in yeast is responsible for spontaneous recombination and the repair of dsb (Ozenberger *et al.*, 1991 and references therein). Mutant yeast cells which are deficient in the RAD52 gene product have been isolated and have been shown to be deficient in mitotic recombination. However, in these RAD52 mutants, ribosomal DNA sequences were shown to spontaneously recombine at approximately the same frequency as the wild type repair proficient cell line (Ozenberger *et al.*, 1991). The repair of the ribosomal DNA (rDNA) is independent of the RAD52 repair mechanism. The ribosomal DNA in the RAD52 mutant contains tandem repeat sequences and

the repair of restriction enzyme induced dsb involved the loss of one or more of the repeat sequences in the rDNA. The more repeats present in the sequence the more efficiently the dsb in the DNA were repaired. A mechanism by which this could occur is shown in Figure 1.7.3. Ozenberger *et al.*, (1991) suggests a mechanism which involve the formation of single stranded (ss) tails followed by reannealing.

b) Misrepair of DNA leading to deletion mutations

Large genetic changes may be correlated with specific features or "landmarks" in the genome in cells which are proficient in DNA repair (Morris and Thacker, 1993). Certain types of DNA sequences are believed to increase the probability of the formation of DNA deletions and insertions. For example, reiterated bases (Streisinger and Owen, 1985; Trinh and Sinden, 1993) and direct repeats (Trinh and Sinden, 1993) are believed to be correlated with spontaneous deletion formation. A mechanism called "slippage mispairing" was proposed by Streisinger and Owen (1985) to explain the high frequency of observed spontaneous frame shifts in the T4 bacteriophage at regions of adenosine homopolymeric runs. The mechanism of slippage mispairing is shown in Figure 1.7.2.

The slippage mispairing mechanism was also used by Farabaugh *et al.*, (1978) to explain the high frequency of loss of segments of tandem repeats in the *lac I* gene. The mechanism of slippage mispairing proposes that during DNA replication one repeat for example, repeat number 1 on one strand slips and aligns with another repeat, for example repeat number 2 on the complementary strand of the DNA double helix. Therefore, as replication proceeds, depending upon the direction of slippage, either a repeat sequence is inserted into the daughter DNA strand or a repeat sequence is deleted. Kimura *et al* (1994) suggested that in the *hprt* gene, may be as many as two thirds of the spontaneously induced deletions may be the result of slippage mispairing. *Alu* repeats are a family of repetitive elements found in the human genome. These repeats have been implicated in the production of DNA deletions. It has been suggested that recombination occurs between these repeat elements resulting in the loss of DNA segments (Lehrman *et al.*, 1985, Nicholls *et al.*, 1987; Huang *et al.*, 1989; Stoppa- Lyonnet, 1990; Morris and Thacker, 1993).

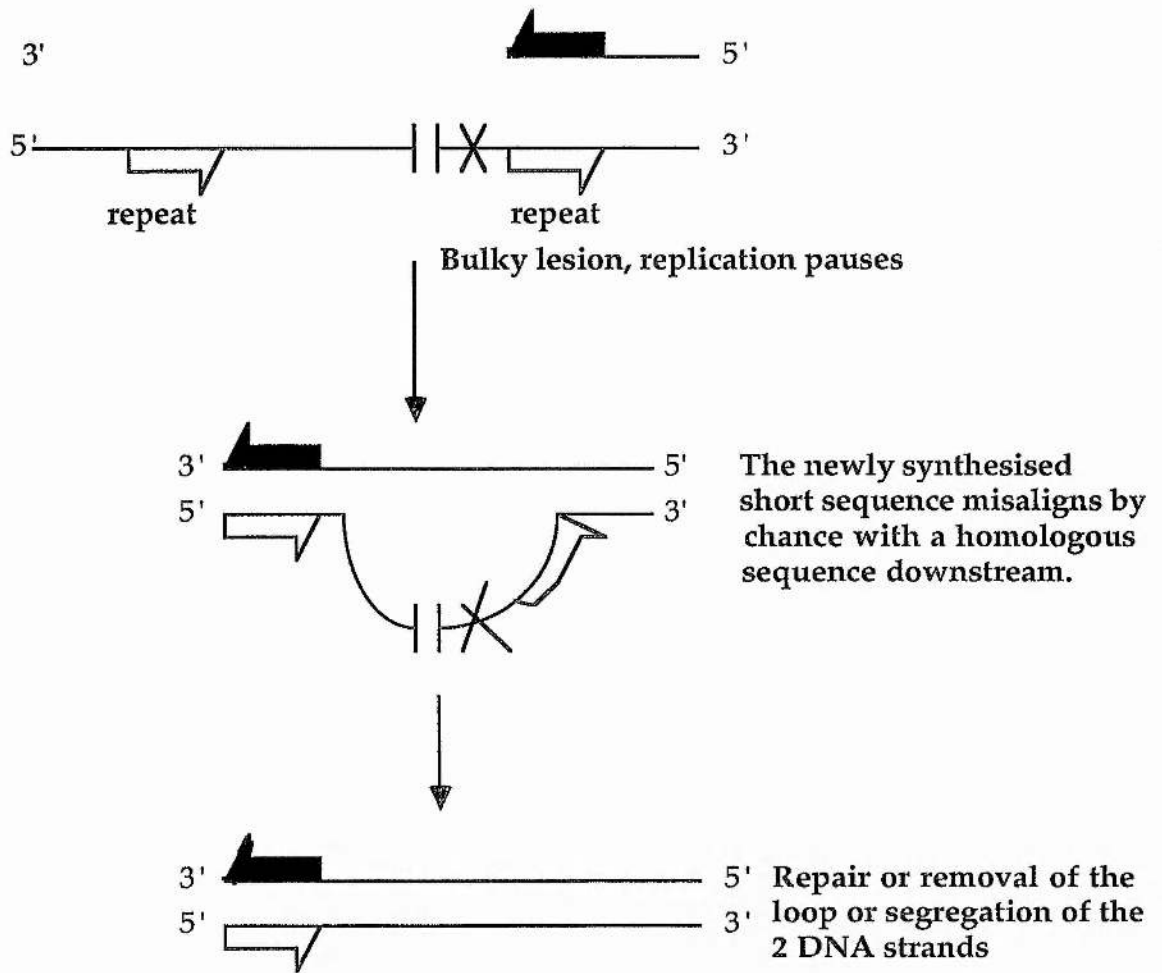


Figure 1.7.2: The mechanism of slippage mispairing. DNA polymerase replicates one of the repeat segments and then pauses when it reaches a bulky lesion. The newly synthesised sequence then misaligns with a homologous sequence downstream causing the looping out of the bulky lesion and one of the repeat segments. DNA replication continues. The loop is then either removed or the two DNA strands separate for the next round of DNA synthesis (diagram redrawn from Kimura *et al.*, 1994).

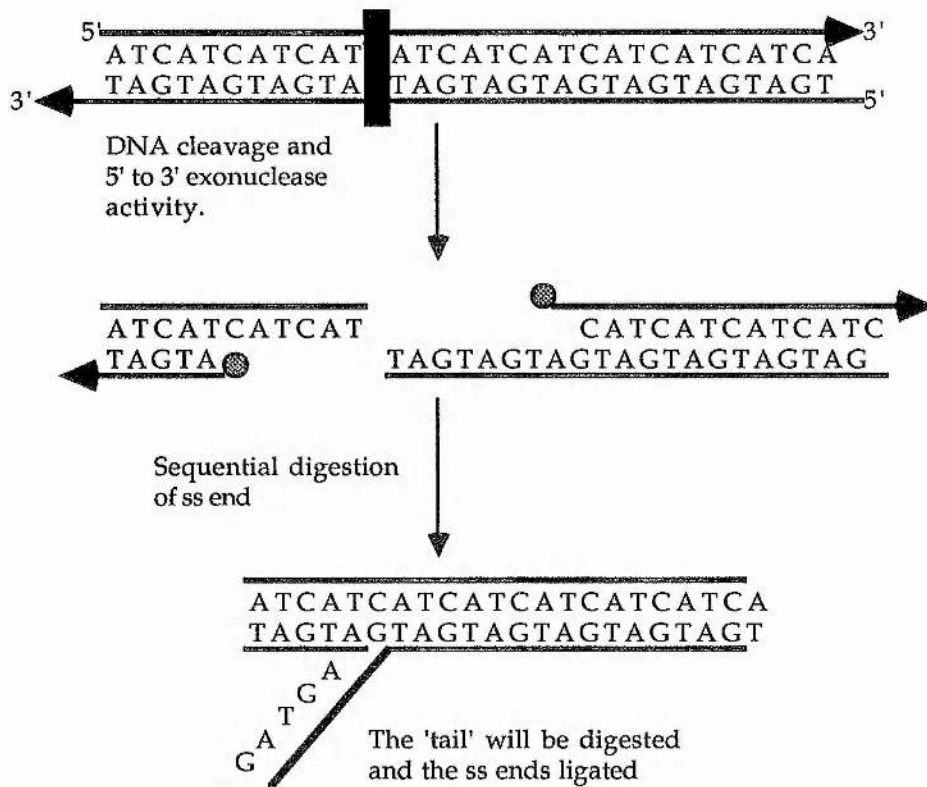


Figure 5.7.3: A possible mechanism of how ribosomal DNA repair can result in the loss of repeat sequences from a segment of repetitive DNA in the RAD52 yeast mutant. Exonucleases "chew" the ends of the dsb in a 5' to 3' direction to produce gaps in both the DNA strands. The DNA would reanneal, the tail would be "chewed back" and the gap repaired (redrawn from Ozenberger and Roeder, 1991).

Palindromic sequences and quasi palindromic sequences are believed to play a part in the production of spontaneous deletion mutations (Trinh and Sinden, 1993; Morris and Thacker, 1993). Evidence suggests (Morris and Thacker, 1993) that these regions in the helix form hairpin loops. Hair pin loops are formed by the pairing of two adjacent complementary stretches of base sequences on the same DNA strand (Watson, 1973). A break may occur at the base of the DNA loop resulting in the loop being deleted. It has also been suggested that misalignment could occur up to the hairpin stem and replication continue after the stem, causing the loss of the DNA sequence in the hairpin loop in the daughter DNA strand (Morris and Thacker, 1993; Trinh and Sinden, 1993). Morris and Thacker (1993) also observed that close to the site of DNA deletions topoisomerase II recognition sites were frequently found. In eukaryotic cells topoisomerase II is an enzyme complex which is responsible for the changing of the DNA conformation from a coiled to a relaxed state. The locations of the DNA deletions are also possibly related to the origins of DNA replication (Morris and Thacker, 1993; Hutchinson, 1993). The significance of these sites to DNA deletion formation has yet to be realised.

Sankaranarayanan (1991), suggested a mechanism of how radiation induced deletions could be formed. It was suggested that the deletions result from two double strand DNA breaks but, was also connected with the organisation of the chromatin within the nucleus and the distribution of energy during irradiation. DNA is packaged into looped structures which are often attached to the nuclear matrix. This packaging is not random, it is highly organised. An ionisation cluster has enough energy to produce more than one dsb. These breaks may occur at the base of the loop and the loop excised at the point where DNA lies next to itself and the free adjacent termini ligated.

It is obvious that there is much still to learn about the mechanism of deletion formation. It is still unclear whether the same mechanisms are involved in the production of spontaneous deletions as in the production of radiation induced deletions and their relationship to the nuclear organisation and particular sequences of nucleotides.

1.8 Changes in phenotype and the relationship to mutation spectrum

It may be argued that the mutational spectra observed from the samples of mutations analysed following mutation assay experiments are not a true representation of the spectrum of the total population of mutations. This is because all the mutations analysed result in a change of phenotype. If there is no change in phenotype, the experimenter is not able to identify a mutational event without the painstaking, tedious and arguably impossible task of analysing the DNA from hundreds of thousands of individual cells. There is a "silent majority" of mutations which do not result in an observable phenotypic change in the cell and hence, are not noticed by the experimenter. This is especially true in higher eukaryotic organisms where there is a high proportion of the genome which is not transcribed and only a fraction of the transcribed DNA is translated into proteins.

Mutations can be categorised into different groups dependent upon the type of genetic change that has taken place and the effect of the change on the cell phenotype. If there is a substitution of one base for another, a point mutation is said to have occurred. Sometimes these mutations are in a non-transcribed part of the gene and produce no change in the protein product. These mutations are silent. If however, the mutation does result in a change in the protein product but this change does not effect the protein function, the mutation is said to be neutral. Most substitutions in protein coding regions which change the amino acid sequence are termed leaky mutations. This is where the protein product has some residual function. The effect of the amino acid substitution may also be dependent upon where in the protein structure the change took place. If the change is located in the catalytic domain of an enzyme it is more likely to result in a profound phenotypic change than if it is in a non-catalytic region. There are other

mutations which result in the formation of a chain termination codon at an inappropriate place. This results in the translation of the amino acids ceasing before the protein is complete and the production of a truncated protein product.

Small additions and deletions to the nucleotide sequence, (for example, the loss or addition of a single base pair) may result in a shift in the reading frame. Frame shift mutations results in the wrong amino acids being incorporated into the protein molecule. This is called a nonsense mutation. This is found in many deletion mutations. In the severe human disorder Duchenne muscular dystrophy, a deletion occurs (often a large deletion) in the *dystrophin* gene. This gene leads to the removal of DNA sequences and the remaining sequences, although in the correct order have undergone a frame shift. Hence, amino acids are absent from the protein product and the amino acid which follow the deletion are not correct. There is therefore no function in the protein product. In the much less severe Beckers muscular dystrophy, there is also a deletion in the *dystrophin* gene but, the remaining bases have not undergone a frame shift. The amino acids proceeding the deletion are correct. The protein product although, having reduced function has some residual activity and the phenotype is less severe (Thacker *et al.*, 1990 and references therein). It can be seen that similar mutational changes can lead to varying degrees of phenotypic change. This is dependent upon the number of bases lost and the location at which the genetic change occurs.

1.9 Thesis Aims

The aim of this thesis is to study the molecular mechanisms of radiation mutagenesis and concentrating on the role of the double strand DNA break on the induction of mutations and the importance of the DNA deletion in the generation of dsb induced mutants.

Chapter 2 explains the general materials and methods used in the experiments outlined in this thesis, including a brief description of the cell lines used and how they are maintained in culture. In Chapter 3, the effects of an increasing dose of gamma radiation (^{137}Cs) upon the surviving fraction and the mutation frequency at the *tk* locus and the *hprt* locus was studied in three Chinese hamster cell lines, CHO-K1, V79-4 and *xrs-5*. The *xrs-5* cells maintained in the St. Andrews laboratory were shown have a *hprt*⁻ phenotype and experiments to ascertain the stability of this phenotype were performed.

Chapter 4 involved the study of the effects of different restriction enzymes on the mutation frequency at the *hprt* and the *tk* loci in the Chinese hamster cell lines. *Streptolysin O* was used as the method of cell poration. This is a relatively new technique which despite having been used as a method to introduce RE into cell in order to produce chromosome aberrations has not been used in the past to introduce RE in order to study mutation induction. Prior to the

restriction enzyme work, the effects of SLO on the cell survival and the background mutation frequency were studied and compared with the effects of another poration technique, electroporation.

The isolation of radiation, SLO and enzyme induced V79-4 *hprt*⁻ mutants and the molecular analysis of the DNA of these mutants via PCR deletion screening is discussed in Chapter 5. The DNA of the *xrs-5 hprt*⁻ cell line maintained in the St. Andrews laboratory was also analysed by the method of PCR deletion screening and the nature of the genetic change was identified.

It is known that the CHO-K1 cell line has only one active *tk* gene. It is not known whether the second copy of this autosomal gene had been inactivated by mutation or deleted. Chapter 6 explains the attempt made to locate the *thymidine kinase* gene(s) by the recently developed method of fluorescent *in situ* hybridisation. A cloned mouse cDNA sequence located in a plasmid vector was cultured in *E.coli*, isolated, labelled with a biotin reporter molecule and hybridised to denatured metaphase chromosomes. Unfortunately, this method was unsuccessful in locating the *tk* gene despite the control experiments working successfully.

Chapter 7 summarises the work performed and the results obtained. The significance of the results are discussed and suggestions for future work given.

Chapter 2

Experimental Materials and Methods

An experiment is a device to make Nature speak more intelligibly

George Wald (1967) Nobel Lecturer (From Wardlaw, 1985)

2.1 Introduction

This Chapter explains the general materials and the methods used in the experimental procedure of the later Chapters. These include a description of the cell lines used, their culture conditions and the assays used to select for phenotypic mutations at the *thymidine kinase (tk)* and the *hypoxanthine guanine phosphoribosyltransferase (hprt)* loci. The varying aspects of the experimental procedure, such as the radiation doses and enzyme concentrations are explained in the appropriate Chapters, as are other methods specific to individual Chapters.

2.2 Cell lines maintained

i) The CHO-K1 cell line

Chinese hamster ovary cells (CHO) were originally subcloned from an ovary cell culture established by Puck in 1957. These cells, like many other cells maintained in culture, have differences in the genome structure compared to normal diploid eukaryotic cells. Both the CHO and the CHO-K1 line have less chromosomes than the standard hamster karyotype (Deaven and Peterson, 1973; Kao and Puck, 1975).

The karyotype of the CHO cells and their daughter cell lines have a DNA content which is close to that of the normal diploid hamster genome. Indeed, G banding reveals many similarities between the genome of the cell line and the wild type hamster (Deaven and Peterson, 1973). The mutation rate in many autosomally recessive genes is anomalously high

(Breslow and Goldsby, 1969; Kao and Puck, 1975). The notion once existed that recessive gene mutants should not be phenotypically expressed in cells which are diploid in nature, since one would expect each genetic locus to be represented twice within the genome. There are several recessive genes within the CHO-K1 and the CHO line which have a higher than expected phenotypic mutation frequency. These frequencies are so high that one must conclude that these genes are demonstrating phenotypic hemizyosity or heterozygosity. Examples of these are resistance to certain lectins such as phytohaemagglutinin (Stanley *et al.*, 1975) and many auxotrophic mutants which are incapable of manufacturing glycine and adenine (Kao and Puck, 1975). Of most significant interest to the research presented in this thesis is the *thymidine kinase* gene (Breslow and Goldsby, 1969; Singh, 1991; Singh and Bryant, 1991).

If however, one is to look at the evolution of the karyotype, the idea of large numbers of recessive genes demonstrating hemizyosity or heterozygosity does not seem so unreasonable (Siminovitch, 1976). The karyotype of cell lines arises through a series of chromosomal and nucleotide rearrangements such as inversions and translocations. In cells maintained in culture, hemizyosity or heterozygosity at many non essential loci would not result in any selective disadvantage. Hence, cells containing changes within the genome resulting in the production of a non functional or absent RNA transcript from one of the two gene copies would be maintained within the cell population and not lost by selection (Siminovitch, 1976).

The main locus of interest in the CHO-K1 cell line is the *thymidine kinase* gene, located on chromosome 7 of the eukaryotic Chinese hamster genome (Stallings and Siciliano, 1981). This gene is hemizygous or heterozygous in the CHO-K1 cell line. The molecular reason for the observed phenotypic hemizyosity or heterozygosity at the *thymidine kinase* locus is not yet fully understood. The *hprt* gene located on the X chromosome is hemizygous. This is due to the loss of an X chromosome from the CHO-K1 genome (Deaven and Peterson, 1973). One would however, expect the *hprt* gene to behave phenotypically as though it was a hemizygous or a heterozygous locus because of X chromosome inactivation, which occurs in all female cells.

ii) The *xrs-5* cell line

Several X-ray sensitive cell lines derived from the CHO-K1 cell line have been isolated. The reason for their sensitivity has been the subject of much research (Jeggio and Kemp, 1983; Jeggio and Holliday, 1986; Johnston, 1994). Evidence also suggests that the reason for the radiosensitivity of the *xrs-5* cells is that it is deficient in the repair of dsb (Kemp *et al.*, 1984). Evidence suggests that the increase in sensitivity is not due to the number of initial DNA strand breakages generated but, due to the inefficiency of the cells to rejoin the dsb (Kemp *et al.*, 1984). Chromatid aberrations are believed to be caused by dsb and are four times more common in *xrs-5* than in the parental CHO-K1 cell line (Bryant *et al.*, 1987; Darroudi

and Natarajan, 1987). Survival assays demonstrate that the proportion of cells killed immediately due to irradiation in *xrs-5* is greater than in the CHO-K1 and an elevated induced mutation frequency per unit dose at the *thymidine kinase* locus has also been observed (Singh, 1991; Mussa *et al.*, 1990). Evidence suggests that the reduced dsb repair capacity of the *xrs-5* gene is due to inactivation of a repair gene by DNA methylation (Jeggo and Holliday, 1986).

Despite the fact that there is an incomplete understanding of the nature of the *xrs-5* mutation, this cell line is invaluable in the study of the causes of different types of genetic mutations. Comparing the effects of radiation and restriction endonucleases upon the mutation frequencies and surviving fraction in the *xrs-5* and the CHO-K1 and a comparison of the molecular changes leading to these mutations may provide a valuable insight into the mechanics of DNA repair.

iii) V79-4 cell line

The V79 cell line is a male Chinese hamster lung fibroblast line which was cultured by Chu and Malling in 1968 and used for mutation assays. The ease of cell maintenance, the rapid growth rate and the single X chromosome makes this cell line the ideal candidate for the study of mutations in X linked genes. Mutation work has been performed analysing the *hprt* gene at the cellular (Van Zeeland and Simons, 1976), and the molecular level. The exon DNA sequences have been cloned and sequenced, facilitating PCR and Southern blot analysis (Thacker, 1986; Xu *et al.*, 1989; Thacker and Ganesh, 1989; Thacker *et al.*, 1990; Koberle *et al.*, 1991).

2.3 Maintenance of the cell lines

Three cell lines were used in the experiments outlined in this thesis, the CHO-K1, *xrs-5* and V79-4. These cells were grown as monolayers in 75cm² tissue culture flasks (Nunc) and maintained in exponential growth in 15ml of MEM/FCS cell medium. 1 litre of MEM/FCS was made up of 100ml Eagles Minimum Essential Medium (Gibco), 10ml of penicillin/streptomycin antibiotic (Gibco), 10ml of glutamine (Gibco), 10ml of non essential amino acids (Gibco), 30ml of sodium bicarbonate, 100ml of foetal calf serum (Flow) and 740ml of double distilled water. The flasks were gassed with carbon dioxide and maintained at 37°C in a dry incubator. In all experiments, the cells were passaged regularly when required in order to maintain them in the exponential growth phase. Passaging involved the removal of the cell medium from the flask. The surface of the flask to which the cells were attached was rinsed twice with 5ml of the trypsin/EDTA and then the trypsin/EDTA solution was removed and the flask of cells was incubated at 37°C for 6 minutes in a dry incubator. Trypsin/EDTA solution was made up of 0.05% trypsin (Difco) diluted in phosphate buffered saline (PBS). 1 litre of PBS contains 8g of

NaCl, 0.2g of KCl, 1.15g of Na₂HPO₄, 0.2g of KH₂PO₄ all dissolved in 1 litre of distilled water. When the cells were detached from the surface of the flask and existing as single cells, 10ml of the cell medium was added and the cell suspension was pipetted up and down several times. This was to ensure that the single cell suspension was homogeneous. The cell concentration was calculated by adding 100µl of the cell suspension to 9.9 ml of isoton in a vial. The cells were counted using a coulter counter (model D) with the settings, threshold, 20; attenuation, 8; aperture current, 0.017. The cells were then diluted appropriately, the excess cells removed and inactivated by pouring into Chloros solution.

2.4 Gamma Irradiation

The cell cultures were irradiated while in exponential growth and while attached to the surface of 75cm² flasks. The flasks were placed in specialised canisters which were then inserted into the gamma irradiator (Cis Bio International Model IBL 437C) and irradiated with ¹²⁶Cs at a rate of 1Gy/13s.

2.5 Cell poration

a) Methods of cell poration with restriction enzymes

There have been many techniques developed for introducing high molecular weight proteins and DNA into cells. Three of the most popular techniques are described below.

i) Sendai virus permeabilization

Sendai virus is a haemolytic paramyxovirus (Bashford *et al.*, 1985). When cells are infected with a virus of this type there is usually a leakage of cell contents into the surrounding medium (Yamaizumi *et al.*, 1979). This indicates the production of cellular pores. If molecules can exit the cell through these cellular holes, it might be expected that molecules from the environment may enter into the cell. It is believed that during viral infection the lipid bilayer of the viral envelope and the cell membrane fuse together and that during the fusion, the cell membrane is somehow distorted (Bashford *et al.*, 1985; 1986). This produces a collapse in the cell membrane potential changing the cell permeability. This change allows the release of monovalent and divalent ions and phosphorylated intermediates of cell metabolism. Sendai virus has been used to successfully introduce T4 endonuclease into mammalian cells (Tanaka *et al.*, 1975). However, there did appear to be a non stochastic distribution of chromosome damage perhaps indicating that there was variability in cell permeability to the enzyme within the experimental cell population (Tanaka *et al.*, 1975). Sendai virus has been used (Singh, 1991) to introduce the restriction enzymes into mammalian cells in an attempt to induce chromosome aberrations and cell mutations.

i) Electroporation

Electroporation can be argued to be the most popular method of cell poration and the most effective in terms of the proportion of cells effected. Electroporation involves treating a population of cells in suspension with a high potential difference. At the critical voltage the cell membrane destabilises and begins to degenerate. During this breakdown transmembrane pores are produced which will allow the entry of foreign molecules. The pore is however, only present for the time of the electrical pulse. One of the problems associated with electroporation is that there is an enormous fraction of the treated cells which are lysed and hence lost from the cell population. Singh (1991) also observed a four fold increase in the mutation frequency at the *tk* locus in CHO-K1 cells as a result of the electroporation treatment.

iii) Streptolysin O poration

One of the most recently developed techniques for porating cells with large protein molecules is by the use of the bacterial toxin Streptolysin O. Streptolysin O is a 69kdalton cytotoxin isolated from the gram positive β haemolytic streptococci strain, *Streptococcus pyogenes* (Bryant, 1992). This thiol activated protein binds to the cholesterol in the target membrane to generate ring type structures which penetrate into the apolar domain of the lipid bilayer (Bhakdi *et al.*, 1985). This generates transmembrane pores which can be visualised as ring type structures using freeze fracture electron microscopy (Duncan and Schlegell, 1975). Experiments were performed in order to ascertain the optimum concentration of streptolysin O required to porate Chinese hamster cells with restriction endonucleases (Bryant, 1992). These experiments demonstrated that SLO does not produce a significant increase above background in the frequency of chromosome aberrations (Bryant, 1992).

b) Restriction enzyme purification and cell poration by SLO

Hanks Balanced Salt Solution (HBSS), 1 litre.

8g, NaCl; 0.4g, KCl; 0.06g, Na₂PO₄.2H₂O; 0.06g, KH₂PO₄; 1.5g, MgSO₄.7H₂O;

1g, D-Glucose, 3.5g, NaHCO₃, 1 litre of sterile double distilled water.

(All chemicals obtained from BDH).

Hanks Balanced Salt Solution+BSA (HBSS+BSA).

Bovine serum albumen (Molecular grade, Sigma), was dissolved in HBSS at 1% (10mg/ml) and then purified by Millipore filtration.

Streptolysin-O, (SLO, Welcome diagnostics)

Streptolysin-O was diluted as directed by the manufacturers specifications to a concentration of 1.81 units/ml in sterile distilled water and then purified by Millipore filtration.

Purification of Restriction Enzymes (RE)

RE, obtained from BRL, were stored at -20°C in the storage buffer in which they were supplied. The storage buffer contains a high concentration of glycerol which was shown by Costa (1990) to produce a high level of background DNA damage. Bryant and Christie (1989) also illustrated that the glycerol storage buffer influenced the chromosome damage. The enzymes were therefore purified from the contaminating storage buffer as previously described by Bryant and Christie (1989). Amicon-10 filters were sterilised by rinsing twice with 70% alcohol and then twice with sterile distilled water. The surface of the filter was covered with $50\mu\text{l}$ of sterile HBSS/BSA. The appropriate volume of RE and 2ml of HBSS was added. The Amicon filter was centrifuged at 6,000rpm in a Beckman Centrifuge (Model JC ME, rotor JA 20; RCF = 2822.4g) for 90 minutes at 4°C . The RE was recovered from the filter by inverting the column and spinning for 10 minutes at 2,000rpm at 4°C in a Chilspin (RCF = 724g). The RE was diluted to a concentration of 1unit/ μl in HBSS/BSA.

Enzyme Activity Assay using the plasmid pBR322

Once the enzyme was purified it was assayed to ascertain whether during the purification procedure the enzyme had lost any of its activity. In the rare cases where substantial activity had been lost the purified enzyme was discarded. pBR322 has only one site which is recognised by the enzyme *Pvu II*. Upon treatment with the *Pvu II* the circular plasmid is linearised. The linear version of the plasmid runs a different distance along the agarose gel during electrophoresis than the circular form. *Pvu II* digestion can therefore be easily assayed. The pBR322 also has only one restriction sites for *EcoRI*. Hence, the activity of this enzyme can also be assayed in this fashion.

$1\mu\text{g}$ of pBR322 was digested with 4 units, 2 units, 1 unit, 0.5 units, 0.25 units and 0.125 units of the restriction enzyme in a total volume of $50\mu\text{l}$ in a 0.5ml sterile microfuge tube. This was repeated for the purified and the unpurified enzyme. An undigested control was also run. The samples were incubated in a water bath for 1 hour at the temperature recommended by the enzyme manufacturer, and the reaction was stopped by the addition of $5\mu\text{l}$ of 50mM EDTA solution, $5\mu\text{l}$ of 20% SDS and $5\mu\text{l}$ of bromophenol blue solution. The samples were run on a 0.8% agarose gel for 5 hours at 60 volts and the digestion products of the purified and the unpurified enzymes compared. In all cases the activity of the purified enzyme was compared to that of the non-purified.

Cell poration with SLO and RE treatment

Flasks of cells were trypsinized and the cells counted. 10^6 cells were added to each of the 10ml sterile V tubes required in the experiment, and centrifuged in a Chilsplin (Fisons, MCR) at 4°C for 10 minutes at 2,000rpm (RCF = 742g). The cell medium was aspirated and the cells resuspended in 10ml of HBSS for washing. The cells were then recentrifuged for 10 minutes at 4°C at 2,000 rpm (RCF = 742g). The HBSS was completely removed by aspiration and the cells resuspended in 1ml of fresh HBSS. The appropriate amount of RE was added to each of the treatment tubes followed by 24 μ l of the SLO solution. The mixture was incubated for exactly 5 minutes before the addition of 9ml of MEM/FCS. The tube was then recentrifuged, the medium removed and the remaining pellet resuspended in 5ml of fresh medium. The cells were then counted in a coulter counter and treated as described in Section 2.6.

2.6 Survival and Mutation assays*Trifluorothymidine, (TFT, (Sigma)).*

TFT powder was dissolved in dimethyl sulphoxide (BDH) at a concentration of 1mg/ml. The TFT solution was later diluted in the MEM/FCS cell suspension to produce a concentration of 3 μ g/ml (MEM/FCS/TFT), the optimum concentration for the selection of *thymidine kinase* deficient mutants (Singh, 1991).

6-Thioguanine, (6-TG, (Sigma)).

6-TG was dissolved in dimethyl sulphoxide at a concentration of 1mg/ml and later diluted in MEM/FCS cell suspension at a concentration of 1 μ g/ml (MEM/FCS/6TG), the optimum concentration for the selection of *hprt*⁻ deficient mutants (Singh, 1991 and references therein).

The term survival may be argued to be misleading, as it does not refer to the ability to live through the mutagenesis treatment, but refers to the ability of the cell to maintain its reproductive capacity. Cell survival, in this thesis, is measured in terms of the ability of a single cell to divide and produce a colony of in excess of 50 cells.

Immediately following the radiation treatment the cells were trypsinized and counted (RE treated cells were just counted) and diluted to a concentration of between 100 and 500 cells per 4ml of MEM/FCS. 4ml of the cell suspension was added to 60mm tissue culture dishes (Corning) and incubated in a wet incubator at 37°C for 8 days prior to fixing and staining (described in Section 2.7). The remaining cells were seeded in 75cm² tissue culture flasks and maintained at exponential growth for the appropriate expression time (see Table 2.6.1).

Cell line	Gene studied	Expression time	Selective agent and concentration used
CHO-K1, <i>xrs-5</i>	<i>Thymidine Kinase</i>	4 days (Singh,1991)	3µg/ml trifluorothymidine.
V79-4, CHO-K1, <i>xrs-5</i>	<i>Hypoxanthine guanine- phosphoribosyl transferase</i>	5 days (Singh 1991 and references therein).	1µg/µl 6-thioguanine

Table 2.6.1: The expression time and the concentration of selective agents required to select for mutations at the *tk* and the *hprt* gene in the CHO-K1, *xrs-5* and the V79-4 cell line.

The expression time is the allowed time to compensate for phenotypic delay. When a genetic change occurs within the cell, a change in phenotype often is not observed immediately. It takes time for the old protein product to be diluted or the new protein product to accumulate.

After the expression time the cells were trypsinized, counted and the cells plated in the appropriate selective medium, 6-TG to select for *hprt* mutations and TFT to select for *tk* mutations. The V79-4 cells were plated at a concentration of 10^5 per 90mm plate, 10 plates per treatment. The CHO-K1 were plated at concentrations of 10^6 per 90mm tissue culture dish. These were calculated to be the maximum concentrations that could be plated without the individual cells interacting with each other (Thacker, 1976; Singh, 1991). When cells are close together they often exchange materials and enzymes through cellular pores, this phenomenon being termed metabolic co-operation (Cox *et al.*, 1972; Subak-Sharpe *et al.*, 1969). Cell overcrowding would thus give rise to an underestimation of the mutation frequency. In conjunction with the mutation plates, cells were seeded in non selective medium in a viability assay. Unless otherwise stated in both experiments with CHO-K1 and the V79-4 cell line, 100-200 cells were plated on 60mm tissue culture plates (Corning) in 4ml of tissue culture medium. The 90mm mutation plates were incubated for 14 days prior to fixing and staining and the 60mm plates were incubated for 8 days.

xrs-5 cells proved to be difficult to maintain in culture and the low plating efficiency dictated that the details of the mutation assay had to be changed. These conditions are described in the appropriate Chapters.

2.7 Fixing and staining plates

The medium was removed from the plates and the plates were then rinsed twice with phosphate buffer pH 6.4 (Gurr buffer) to remove any medium and unattached cells. After air drying, the colonies were fixed with methanol for 10 minutes and when the methanol had been removed the plates were allowed to dry for 30 minutes. A concentrated solution of Geimsa stain (BDH) was added to the plates and left for 10 minutes. The blue dye was removed by dipping the plates gently in warm water and the plates allowed to dry in the air. Only colonies of greater than 50 cells were scored.

2.8 Calculation of mutation frequency and surviving fractions

The plating efficiency which may be defined as the proportion of the viable cells in an untreated cell population was calculated by

$$\frac{\text{Number of colonies scored on non selective plates}}{\text{Number of cells seeded}} \times 100\%$$

The surviving fraction which can be described as the proportion of viable cells in a treated cell population was calculated using the following equation;

$$\frac{\text{Proportion of viable cells in treated population}}{\text{Proportion of viable cells in untreated population}}$$

The mutation frequency which is the proportion of observed mutant colonies per viable cell was calculated by

$$\frac{\text{Mean number of mutant colonies counted per X amount plated}}{\text{Proportion of viable cells}}$$

The induced mutation frequency can be defined as the observed frequency of mutations produced as a result of mutagenic treatment above the observed background mutation frequency. It was calculated as,

$$(\text{Mutation freq in treated population}) - (\text{mutation freq in untreated population})$$

2.9 Cryopreservation of cells

Cells were trypsinized and resuspended in MEM/FCS medium at a concentration of 10^7 /ml. 100 μ l of dimethyl sulphoxide was added per 1ml of cell suspension. 1ml of the cell suspension was added to each of the sterile freezer vials. The vials were immediately placed at -70°C and after 24 hours placed at -196°C in the liquid nitrogen storage tank. Cells can be stored for many years in this way.

2.10 The preparation of water saturated phenol

Redistilled phenol (Sigma) was stored at -20°C in a crystallised state. Prior to preparation it was allowed to warm up to room temperature and then melted in a 60°C water bath. The phenol was shaken with an equal volume of distilled water and the two phases allowed to separate. The lower water saturated phenol phase was transferred to a glass container and 8 hydroxyquinolene added to produce a concentration of approximately 0.1mg/ml. The now yellow liquid was shaken with an equal volume of 0.1M tris-HCl (pH 8), mixed and centrifuged at 1,000rpm for 20 minutes in a Chilspin centrifuge (RCF = 185g). The colourless aqueous layer on top was discarded and the remaining saturated phenol stored at 4°C until use.

2.11 The isolation of DNA from mammalian cells

10^6 cells were resuspended in MEM/FCS and centrifuged for 10 minutes at 2,000rpm (RCF = 742g). The remaining pellet was resuspended in 0.5ml of 1M tris-HCl pH8. The cells were lysed by the addition of 0.5ml of 5% SDS and 0.5ml of 10mM EDTA and incubated for 10 minutes at 60°C . To digest the cellular protein, 100 μ l of self digested pronase stock (4mg/ml; Sigma) solution was added and the resulting mixture incubated at 37°C overnight. The DNA solution was separated into 600 μ l aliquots in sterile microfuge tubes (Ependorf) and an equal volume of phenol added. The mix was inverted several times to produce an emulsion and then centrifuged for 5 minutes at 12,000rpm (RCF = 5644g) in a microcentrifuge. The upper aqueous phase was transferred to a clean tube and the lower phenol layer discarded. An equal volume of phenol : chloroform mix (1:1) was added and the resulting emulsion centrifuged at 12,000 rpm (RCF = 5644g) for 5 minutes. This method of phenol treatment followed by phenol : chloroform treatment was repeated three times on each DNA sample. 0.4ml of the DNA solution was added to a clean microfuge tube followed by the addition of 40 μ l of 3M sodium acetate solution (pH 5.8), and 800 μ l of ice cold ethanol. To aid the DNA precipitation, the tube was placed at -20°C for 1 hour and then centrifuged for 5 minutes at 12,000rpm. The supernatant was discarded and the remaining pellet dried in a vacuum tank. Once the pellet

was dry it was dissolved in 50 μ l of TE buffer and stored at 4 $^{\circ}$ C. The purity and the concentration of the DNA was calculated by measuring the absorbency of the TE (10mM tris-HCl; 1mM EDTA in sterile distilled water) diluted sample at 260nm and 280nm in a spectrophotometer using quartz vials (after adjusting the absorbency to zero with TE buffer). The purity of the DNA is measured as a ratio of the absorbency at 260nm divided by the absorbency at 280nm, the ratio for a totally pure sample being 2. If the ratio of the absorbencies was less than 1.75 then the phenol/chloroform extraction procedure was repeated.

The concentration of the nucleic acids was calculated by

$$\text{Absorbency at 260nm} \times 50 \times \text{dilution factor} = \mu\text{g/ml.}$$

Chapter 3

The genetic and cellular effects of gamma radiation upon Chinese hamster cell lines

One unusually frank advocate of the human genome project, while agreeing that it makes more scientific sense to survey the DNA of mice than men, points out that the human genome is unique as it belongs to the only species that is willing to pay the bill

Steven Jones, *The Language of the Genes*.

3.1 Introduction

In order to study the effects of radiation and the importance of the dsb upon mutation induction and cell death, a series of experiments were performed on the CHO-K1 cell line, a radiosensitive dsb repair deficient mutant of CHO-K1, *xrs-5* and the male fibroblast cell line V79-4. The genes of interest in the mutation assays were the autosomally located *thymidine kinase* gene (*tk*) and the *hypoxanthine guanine phosphoribosyl transferase* gene (*hprt*) located on the X chromosome.

A single experiment was performed to illustrate that the optimum plating concentration to select for CHO-K1 *tk*⁻ mutant cells is 10⁶ cells per 10ml per 90mm tissue culture plate as predicted by Singh (1991). Experiments were performed on the CHO-K1 cell line to study the effects of expression time on the mutation frequency at the *tk* locus. Also, experiments to find the frequency of spontaneous *tk* and *hprt* mutations within the CHO-K1, *xrs-5* and V79-4 cell populations were carried out. In all three cell lines the lethal effects of different doses of gamma radiation, where cells were plated immediately following irradiation, were studied using clonal survival assays. The effect of increasing doses of radiation on the mutation

frequency at the *tk* locus were analysed in CHO-K1 cells and *xrs-5* cells and at the *hprt* locus in the CHO-K1 and V79-4 cell lines and the results obtained from the different cell lines were compared.

3.2.1 The optimum plating concentration of CHO-K1 cells to select for *tk* mutations

It has been shown that the density at which cells are plated effects the recovery of mutants cells (Chu and Malling, 1968). Above a certain cell density mutant cells and wild type cells will come into contact and materials will be exchanged between cells through gap-junctions in the cell membrane. The wild type cells will "poison" the mutant cells, with for example *tk* gene products and as a consequence, there will be an underestimate of the mutation frequency. Also, if not enough cells are plated no mutants will be observed.

It was calculated by Singh (1991) using a *tk*⁻ CHO-K1 cell line that the maximum plating concentration for CHO-K1 cells should be 10⁶ per 90mm tissue culture dish. A single experiment was performed to illustrate this observation using CHO-K1 cell line (*tk*⁺). 2 × 10⁶ cells were set up in MEM/FCS in a 75cm² tissue culture flask and irradiated (24 hours later) with 4Gy gamma radiation. The cells were cultured in non selective MEM/FCS for 4 days (the expression time suggested by Singh and Bryant, 1991) passaging when required. The excess cells were returned to another 75cm² flask and culturing in non selective MEM/FCS continued. The cells were then plated in MEM/FCS/TFT at cell concentrations of 10⁵ (30 plates), 5 × 10⁵ (10 plates), 10⁶ (3 plates), 2.5 × 10⁶ (3 plates) and 5 × 10⁶ (3 plates) in 90mm tissue culture dishes. A clonogenic assay was performed on the same cell population to assay for survival in non-selective medium. 200 cells were plated in 4ml of MEM/FCS in 60mm tissue culture dishes. The 90mm tissue culture dishes were incubated for 14 days and the 60mm tissue culture dishes were incubated for 8 days. Figure 3.2.1.1 shows the effect of different cell plating concentrations on the observed frequency of *tk*⁻ mutants in 4Gy irradiated CHO-K1 cells. Above 10⁶ cells the observed mutations per 10⁵ survivors decreases dramatically, presumably due to metabolic co-operation. Hence, plating above this calculated concentration will result in an underestimate of the mutation frequency.

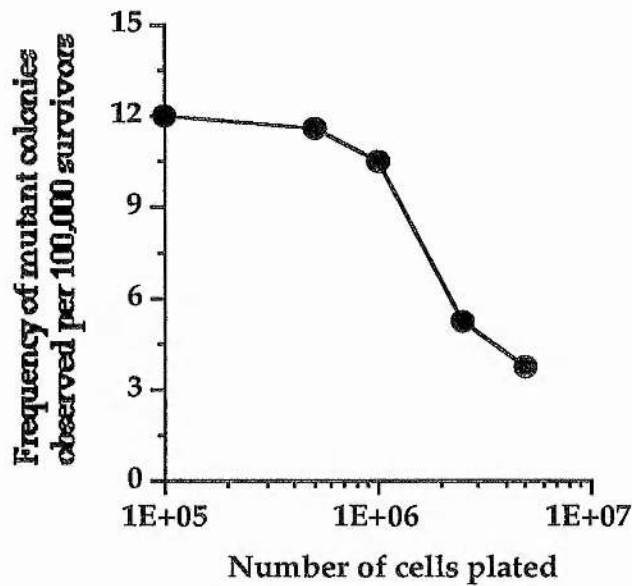


Figure 3.2.1.1: The effect of the cell plating number (in 90mm tissue culture dishes) on the observed mutation frequency at the *tk* locus in CHO-K1 cells following 4Gy gamma irradiation and an expression time of 4 days. The data represent the results of a single experiment.

3.2.2 The optimum expression time for CHO-K1 cells at the *tk* locus

Singh (1991) noticed that the observed mutation frequency at the *tk* locus was dependent upon the expression time. He showed that the optimum expression time for the *tk* locus in CHO-K1 cells was 4 days. This experiment was repeated in the following way. 9 flasks of CHO-K1 cells were set up at cell densities varying from 4×10^6 to 1×10^6 per 75 cm² flask (the density used was dependent upon the expression time) and 24 hours later irradiated with 4Gy of gamma radiation. Immediately following irradiation the flask with the highest cell concentration was trypsinized and the cells plated at a concentration of 10^6 per 90mm tissue culture dish (3 dishes) in 10ml of MEM/FCS/TFT selective medium. 100-200 cells were then plated in a clonogenic assay in 4ml of non selective MEM/FCS in 60mm tissue culture dishes (3 dishes). Each day for 8 days, this plating procedure was repeated each day, using cultures that had been set up at a lower concentration than the previous day. This procedure helped to ensure that there were enough cells for plating during the first few days. The 90mm tissue culture dishes were incubated for 14 days and the 60mm tissue culture dishes were incubated for 8 days prior to fixing and staining. Figure 3.2.2.1 shows the effect of different expression times on the observed induced mutation frequency at the *tk* locus in CHO-K1 cells. The data suggests that the optimum expression time is between 2-4 days and that above 5 days the observed mutation frequency at the *tk* locus decreases. An optimum expression

time of 4 days was also found by Singh (1991). An expression time of 4 days was therefore used in the *tk* mutation experiments.

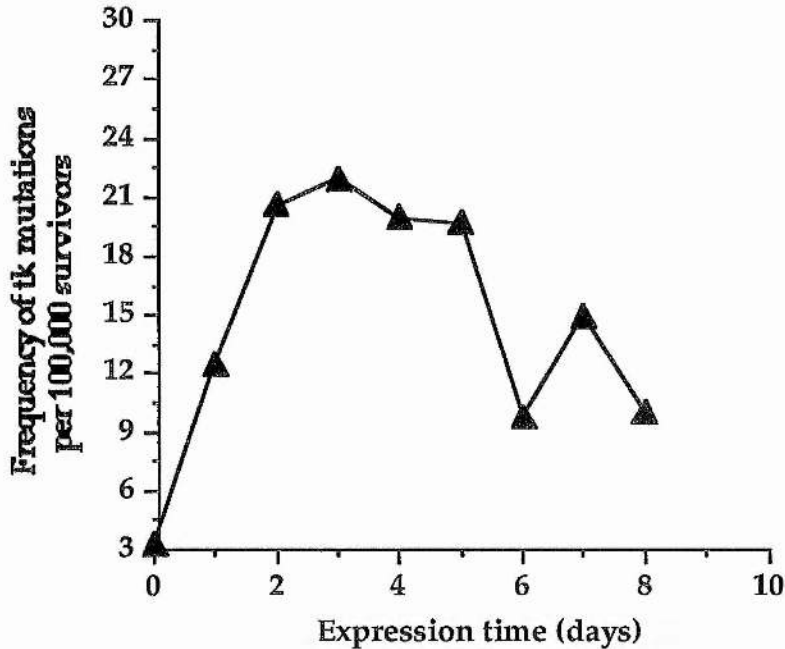


Figure 3.2.2.1 : The effect of expression time on the observed frequency of *tk* mutations in the CHO-K1 cell line. The results represent the mean of two independent experiments.

3.2.3 The spontaneous mutation frequency at the *tk* and the *hprt* locus in the different Chinese hamster cell lines

Within a population of cells, not all cells are identical. Errors in the process of DNA replication occur spontaneously. This results in a proportion of the cells having a different genotype to other cells in the population. If the change in the DNA sequence is in a dominant gene or in a heterozygous or hemizygous gene then a change in the cell phenotype may be produced.

Large numbers of cryogenic vials containing identical stock cell cultures were stored in liquid nitrogen. These cells were stored in non selective MEM/FCS medium and thawed and grown in 75 cm² tissue culture flasks (Nunc) in MEM/FCS medium and passaged (when required) to maintain the cells in the exponential growth phase. A passage line was maintained for three months and it was from this passage line that cells for experiments were taken. After three months a new stock of cells would be thawed. This procedure was adopted because

cells grown for extended periods of time in culture tend to change their genetic characteristics due to genetic drift. This ensured that successive experiments were carried out on cell populations with the same genetic characteristics. These fresh cells were grown for several passages prior to use in experiments.

To check the frequency of *hprt*⁻ cells within the V79-4 cell population 5×10^5 cells were set up in a 75cm² tissue culture flask, maintained in exponential growth for 5 days and then 10^5 cells plated in 10ml of 6TG selective medium (MEM/FCS/TG) on 90mm tissue culture plates, 10 plates per experiment. In order to check the plating efficiency, a clonogenic assay was performed. 100-200 cells were plated in 4ml of MEM/FCS in 60mm tissue culture dishes (Corning). CHO-K1 cells show less metabolic co-operation than the V79-4 cell line (Singh, 1991 and references therein) and were therefore plated in TFT selective medium (MEM/FCS/TFT) to assay for *tk*⁻ mutations at the higher concentration of 10^6 per 10ml of TFT selective medium in 90mm tissue culture plates. A clonogenic assay was again performed in order to measure the plating efficiency. 100-200 cells were plated on 60mm tissue culture plates in 4ml of non selective MEM/FCS. The CHO-K1 and the V79-4, 60mm plating efficiency plates were incubated for 8 days and the mutation plates were all incubated for 14 days prior to fixing and staining with giemsa (Sigma) as described in Chapter 2. The plating efficiency and the mutation frequencies were calculated as described in Chapter 2.

When the initial experiments were performed with *xrs-5* cells, 10^6 cells were plated in 90mm dishes in TFT selective medium and no mutants were observed. It was possible that due to the reduced plating efficiency (between 15% and 25%), 10^6 cells were inadequate for mutation assays. Plating a greater number of cells in 90mm dishes would be unsatisfactory since chemicals would be released from the wild type cells poisoning the adjacent mutant cells, thus producing an underestimate of the mutation frequency. To rectify this situation, the *xrs-5* cells were plated into 150mm diameter dishes having approximately three times the area of the 90mm dishes. 3×10^6 cells were plated into these dishes in a total volume of 30ml of MEM/FCS/TFT selective medium. The clonogenic assay measuring the plating efficiency involved plating 500 cells in 90mm dishes in 10ml of non selective MEM/FCS medium. To demonstrate that the plate size and manufacturers types (90mm plates were from Nunc and 150mm plates were from Corning) were not responsible for any difference in the mutation frequency observed between CHO-K1 and *xrs-5* cells, a single mutation assay experiment involving plating CHO-K1 in the 150mm plates was performed. Approximately the same spontaneous mutation frequency in the CHO-K1 cells at the *tk* locus was observed when the assay was performed with the different sized tissue culture dishes.

Various cell lines cultured independently show different spontaneous mutation frequencies. The background mutation frequency at the *tk* and the *hprt* locus for the different cell lines are shown in Table 3.2.3.1. The *xrs-5* cell line has a higher *tk* mutation frequency compared

with the parental CHO-K1 cell line. In the results obtained the *xrs-5* and CHO-K1 had a similar *tk* mutation frequency. The experiments however suggest, that the *xrs-5* cells maintained in this laboratory are *hprt*⁻, having reduced or absent HPRT enzyme activity.

Cell line	Locus	Mutation frequency (10 ⁻⁵)	Standard error	Number of experiments
V79-4	<i>hprt</i>	1.36	0.27	3
CHO-K1	<i>tk</i>	2.9	0.8	3
	<i>hprt</i>	0.567	0.2	3
<i>xrs-5</i>	<i>tk</i>	3.73	2.8	3
	<i>hprt</i>	<i>hprt</i> ⁻	-	3

Table 3.2.3.1: The different spontaneous mutation frequencies at the *tk* and the *hprt* locus observed in the CHO-K1, V79-4 and the radiation sensitive *xrs-5* cell lines.

Singh (1991) reported that the spontaneous mutation frequency of the *xrs-5* cells was higher than in the parental CHO-K1 cells. This was tested by analysing the data statistically with a t-test (performed as described by Wardlaw, 1985). As the data (shown in Table 3.2.3.2) for the *xrs-5* and the CHO-K1 cell line was not collected at the same time and the experiments were performed independently, an unpaired t-test was performed. A t-test is used to assess the significance of the difference in the means of two groups of experiments whose standard deviations or variances are not significantly different. It was used to see if the spontaneous mutation frequency in the *xrs-5* cells at the *tk* locus was significantly different to the spontaneous mutation frequency at the *tk* locus in the parental CHO-K1 cells.

	CHO-K1	<i>xrs-5</i>
	4.36	1.3
	1.56	7.7
	2.22	2.2
mean	2.9 = y ₁	3.73 = y ₂
standard deviation	0.8 = S ₁	2.80 = S ₂

Table 3.2.3.2: The results show the spontaneous mutation frequencies at the *tk* locus in the CHO-K1 and the *xrs-5* cell line. Each of the values in the data are the mean of an independent experiment. The mean and the standard deviation of the experiments were calculated and are also tabulated.

Before the t-test was performed an F test was performed on the standard deviations, S_1 and S_2 . This was to assess whether or not the standard deviations of the two groups were significantly different.

$$[S_1 \times S_1] \times [S_2 \times S_2]^{-1} = F \quad (\text{Eq.3.2.3.1})$$

The degrees of freedom were calculated by (n-1) for each set of experiments, hence the degrees of freedom were (2, 2). The F value calculated (12.25) was lower than the value tabulated at the 5% level (19.00). Therefore the difference in the variances between the two groups is suggested not to be significant. A comparison of the data collected by a t-test is therefore valid

$$[(y_2 - y_1) (N)^{1/2}] \times [(S_1^2 + S_2^2)^{1/2}]^{-1} = t \quad (\text{Eq.3.2.3.2})$$

The t value obtained by substituting in the above equation (taken from Wardlaw, 1985) where y_1 and y_2 are the mean values and S_1 and S_2 were the standard deviations. N was the number of experiments. The t value was calculated to be 0.49. This value is lower than the tabulated value for 2 degrees of freedom. It is therefore suggested that there is no significant difference between the spontaneous mutation frequencies at the *tk* locus in the *xrs-5* cell line and the parental CHO-K1 cell line that can not be explained in terms of random sampling fluctuations.

3.2.4 The effects of gamma radiation on the mutation frequency

5×10^5 V79-4 cells and 10^6 CHO-K1 cells were seeded in 75cm^2 flasks in non selective MEM/FCS medium and incubated for 24 hours prior to gamma irradiation (see Chapter 2) at 0Gy, 2Gy, 4Gy and 6Gy. Immediately following irradiation the cells were trypsinized, counted and were plated in a survival assay at concentrations ranging from 100 to 500 cells per 4ml of MEM/FCS in 60mm dishes. The remaining cells were returned to the appropriate flasks and cultured for the required expression time (passaging when necessary) and the mutation assay performed as described in the Chapter 2.

xrs-5 cells are much more sensitive than either V79-4 cells or CHO-K1 cells to the lethal effects of gamma radiation. *Xrs-5* cells were therefore treated with lower radiation doses. 10^6 cells were seeded in 75cm^2 flasks and irradiated approximately 24 hours afterwards with radiation doses of 0Gy, 0.5Gy, 1Gy and 1.5Gy. A survival assay was performed which involved the plating of cells at concentrations of 500, 800, 1,000 and 1,500 in 90mm tissue culture plates in 10ml of non selective MEM/FCS. The remaining cells were returned to the 75cm^2 flasks and further incubated for the appropriate expression time prior to plating at 3 x

10^6 cells per 150mm tissue culture dish in 30ml of TFT (3 replicate plates). Three replicate plates of 500 cells diluted in 10ml of non selective MEM/FCS medium were plated in 90mm tissue culture dishes for a plating efficiency assay. The 150mm plates were incubated for 16-20 days prior to fixing and staining and the 90mm plates were incubated for 10 days.

As the radiation dose increases so does the probability of reproductive cell death. This is illustrated in Figures 3.2.4.1 and 3.2.4.2. These graphs suggest that at the doses studied, the CHO-K1 cell line is more sensitive to the lethal effects of gamma radiation than the V79-4 cell line. Up to 4Gy the standard errors overlap. Hence, more experiments and perhaps some at higher doses would need to be performed before a firm conclusion could be drawn. The *xrs-5* cells however, can be seen to be substantially more sensitive than CHO-K1. Similar results were also obtained by Jeggo and Holliday (1986), Costa and Bryant (1988), Singh (1991), Singh and Bryant (1991) and Shadley *et al.*, (1991). Bryant *et al.*, (1987) and Shadley *et al.*, (1991) also observed a greater induction of chromosome and chromatid aberrations per unit X-ray dose in the *xrs-5* cells as compared to the CHO-K1 cells. Chromosome and chromatid aberrations could possibly be the cause of reproductive cell death.

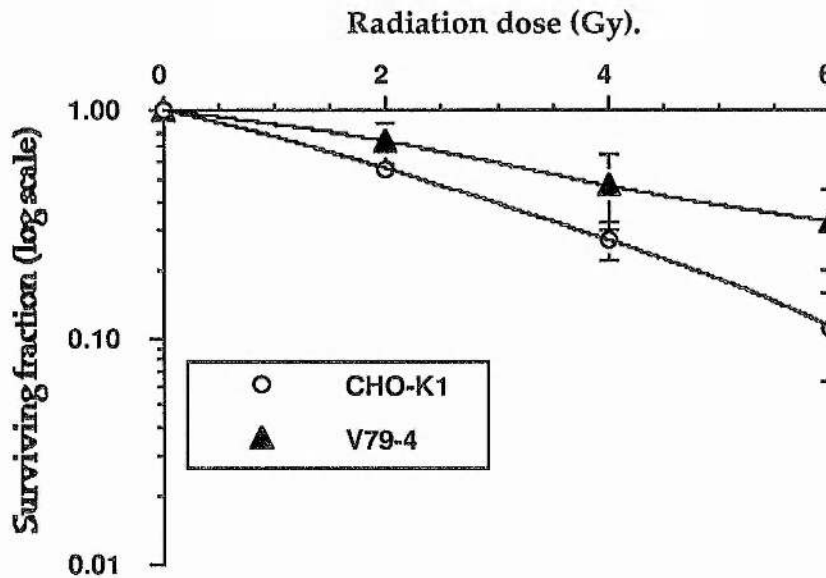


Figure 3.2.4.1: The relationship between the radiation dose and the surviving fraction (plotted on a log scale) in V79-4 and CHO-K1 cells. Each data point is the mean of three independent experiments and the vertical lines represent the standard errors of the mean.

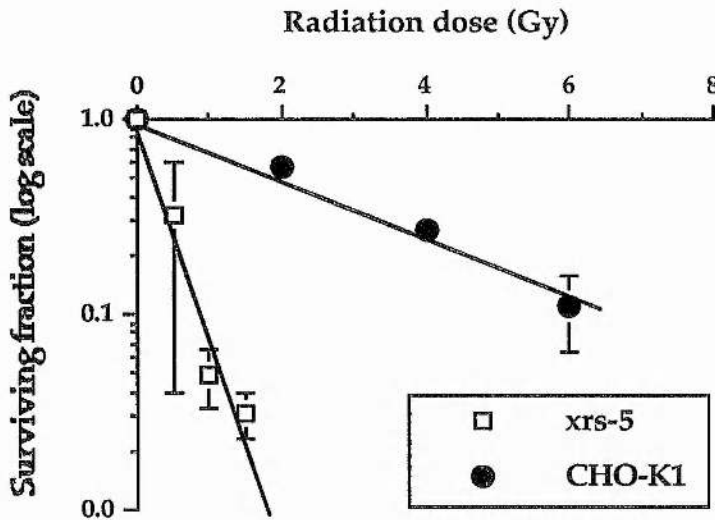


Figure 3.2.4.2: The relationship between gamma irradiation and the surviving fraction plotted on a log scale in CHO-K1 cells and the X-ray sensitive *xrs-5* cells. Each data point for the CHO-K1 and *xrs-5* represents the mean of three independent experiments and the vertical lines represent the standard error of the mean .

Figure 3.2.4.3 shows the induction of mutations at the *hprt* locus in V79-4 cells and at the *tk* locus in CHO-K1 cells as a result of gamma irradiation. The frequency of induced mutation at the *tk* locus in CHO-K1 cells is similar to the frequency of *hprt* mutations in the V79-4 cell line at the doses studied. This is surprising since the induced *hprt* mutation frequency in the CHO-K1 cell line is much lower than that at the *tk* locus (Figure 3.2.4.5). The effects of gamma irradiation upon the induction of *tk* mutations in the CHO-K1 cell line and the daughter *xrs-5* cell line is shown in Figure 3.2.4.4.

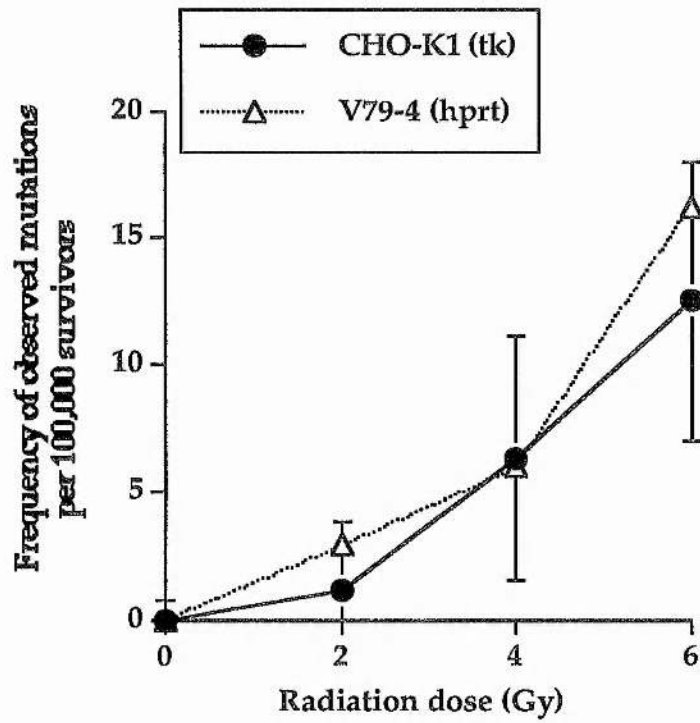


Figure 3.2.4.3: The effects of gamma irradiation upon the induced mutation frequency at the *tk* locus in the CHO-K1 cell line and at the *hprt* locus in the V79-4 cell line. All data points are the mean of three independent experiments and the vertical lines represent the standard error of the mean of three independent experiments.

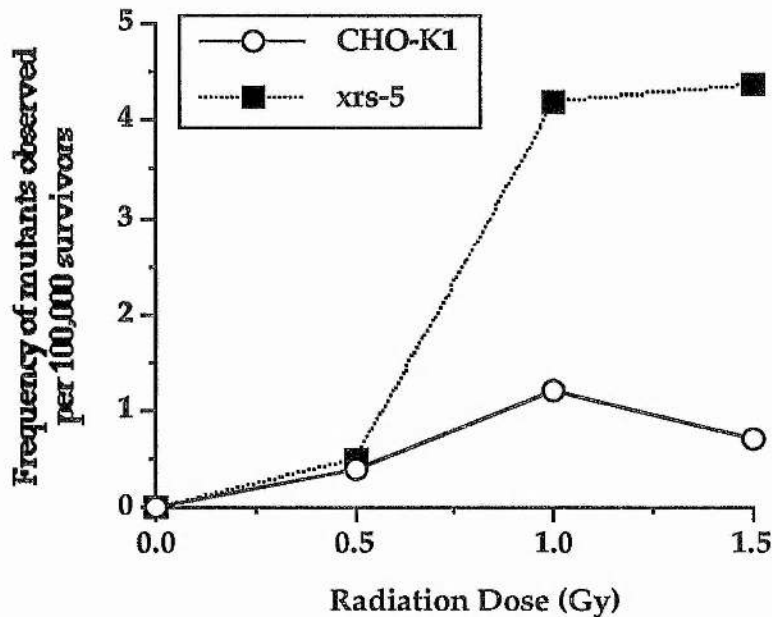


Figure 3.2.4.4: The effects of gamma irradiation (at doses between 0Gy and 1.5Gy) upon the induced mutation frequency at the *tk* locus in the *xrs-5* cell line and in the parental CHO-K1 cell line. Each data point in the *xrs-5* represents the mean of two independent experiments and CHO-K1 data represents a single experiment.

Mussa *et al.*, (1990) and Singh (1991) first showed that *xrs-5* was hypermutable at the *tk* locus as compared to the parental CHO-K1 cell line. Only small radiation doses were used for this study. This is because in *xrs-5* cells at high radiation doses, there is a large amount of cell death. At higher doses an inconveniently large number of cells would need to be irradiated for there to be an adequate number of cells available for plating in a mutation assay. At 0.5Gy a similar mean induced mutation frequency at the *tk* locus was observed for both CHO-K1 and *xrs-5* cells however, at 1Gy and 1.5Gy the mean induced mutation frequency in the *xrs-5* cells was almost 4 times that of the CHO-K1 cells, thus supporting the observations of Mussa *et al.*, (1990) and Singh (1991). Darroudi and Natarajan (1989) also found the *xrs-5* cell line to be hypermutable at the *hprt* locus as compared to the CHO-K1 cell line when treated with X-rays. Shadley *et al.*, (1991) however, found little difference in the induced mutation frequency at the *hprt* locus per unit of X-ray dose between the *xrs-5* cells and the CHO-K1 cells. However, it was found that the *xrs-5* cells maintained in the St. Andrews laboratory are *hprt* - Induced mutations at this locus could therefore not be studied.

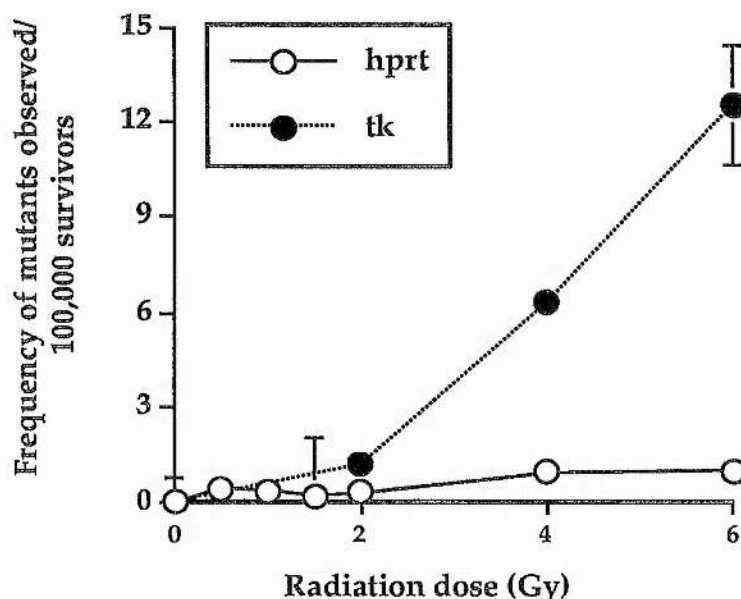


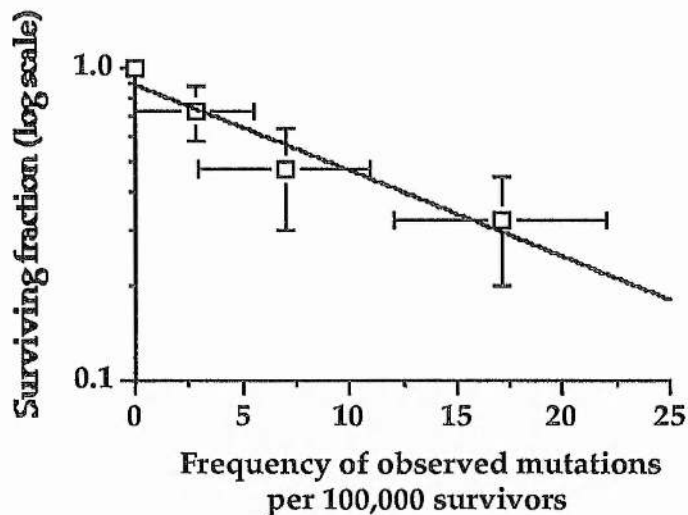
Figure 3.2.4.5: The effect of different doses of gamma radiation on the induced mutation frequency at the *tk* and the *hprt* locus in the CHO-K1 cell line. The results are the mean of three independent experiments and the vertical lines represent the standard error of the means. In some cases the standard error is too small to be observed on the graph.

Cell lines may be expected to differ in terms of the immediate surviving fraction and mutation frequency following treatment with gamma rays. This response is dependent upon many variables such as the organism from which the cells were initially derived and the method by which the cells were immortalised and the general evolution of the cell line. Also of importance when studying a specific locus is the genetic background. When cells are immortalised the chromosomes are often rearranged, for example, chromosomal translocations and inversions frequently occur. The location of the genetic locus of interest in relation to other genes may be different in the different cell lines as compared to the genes of the diploid eukaryotic hamster. This may explain the differences observed in the induced mutation frequency at the *hprt* locus in the CHO-K1 and the V79-4 cell lines. The different cell lines may be under different genetic constraints. In CHO-K1 cells there could be essential genes flanking one or both sides of the *hprt* gene hence limiting the survival and proliferation of *hprt* mutations. The selective pressure on the *hprt*⁻ cells in the V79-4 cell line may not be so great as in the CHO-K1 cells. It is also possible that there is a different amount of damage induced in the *hprt* gene of the two different cell lines per unit radiation dose. It is easy to see

how some areas of the genome may be more susceptible to DNA damage than other areas of the genome. This is related to the chromosome organisation. DNA which is tightly coiled and packed will be more protected from free radicals and perhaps direct damage, than DNA which is exposed. The different cell lines may have different chromosomal organisation hence, not only the position of the gene in relation to other genes could have changed but also the three dimensional coiling of the DNA and their association to histone and non histone proteins could have altered. All these changes may effect the spontaneous and induced mutation frequencies.

3.2.5: The relationship between the surviving fraction (plating immediately after treatment) and the mutation frequency (following the required expression time).

Figure 3.2.5.1 shows the relationship between the immediate surviving fraction and the *hprt* mutation frequency following a 5 day expression time in the V79-4 cell line. As the surviving fraction decreases the mutation frequency increases. This suggests that the mechanism responsible for cell killing is also responsible (or related to) the mechanism by which mutations are induced.



3.2.5.1: The relationship between the surviving fraction immediately following irradiation and the induced mutation frequency at the *hprt* locus after a 5 day expression time. The vertical and the horizontal bars represent the standard error of the mean of three independent experiments.

The relationship between the cell surviving fraction (cells plated immediately following irradiation) and the *tk* mutation frequency following a 4 day expression time in the *xrs-5* cell line and the parental CHO-K1 cell line is illustrated in Figure 3.2.5.2. The Figure suggests that in CHO-K1 and *xrs-5* cells the relationship between the surviving fraction and the mutation frequency is different.

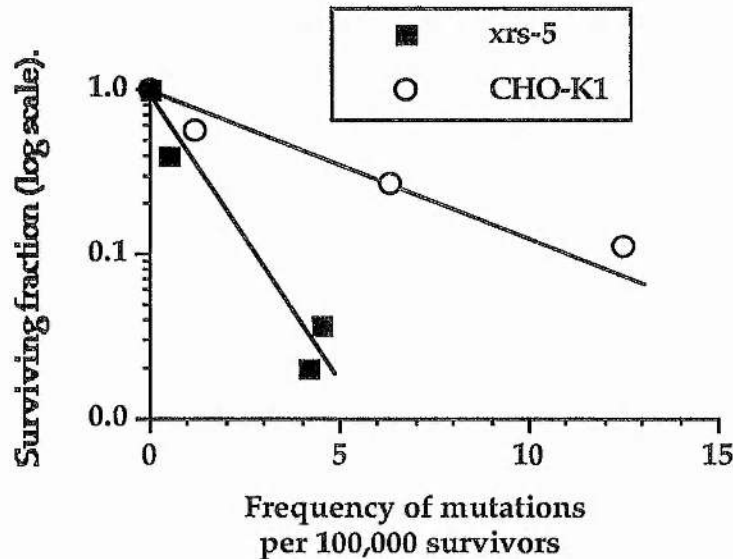


Figure 3.2.5.2: A comparison of the relationship between the surviving fraction immediately after radiation treatment and the mutation frequency at the *tk* locus after a 4 day expression time in CHO-K1 and *xrs-5* cells.

At a surviving fraction of 0.1 it can be estimated (from Figure 3.2.5.2) that there would be approximately 3 *tk*⁻ mutants observed per 10⁵ survivors in the *xrs-5* cell population. However, in CHO-K1 cells at an equitoxic dose it would be expected that there would be approximately 10-12 *tk*⁻ mutants observed per 10⁵ survivors. A similar relationship was also observed by Shadley *et al.*, (1991) at the *hprt* locus when treated with both X-rays and ²¹²Bi. This is consistent with the idea that the *xrs-5* cell line is deficient in DNA repair. The *xrs-5* cells are more likely to die following radiation exposure than be repaired. Erroneous DNA repair is one of the causes of DNA mutations. When the CHO-K1 genome is damaged by radiation exposure the DNA will be repaired. However, DNA repair is not 100% efficient. Although the lethal lesions are removed, allowing the cells to reproduce and proliferate, errors in the nucleotide sequence may be introduced. It therefore supports the hypothesis that mutation is often the result of aberrant DNA repair. In the *xrs-5* cells a much smaller percentage of the damage is repaired, more of the lesions will therefore be allowed to remain in the genome sentencing a greater proportion of the cells to reproductive death. As there is a smaller proportion of the cells repaired in *xrs-5*, there is less opportunity for mutations to

accumulate, hence a smaller number of *tk* mutations per unit surviving fraction is observed. Shadley *et al.*, (1991) interpreted such an observation to mean that the *xrs-5* cells are hypomutable. This interpretation is somewhat controversial. If CHO-K1 and *xrs-5* cells were treated with equitoxic doses, CHO-K1 would be treated with almost four times the radiation dose of *xrs-5* cells. Under these circumstances it would not be surprising to observe a high induced mutation frequency at the *tk* locus in CHO-K1 cells as compared to *xrs-5* cells.

3.2.6 The *xrs-5* cell line and the *hprt* gene

The *xrs-5* cell line maintained in this laboratory was shown to survive and proliferate in 6TG selective medium, suggesting that this cell population was phenotypically *hprt*⁻. This observation was studied further in the following series of experiments.

i) The ability of xrs-5 cells to grow in different selective media

xrs-5 cells were taken from a frozen stock and grown exponentially for several days prior to trypsinizing, counting and the plating of 500 cells per 90mm dish in the three different media; FCS/MEM, FCS/MEM/6TG and FCS/MEM/HAT. MEM/FCS/HAT medium was made up as follows, for 1 litre; 100ml of Eagles MEM (Gibco), 10ml penicillin/streptomycin (Gibco), 10ml of glutamine, 10ml non essential amino acids (Gibco), 30ml of sodium bicarbonate solution, 100ml of foetal calf serum (Flow), 20ml of HAT (x50 concentrate; Sigma) and 720ml sterile distilled water. There were three replicate plates for each of the different selective media set up. The dishes were incubated for 10 days prior to fixing and staining.

HAT medium contains hypoxanthine, aminopterin and thymidine. Tetrahydrofolate is a co-enzyme which is necessary for the insertion of 2 of the carbon moieties into the purine ring in the *de novo* biosynthesis pathway. During the insertion of the carbon atoms into the purine ring tetrahydrofolate is converted to dihydrofolate. The tetrahydrofolate is regenerated by the enzyme dihydrofolate reductase (see Figure 3.2.6.1) and used again in further purine ring manufacture. Aminopterin is a competitive inhibitor of the enzyme dihydrofolate reductase, hence inhibiting the recycling of dihydrofolate thus inhibiting the *de novo* biosynthesis of purines. Hypoxanthine and thymidine are substrates in the salvage pathway of purines and pyrimidines. Cells cultured in HAT-supplemented medium therefore have to manufacture all their nucleic acids by the salvage pathway. Cells with functional purine and pyrimidine salvage pathways are therefore able to survive and proliferate, cells with inefficient or inactive salvage pathways will not be able to replicate or reproduce.

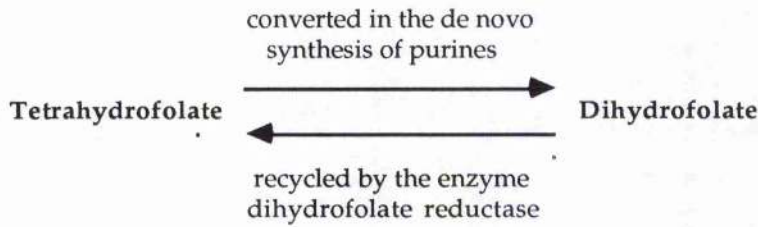


Figure 3.2.6.1: The conversion of tetrahydrofolate to dihydrofolate during the synthesis of the purine ring and the recycling of dihydrofolate by the enzyme dihydrofolate reductase.

Figure 3.2.6.2 illustrates the cloning efficiency of the *xrs-5* cells in MEM/FCS and in the selective media MEM/FCS/HAT and MEM/FCS/6TG. The results suggest the *xrs-5* cells can grow as efficiently in MEM/FCS/TG as in non selective MEM/FCS. The *xrs-5* cells however, do not grow at all in the selective MEM/FCS/HAT medium. These results suggest that the HPRT enzyme activity is reduced or the enzyme is absent.

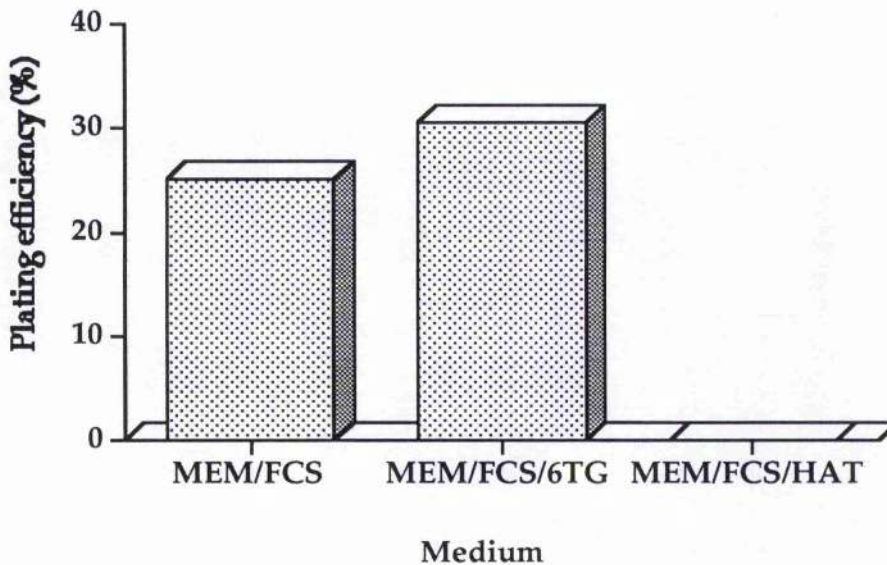


Table 3.2.6.2: The cloning efficiency of the *xrs-5* cells in different selective media. The results are the mean of 3 replicate plates of a single experiment.

ii) 5-Azacytidine treatment

To ascertain whether the *hprt*⁻ phenotype was the result of a genetic or an epigenetic change involving DNA methylation, the *xrs-5* cells were treated with 5-azacytidine (5-AZ) as described by Jeggo and Holliday (1986). 5-AZ is an analogue of the purine nucleotide base cytosine. The structure of the base moiety is shown in Figure 3.2.6.3. 5-AZ was initially

synthesised in Czechoslovakia in 1964 and was used in cancer treatment (Walker *et al.*, 1979). At the position 5 of the pyrimidine ring in 5-AZ the carbon in the cytosine is substituted for a nitrogen. It is incorporated into the DNA molecule in the place of cytosine. It is however, resistant to DNA methylation.

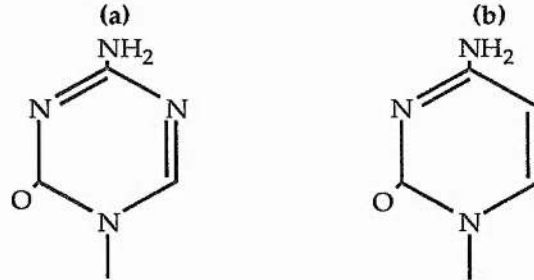


Figure 3.2.6.3: The structure of the base moiety of the nucleotide analogue (a) 5-Azacytidine (5-AZ) and the base moiety of the nucleotide (b) cytosine. Notice in 5-AZ the carbon at position 5 has been substituted for a nitrogen (diagram redrawn from Walker *et al.*, 1979)

Cells were seeded at a concentration of 10^6 per 75cm^2 flask, one experimental flask and one control. 5-AZ is unstable in aqueous solution and thus was made up at a concentration of 1mg/ml immediately prior to use. The 5-AZ solution was added to the treatment flask to produce a final concentration of $1\mu\text{g/ml}$ and incubated for 15 hours. After this time period the 5-AZ supplemented medium was replaced with MEM/FCS and cultured for a further 5 days, passaging when necessary. 500 cells were plated in 90mm dishes in each of the following different media MEM/FCS, MEM/FCS/6TG and MEM/FCS/HAT, 3 replicate plates for each growth treatment.

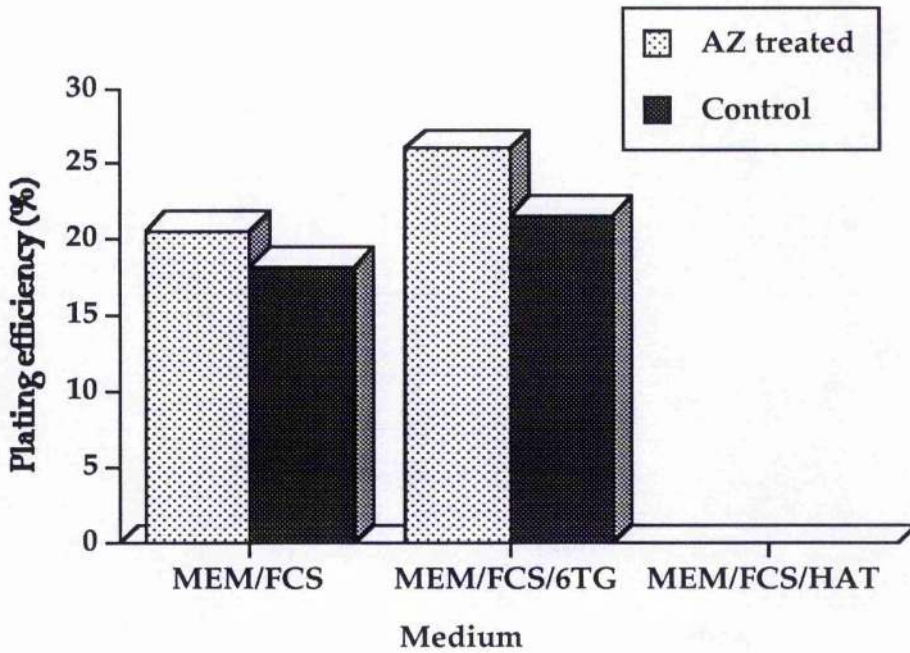


Figure 3.2.6.4: The cloning efficiency of the *xrs-5* cells in different selective media following treatment with 5-Azacytidine. No colonies were observed following incubation in MEM/FCS/HAT medium. The results represent the mean of three replicate plates in a single experiment.

Figure 3.2.6.4 shows the effect of 5-AZ upon the cloning efficiency of the *xrs-5* cell line. As before the cells grow and proliferate in the MEM/FCS/6TG selective medium but not in the MEM/FCS/HAT medium. Genes can be switched on and off by DNA methylation. The *hprt* gene is a type II gene having a promoter which has a high GC content (Alberts *et al*, 1991). The methylation of cytosine residues at the promoter region of a gene can eliminate gene expression. 5-AZ is an analogue of cytosine which can be incorporated into the DNA but cannot be methylated. Hence, if the *hprt* gene was inactive as a result of DNA methylation it would be expected that a proportion of the cells (following incubation in 5-AZ for the period of time it takes for the population to double) would revert back to the *hprt*⁺ phenotype. Cells which are *hprt*⁺ are capable of growing and proliferating in MEM/FCS/HAT selective medium. No colonies were observed on the MEM/FCS/HAT medium cultured plates. Jeggo and Holliday (1986) have shown that the concentration of AZ used can cause changes in the pattern of DNA methylation. At this concentration there was no reversion of the *hprt*⁻ phenotype to *hprt*⁺.

ii) Reversion Analysis

The *xrs-5* cells were grown up and plated in 150mm dishes, 3×10^6 per dish in FCS/MEM/HAT medium (6 replicate plates) and 500 cells diluted in 10ml of non selective

FCS/MEM medium were plated in 90mm dishes for a plating efficiency assay. This experiment was repeated twice with two independently thawed vials of cells.

Table 3.2.6.1 shows the spontaneous reversion frequency at the *hprt* locus. Base pair substitutions in the DNA can cause an altered phenotype and an inactive protein product if they are in a critical part of the DNA sequence. Base pair substitutions occur spontaneously in the DNA molecule as a result of errors in the DNA replication machinery. Reversions occur as a result of a back mutation, that is another spontaneous mutation which restores the original sequence. If the *hprt*⁻ phenotype was a result of a base substitution it would be expected that there would be a certain proportion of revertants. There were in the above described assay no *hprt*⁺ revertants observed giving a frequency of back mutation of less than 0.69×10^{-6} .

Cell line	Gene of interest	Mean Cloning efficiency	Spontaneous Reversion frequency.
<i>xrs-5</i>	<i>hprt</i>	23%	No mutants seen < 0.69×10^{-6}

Table 3.2.6.1: Table shows the mean cloning efficiency and the spontaneous reversion frequency of the *hprt*⁻ phenotype in the *xrs-5* cell line to the *hprt*⁺ phenotype. The results represent the mean of two independent experiments.

3.3 Summary

The optimum plating density of the CHO-K1 cells to select for *tk*⁻ mutations was 10^6 per 90mm tissue culture dish and the optimum expression time approximately 4 days as found by Singh (1991). As the radiation dose increases so does the probability of cell death but, the sensitivity of the different cell lines to the lethal effects of gamma radiation is not the same. It is suggested that the CHO-K1 cells are slightly more sensitive to the lethal effects of gamma radiation than the V79-4 cells at the dose range studied. The *xrs-5* cells were observed to be substantially more sensitive to the lethal effects of gamma radiation than the parental CHO-K1 cells. This supports the observations of Jeggo and Holliday, (1986), Costa and Bryant, (1988), Mussa *et al.*, (1990), Singh (1991) and Shadley *et al.*, (1991).

Gamma rays are equally effective at producing *hprt*⁻ mutations in V79-4 cells as they are *tk*⁻ mutations in CHO-K1 cells. However, gamma rays are relatively ineffective at inducing *hprt*⁻ mutations in the CHO-K1 cell line, producing approximately only 2 per 10^5 survivors

following a 6Gy gamma radiation dose as compared to approximately 12.5 per 10^5 survivors at 6Gy induced at the *tk* locus (estimated from Figure 3.2.4.5). The reason for this observation is unknown.

There is a relationship between the surviving fraction (cells plated immediately following irradiation) and the induced mutation frequency. As the surviving fraction decreases the induced mutation frequency (following the required expression time) increases. This observed relationship between mutation induction and surviving fraction suggests that the mechanism by which mutations are induced is the same as the mechanism by which radiation induces reproductive cell death. The induced mutation frequency at the *tk* locus in *xrs-5* cells is greater per unit dose of gamma rays than in the CHO-K1 cells. However, in the *xrs-5* cells the mutation frequency at the *tk* locus is less than in CHO-K1 cells at an equitoxic dose of radiation. This suggests that a greater proportion of the DNA damage is lethal in the *xrs-5* cells than in the CHO-K1 cells and supports the hypothesis that some mutations are the result of unrepaired double strand breaks. Shadley *et al.*, (1991) also found a similar relationship with the CHO-K1 and *xrs-5* cells at the *hprt* locus but interpreted the differences in the surviving fraction-mutation induction relationship to mean that the *xrs-5* cells were hypomutable. Zdzienicka *et al.*, 1988 made a similar interpretation concerning an X-ray sensitive V79 cell line. However, if one were to treat CHO-K1 and *xrs-5* cells with equitoxic doses for example, the dose which causes a reduction in the surviving fraction to 0.1, this means that we treat *xrs-5* cells with 1Gy and CHO-K1 with approximately 6.25Gy gamma radiation (estimated from Figure 3.2.4.2). The 6.5Gy radiation will cause substantially more damage to the genome than the 1Gy dose, hence such a comparison is not valid. In conclusion the *xrs-5* cells are deficient in the repair of dsb and the cytotoxic effects of radiation on these cell lines supports the hypothesis that the unrepaired dsb are lethal lesions. The mutation experiments support the observations of Mussa *et al.* (1990) and Singh (1991) that the *xrs-5* cells are hypermutable at the *tk* locus as compared to the parental CHO-K1 cell line and provides more evidence that the dsb is involved in the induction of gene mutations.

At the concentration used AZ did not revert the *hprt*⁻ phenotype of the *xrs-5* cell line to *hprt*⁺. In the spontaneous reversion experiments, no revertants were observed indicating that if back mutation occurred it did so with a frequency of less than 0.69×10^{-6} .

Chapter 4

The effects of restriction enzymes on cell viability and the mutation frequency in Chinese hamster cell lines

Restriction enzymes can be used to slice genes into pieces. Dozens are available, each cutting a specific group of letters. The length of the cut letters depends on how frequently that group is repeated. If each sentence in this volume was cut when the word "and" appeared, there would be thousands of short fragments. If the enzyme recognised the word "but" there would be fewer longer sections; and the enzyme cutting the word "banana" (which does appear occasionally) would just produce a few fragments thousands of letters long.

-Steven Jones, The Language of the genes

4.1 Introduction

Radiation produces a multitude of DNA lesions (see, Figure 1.5.1), each of which might reduce the probability of cell survival and increase the probability of the surviving cells producing progeny cells with a genetic mutation, that is if it was not for the highly efficient DNA repair mechanisms within the cell. With such a wide spectrum of DNA lesions produced by irradiation it is usually impossible to attribute any particular phenotypic change to any specific primary lesion. There is accumulating evidence to suggest that the double strand DNA break (dsb) is the primary lesion responsible for the effects of radiation, such as chromosome aberration formation (Bryant 1984; 1988; 1989; Liu and Bryant, 1993a; 1993b), mutation induction (Singh and Bryant, 1991; Singh, 1991; Kinashi *et al.*, 1992) and

oncogenic transformation (Bryant and Riches, 1990). Type II restriction enzymes (RE) mimic the cellular effects of radiation but, produce dsb with known characteristics (Bryant, 1984; 1988; 1989; Winegar *et al.*, 1988; 1989). Restriction enzymes can be introduced into the cell using streptolysin O (SLO) poration and it was shown Bryant (1992) that the optimum concentration of SLO needed for poration of Chinese hamster cells was 0.045 units/ml. In the experiments reported in this section, the effect of varying the SLO concentration on the induced mutation frequency was studied. SLO poration is a new technique and has not in the past been used for the introduction of RE into cells in order to study the induction of genetic mutations. Therefore, prior to any RE experiments the effect of SLO alone on the cell viability and the mutation frequency in CHO-K1, V79-4 and *xrs-5* cells lines was studied. These results were compared to those obtained with the previously used technique, electroporation. The plating efficiency data was analysed statistically and the influence of different poration buffers on the plating efficiency of cells plated immediately following treatment, and the mutation frequency was also studied. The effects of the restriction enzymes *Pvu II* and *EcoRI* (introduced into the cell by SLO) on the surviving fraction of the different cell lines and the mutation frequency at the tk locus in the CHO-K1 and *xrs-5* cells, and at the *hprt* locus in the V79-4 cells were studied and compared.

4.2 Method and Results

4.2.1 Optimum concentration of SLO for cell poration

Dehydrated streptolysin O (Welcome diagnostics, UK) was made up according to the manufacturers specifications to produce a stock solution with a concentration of 1.81 units/ml. *Pvu II* was purified from the storage buffer in which it was supplied and the enzyme activity assayed as described in Section 2.5. V79-4 cells were suspended in HBSS at a concentration 10^6 cells/ml (as described in Section 2.5) and 10 sterile V-tubes each containing of 1ml of HBSS cell suspension were prepared. The 5 control tubes were treated with SLO alone, to produce final concentrations of 0, 0.01, 0.02, 0.04 and 0.06 units/ml. The remaining samples were treated at the above SLO concentrations and also with 10 units of the purified restriction enzyme *Pvu II*. The treatment procedure was as described in Section 2.5. Following treatment, the cells were incubated in 75cm² flasks in MEM/FCS for the required expression time prior to plating in a mutation assay (as described in Section 2.6).

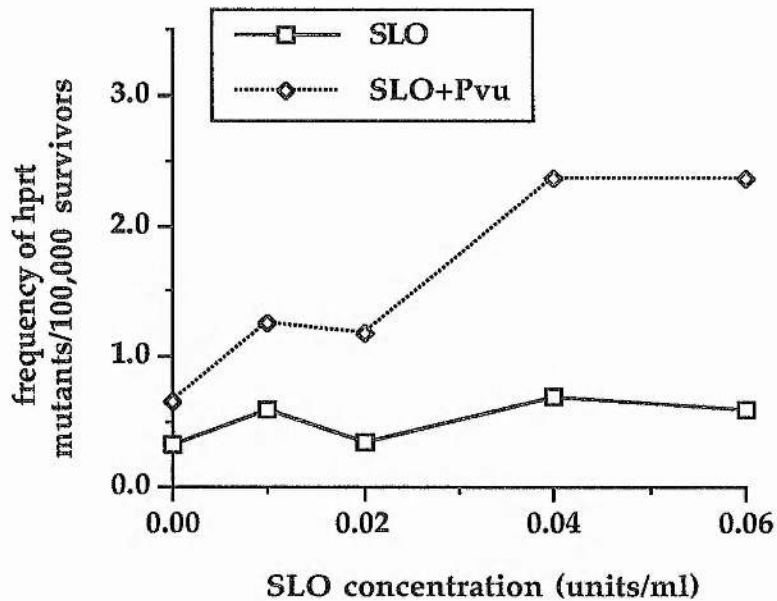


Figure 4.2.1.1: The effect of various concentrations of SLO with or without restriction endonuclease, on the frequency of *hprt* mutations in V79-4 fibroblast cells when used as a method of cell poration. The results represent the mean of 2 independent experiments.

Figure 4.2.1.1 shows the effect of various concentrations of SLO on the frequency of *hprt* mutations in Chinese hamster V79-4 cells. The graph suggests that the various concentrations of SLO on their own, all produce the same response in terms of mutation induction in V79-4 at the *hprt* locus. However, when the SLO was used at 0.04 units/ml to introduce *Pvu II* into the cell, there was a clearly observable increase in the mean *hprt* mutation frequency. There was no further increase in the mean mutation frequency when the concentration was increased to 0.06 units/ml. This supports the data obtained by Bryant (1992) that the optimum SLO concentration for the poration of Chinese hamster cells in culture is 0.045 units/ml. This concentration of SLO was used in all subsequent cell poration experiments.

4.2.2 The effect of SLO on the surviving fraction and the mutation frequency at the *tk* and the *hprt* loci in Chinese hamster cells

2×10^6 cells from an exponentially growing cell population were suspended in MEM/FCS medium and centrifuged in a Chilsplin centrifuge at 2,000rpm for 10 minutes (RCF = 742g). The pellet was resuspended in HBSS at a concentration of 10^6 cells per ml. 1ml of the cell

suspension was treated with 24 μ l of the SLO stock solution and incubated for 5 minutes. Another 1ml of sample was reserved as the untreated control. Following incubation, 5ml of MEM/FCS medium was added to the test sample and the control tubes to dilute the SLO. The samples were centrifuged, the pellet of cells resuspended in 5ml of MEM/FCS medium and the cell number counted. 100-200, CHO-K1 or V79-4 cells were plated in 4ml of MEM/FCS medium in 60mm tissue culture dishes (3 replicate dishes) and 500 *xrs-5* cells were plated in 10ml of MEM/FCS in 90mm tissue culture plates (3 replicate plates for each sample). The remaining cells were transferred to 75cm² tissue culture flasks and incubated for the required expression time. The CHO-K1 cells and the V79-4 cells were plated as described in Section 2.6 and the *xrs-5* cells as described in Section 3.2.4.

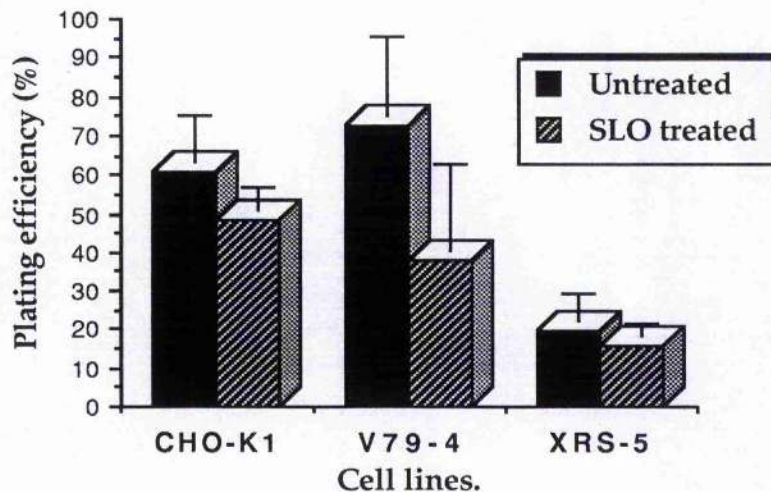


Figure 4.2.2.1: The effects of SLO on the plating efficiency of the different Chinese hamster cell lines following SLO treatment. The data represents the mean of three independent experiments and the vertical lines show the standard error of the mean.

Figure 4.2.2.1 shows the effect of SLO treatment on the plating efficiency of the different cell lines, which was reduced in the SLO treated cells. Table 4.2.2.1 shows the effects of SLO on the mutation frequency in the different cells lines in the gene of interest following the required expression time. In CHO-K1 and V79-4 the SLO treated cells had higher mean mutation frequencies than the untreated controls. In *xrs-5* cells the mean mutation frequency at the *tk* locus was approximately the same in the control and the poration samples.

Cell lines	Mutation frequency per 10 ⁵ survivors.	
	Control	SLO treated
V79-4 (<i>hprt</i>)	0.52 (0.144)	1.44 (0.936)
CHO-K1 (<i>tk</i>)	4.14 (3.18)	9.49 (2)
<i>xrs-5</i> (<i>tk</i>)	7.99 (4.5)	7.751 (5.195)

Table 4.2.2.1: Shows the effect of SLO on the mutation frequency at the *tk* locus in CHO-K1 and *xrs-5* cells and at the *hprt* locus in V79-4 cells. In all cases the results represent the mean and standard error (in brackets) of three independent experiments.

4.2.3 The effect of electroporation on the surviving fraction and the mutation frequency at the *hprt* locus in Chinese hamster V79-4 cells

V79-4 cells from an exponentially growing cell population were pelleted by centrifugation and then resuspended at a concentration of 10⁶/ml in HBSS. A cell porator (BRL) was set up with the following parameters; field strength: 750v/cm, capacitance: 1600µf, resistance: low and temperature: ambient. The cells (10⁶) were electroporated while suspended in 1ml of HBSS in a disposable electroporation chamber. Prior to cell poration these sterile chambers were rinsed with HBSS/BSA at least twice. Proteins will attach to the surface of the electroporation chamber, the surfaces of the chamber were therefore saturated with BSA proteins prior to cell poration. Following cell poration the cells were resuspended in MEM/FCS medium and counted. 500 cells were diluted in 10ml of MEM/FCS medium and plated on 90mm tissue culture plates (3 replicate plates) and incubated for 8 days prior to fixing and staining. The remaining cells were then placed in 75cm² tissue culture flasks and incubated for an expression time of 5 days prior to plating as described in Section 2.6. For each experiment there was also an untreated control in which the same procedure was followed for the control except for the cell poration.

The effect of cell poration with SLO on the plating efficiency of cells was compared with the untreated controls using a paired t-test. The data obtained was analysed as described by Wardlaw (1985). The results suggested that the treatment of CHO-K1, *xrs-5* and V79-4 cells

with SLO did not significantly reduce the cell viability. The statistics are shown in Table 4.2.3.2.

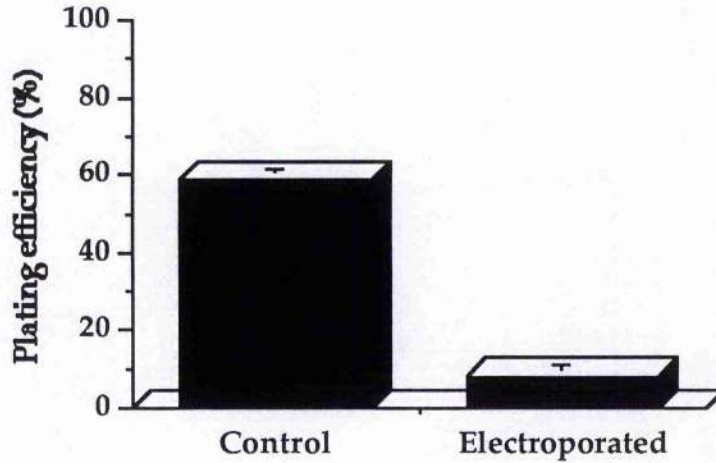


Figure 4.2.3.1: The effects of electroporation on the plating efficiency of V79-4 cells, cells having been plated immediately following treatment. The results represent the mean of three independent experiments and the vertical bars represent the standard error of the mean.

<i>hprt</i> mutation frequency per 10 ⁵ survivors		
Cell line	Control	Electroporated
V79-4	1.8 (1.9097)	7.0 (2.8)

Table 4.2.3.1: Shows the effects of the electroporation treatment on the mutation frequency at the *hprt* locus in V79-4 cells. The results represent the mean and the standard error (in brackets) of three independent experiments. The standard error values are shown in brackets.

Electroporation treatment reduced the plating efficiency substantially more than the SLO treatment. The t-test suggested that the differences in the plating efficiency of the electroporation treated V79-4 cells and the untreated control were significantly different at the 5% level. The statistical values are shown in Table 4.2.3.2.

Cell line	Poration treatment.	t-value	Significance
V79-4	SLO	2.04 (df=2.)	Not significant
V79-4	Electroporation	3.7 (df=2)	Significant at the 5% level.
CHO-K1	SLO	2.35 (df=2)	Not significant
<i>xrs-5</i>	SLO	0.71 (df=2)	Not Significant.

Table 4.2.3.2: The results of the paired t-tests comparing the effects of SLO and electroporation on the plating efficiency of cells plated immediately after treatment as compared with the untreated controls.

In the experiments described in this Chapter, the results suggested that cell poration with SLO leads to an increase in the background mutation frequency and although a decrease in the cell viability was observed in all cell lines, this decrease was not shown to be significant. The increase in background mutation frequency and the decrease in the cell viability have also been observed following electroporation (Singh, 1991). Cells are porated in order to allow the access into the cell of high molecular weight protein molecules. These pores will allow protein to diffuse from the surrounding poration buffer into the cell. However, these membrane pores also allow chemicals to diffuse out of the cell into the surrounding medium. These chemicals may include nucleotides and nucleotide precursor molecules. The fidelity of DNA replication is strongly dependent upon the correct balance of nucleotides and nucleotide precursor molecules (Kunz, 1982). This balance is carefully maintained within the cell by a series of complex and interconnected biochemical pathways. Evidence suggests that thymidine deprivation is mutagenic in prokaryotes and in organelle genomes of eukaryotes (Kunz, 1982 and references therein). There is however, no evidence to suggest that thymine deprivation causes mutations in the genomic DNA of eukaryotic cells. However, evidence does suggest that thymine deprivation results in an elevated frequency of chromosomal and chromatid aberrations in Chinese hamster cells (Lozzio, 1968). A possible way to reduce the cellular and the molecular changes produced as a result of cell poration due to nucleotide pool imbalances would be to use a poration buffer with the same nucleotide and nucleotide precursor concentrations as is usually present in the cell. This unfortunately would be impractical. There are a large number of chemical substrates involved in DNA synthesis and nucleotide metabolism and the concentrations of nucleotides and their precursors within the cell is cell cycle dependent.

4.2.4 The effects of various poration buffers on the plating efficiency of cells plated immediately following SLO treatment and the mutation frequency at the *tk* locus in CHO-K1 cells and the *hprt* locus in V79-4 cells.

HBSS was prepared as described in Chapter 2. The potassium (K) buffer was made up by dissolving 4.47g, KCl; 0.0083g, CaCl₂; 0.87g, K₂HPO₄; 0.68g, KH₂PO₄; 12.5ml, Hepes medium; 0.375g of EGTA and 0.38g of MgCl₂ in 500ml of sterile distilled water and sterilising by Millipore filtration. The K+ATP buffer was made up by dissolving ATP (molecular grade, Sigma) in the K buffer to produce a final concentration of 2mM. The solution was sterilised by Millipore filtration.

The cellular effects of SLO when porating cells while suspended in various buffers was studied in CHO-K1 and V79-4 cells. Cells were plated immediately following treatment in a clonal assay in order to measure the plating efficiency. The remaining cells were further cultured in 75cm² tissue culture flasks for the appropriate expression time, prior to a *tk* mutation assay in CHO-K1 cells and a *hprt* mutation assay in V79-4 cells. The cell poration was performed as described in Section 2.5, substituting HBSS for the appropriate poration buffer. For each treatment there was also a non-porated control, where the cells were treated in the same way except SLO was not added. The mutation assay was performed as described in Section 2.6.

The effects on the plating efficiency of porating cells while suspended in different buffers are shown in Figures 4.2.4.1 (a) for V79-4 cells and in 4.2.4.2 (a) for CHO-K1 cells. In V79-4 cells the mean plating efficiency was reduced in the SLO treated cells as compared with the untreated controls. This was true for cells treated in all three poration buffers. The data shows that there is little difference in the plating efficiency of V79-4 cells porated with SLO in the different poration buffers. In CHO-K1 cells treated while suspended in the K+(ATP) buffer there appeared to be an elevated mean plating efficiency (Figure 4.2.4.1a).

The V79-4 cells treated while suspended in HBSS had a lower *hprt* mutation frequency as compared to the cells suspended in other buffers. However, the mean *hprt* mutation frequency was increased in all cell buffers following cell poration with SLO. Treatment of CHO-K1 cells in the different buffers had little obvious effect on the mean mutation frequency at the *tk* locus.

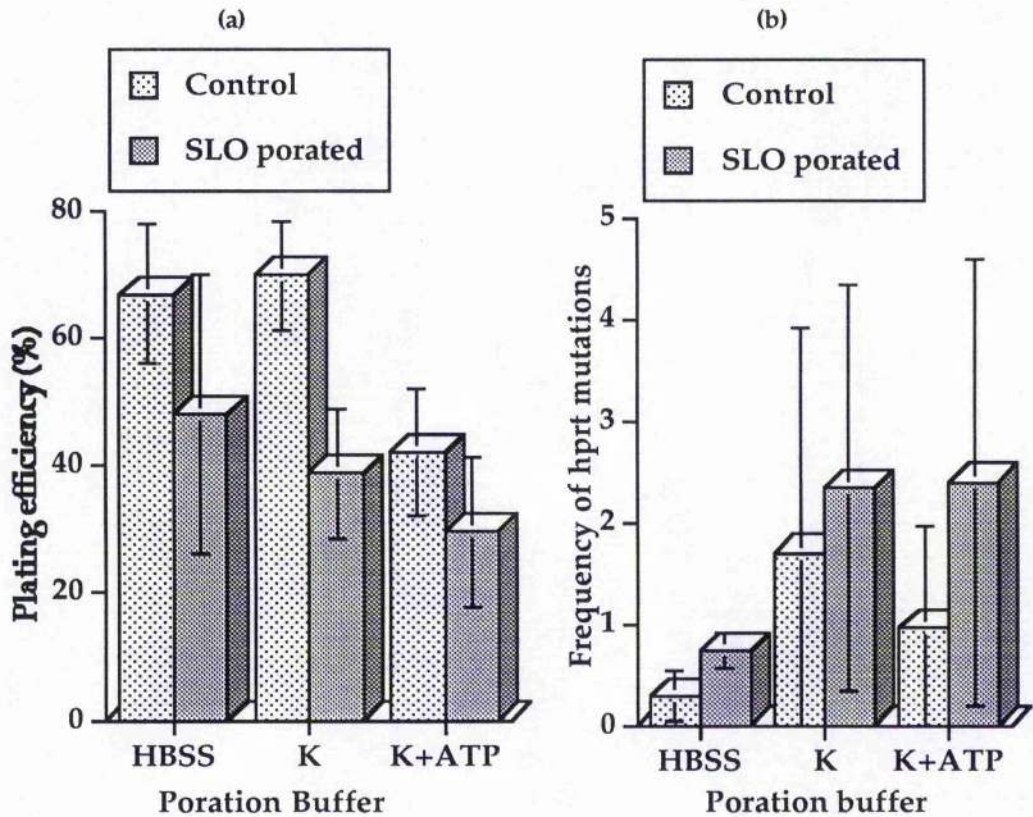


Figure 4.2.4.1: In (a), the effects of various poration buffers on the plating efficiencies of V79-4 cells are shown. In (b), the effects of the various different poration buffers on the mutation frequency per 10^5 survivors at the *hprt* locus in V79-4 cells are shown. The bar chart illustrates the mean plating and mean mutation frequencies observed with and without SLO treatment. The results illustrated in both of the above figures represent the mean of three independent experiments and the vertical lines represent the standard errors of the mean.

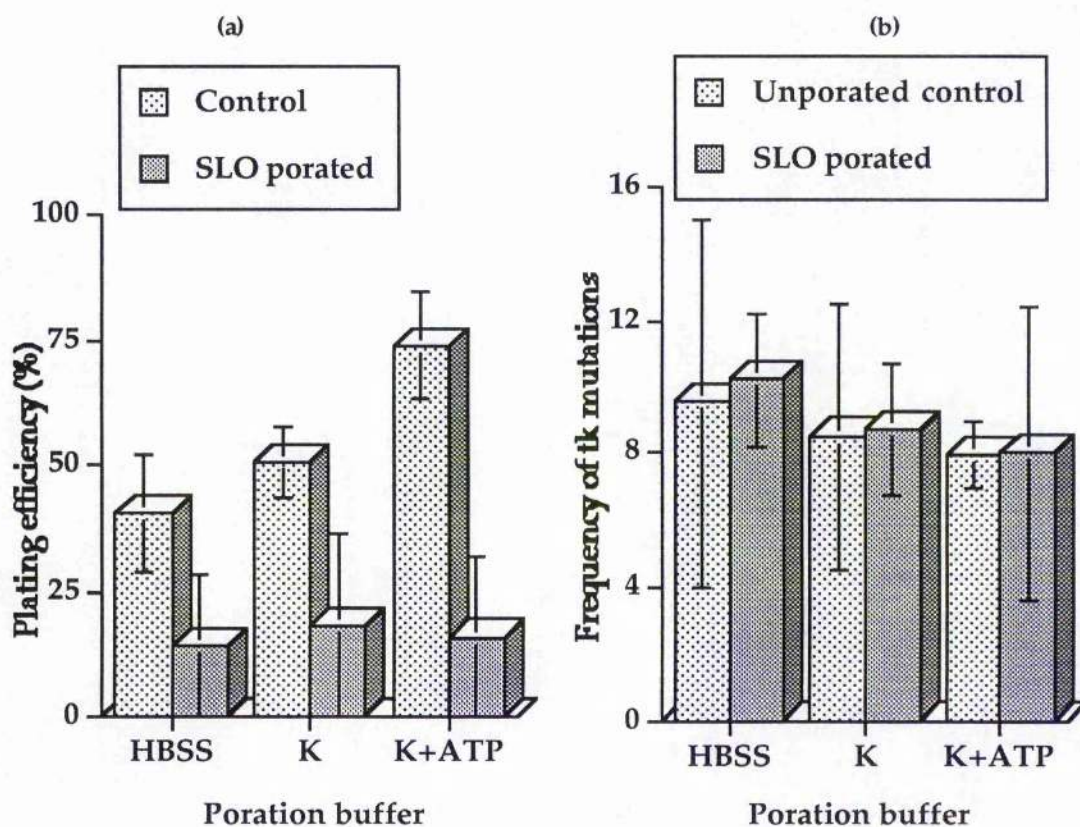


Figure 4.2.4.2: In (a), the effect of different poration buffers on the plating efficiency of CHO-K1 cells plated immediately following SLO treatment and of the untreated controls are shown. In (b), the effects of different poration buffers on the mutation frequency per 10^5 survivors at the *tk* locus in CHO-K1 cells following a 4 day expression time are shown. The bar chart represents the data obtained with and without SLO treatment. The results in both the illustrated Figures represent the mean of three independent experiments and the vertical lines represent the standard errors of the mean.

In general the poration of CHO-K1 and V79-4 cells in the presence of the intracellular K buffer and the K + (ATP) buffer had little effect on reducing the background mutation frequency at the *tk* locus in CHO-K1 cells and at the *hprt* locus in V79-4 cells as compared with the standard HBSS buffer. Indeed treatment of cells in HBSS buffer appeared to be less mutagenic than treatment in the intracellular potassium based buffers. Van den Hoff *et al.*, (1992) observed that the K intracellular buffer improved the survival of rat hepatoma cells following electroporation. In the experiments described in this Chapter, there was little difference in the plating efficiency of the cells treated with SLO while suspended in the different poration buffers. However, the recovery and the plating efficiency of cells following

SLO treatment is intrinsically much higher than in the electroporation treatment (Bryant, 1992).

4.2.5 The effects of *Pvu II* and *EcoRI* on the surviving fraction and mutation frequency of Chinese hamster cell lines

The ability of RE to produce varying and predictable types of dsb has been exploited in the past in order to study the effects of different types of dsb on the formation of chromosome aberrations, chromatid aberrations, cell death and the induction of gene mutations. It has been found (Bryant, 1984; 1988; Moses *et al.*, 1990; Singh and Bryant, 1991; Singh, 1991) that blunt ended dsb are more effective at inducing the mentioned above cellular responses than cohesive termini.

Pvu II and *EcoRI* were purified from the contaminating storage buffer by Amicon filtration, as described in Section 2.5. The RE were assayed for activity (Section 2.5) and diluted in HBSS/BSA to produce a concentration of 1 unit/ μ l. Suspending the RE in HBSS/BSA helps maintain enzyme activity. V79-4, CHO-K1 and *xrs-5* cells were grown exponentially, counted and treated as described in Section 2.5. CHO-K1 cells were treated with *Pvu II* or *EcoRI* to produce final concentrations of 0, 3, 6, and 9 units/ml. The V79-4 cells were treated with *Pvu II* and *EcoRI* of concentrations 0, 5, 10 and 15 units/ml. In all cases SLO was the chosen method of cell poration. Immediately following treatment a clonogenic assay was performed. 100-200, CHO-K1 or V79-4 cells were plated in 60mm tissue culture dishes in 4ml of MEM/FCS (3 replicate plates). 500 *xrs-5* cells were plated on 90mm tissue culture dishes in 10ml of MEM/FCS. The remaining cells were returned to the tissue culture flasks and grown exponentially for the required expression time prior to a mutation assay being performed. The CHO-K1 and the V79-4 assay is described in Section 2.6 and the *xrs-5* mutation assay is described in Section 3.2.4.

Figures 4.2.5.1 show a photograph of an agarose gel. Shown are the digestion products of 1 μ g pBR322 plasmid when treated with different concentrations of *Pvu II*. The first seven lanes are of plasmid digestion with different concentrations of purified enzyme and the following seven when the plasmid is treated with the non-purified *Pvu II*. The enzyme is shown not to have lost its activity following purification.

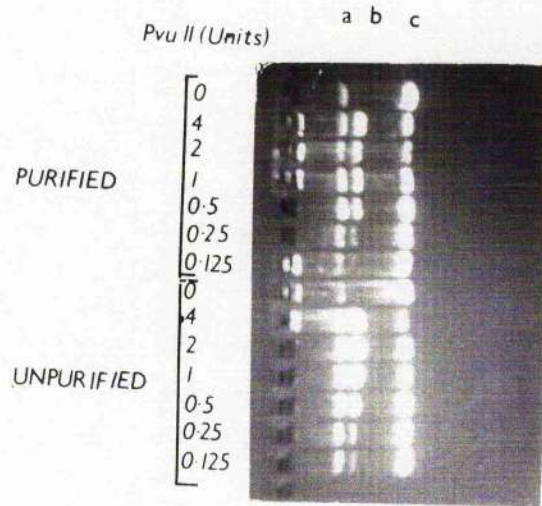


Figure 4.2.5.1: A photograph of an agarose electrophoresis gel illustrating the assay of the activity of the restriction enzyme *Pvu II*. The photograph shows the digestion products of the plasmid pBR322 when treated with varying concentrations of *Pvu II*. The top seven lanes are the digestion products of the purified enzyme and the lower seven lanes represent the digestion products of the non-purified enzyme. The symbols a, b, and c show the positions of the plasmid along the gel when it is in the open circle (a), linear (b) and closed circle (c) forms.

Figure 4.2.5.2 shows the effect of different concentrations of *Pvu II* and *EcoRI* on the surviving fraction of V79-4 cells, the cells having been plated immediately following treatment. The effect of various concentrations of *Pvu II* on the surviving fraction in *xrs-5* cells and parental CHO-K1 cells are shown in Figure 4.2.5.3. The trends in the graph suggest that *xrs-5* are more sensitive to the lethal effects of *Pvu II* than CHO-K1. However, the standard errors of the means of the three experiments do overlap, hence more experiments must be performed before any conclusions can be made.

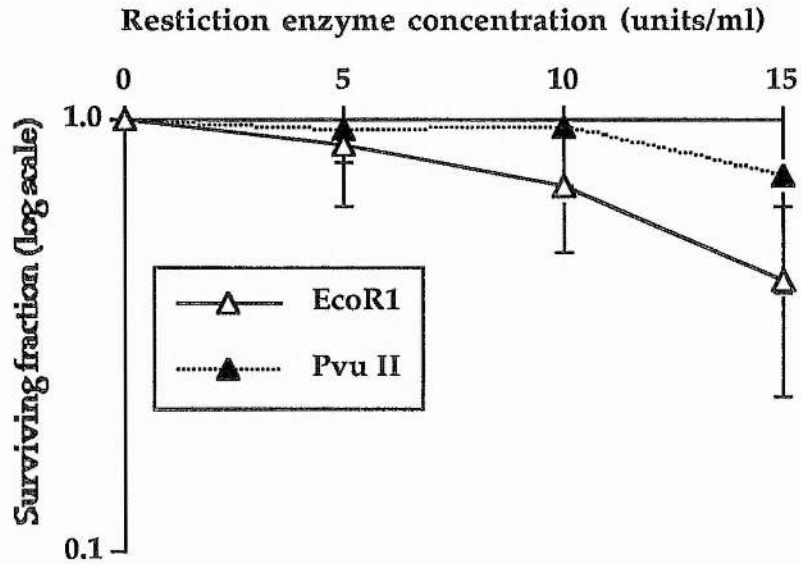


Figure 4.2.5.2: The effect of various concentrations *EcoRI* and *Pvu II* on the surviving fraction (log scale) of the V79-4 cells. The results represent the mean of three independent experiments and the vertical lines represent the standard errors of the mean.

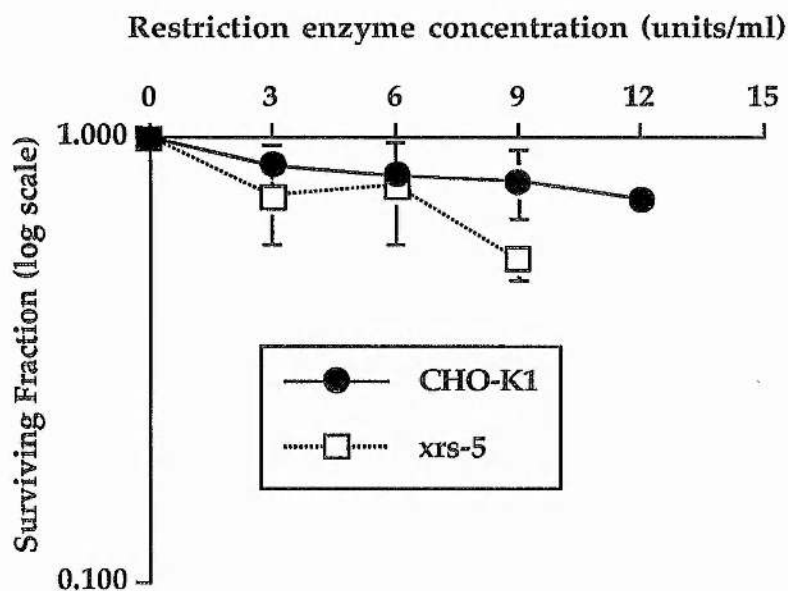


Figure 4.2.5.3: The effect of various concentrations of *Pvu II* on the surviving fraction of CHO-K1 and *xrs-5* cells. The data of both graphs represent the mean of three independent experiments and the vertical lines represent the standard errors of the mean.

The surviving fraction of the CHO-K1 cells immediately following treatment with *Pvu II* (generating blunt ends) and *EcoRI* (generating cohesive termini) are shown in Figure 4.2.5.4.

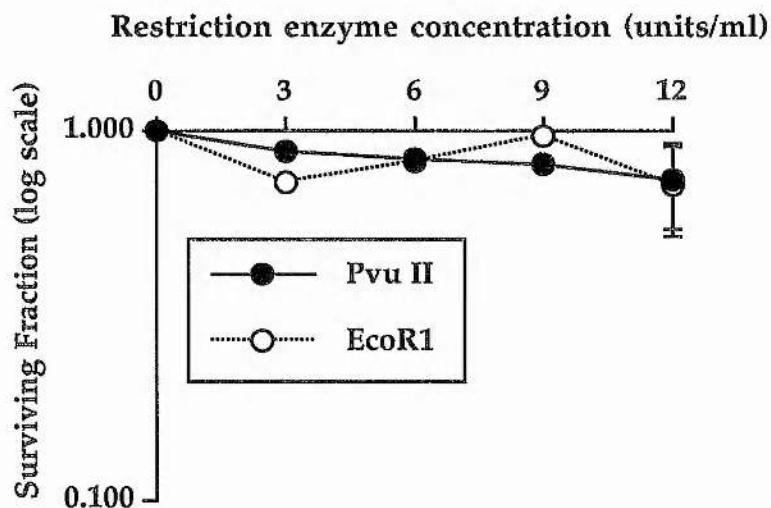


Figure 4.2.5.4: The effect of various concentrations of *Pvu II* and *EcoRI* on the surviving fraction of CHO-K1 cells. The data represents the mean of three independent experiments and the vertical lines represent the standard error of the mean.

Treatment of Chinese hamster CHO-K1, V79-4 and *xrs-5* cells with purified RE produced a reduction in the cell viability. Obe *et al.* (1986) found that treatment of V79-4 cells with *Alu I* (blunt-ended dsb) caused a reduction in the viability of cells but no obvious dose response relationship was observed. Giaccia *et al.* (1990) however, found a close correlation between the concentration of *Alu I* and the reduction in viability of CHO-16B33 cells and in the X-ray sensitive XR-1 cell line. Experiments outlined in this Chapter illustrate that treatment of CHO-K1 cells and *xrs-5* cells with *Pvu II* produced a decrease in the surviving fraction which appeared to be linearly related to the RE concentration. Kinashi *et al.*, (1992) also found a similar response with CHO-K1 cells electroporated with *Pvu II*. The dsb repair deficient *xrs-5* cell line appeared to be more sensitive to the lethal effects *Pvu II* than the CHO-K1 cells. The overlapping standard error bars however, do suggest that more experiments, perhaps at higher RE doses, must be performed before any conclusions can be drawn. *EcoRI* produced a greater reduction in the surviving fraction in the V79-4 cells than the *Pvu II*. This is surprising since it is widely believed that cohesive-ended dsb are more effectively repaired than blunt ended dsb. For CHO-K1 there appeared to be little correlation between the *EcoRI* concentration and the reduction in the cell surviving fraction. Giaccia *et al.* (1990) found that *Sau3a* (producing cohesive termini) was less effective at reducing the surviving fraction than *Alu I*. This was also observed by Kinashi *et al.*, (1992). These RE cut at the same nucleotide sequence. However, analytical pulse field gel electrophoresis analysis of the DNA suggested that the *Sau 3A* produced less dsb *in vivo* than *Alu I* per unit concentration (Giaccia *et al.*, 1990).

Figures 4.2.5.5, 4.2.5.6 and 4.2.5.7 show the effects of various concentrations of RE on the mutation frequency at the *tk* and the *hprt* loci in the different Chinese hamster cell lines. Figure 4.2.5.5 shows the effect of varying concentrations of *Pvu II* and *EcoRI* on the mutation frequency at the *hprt* locus in the V79-4 cell line. The graph indicates that in V79-4 cells, at the *hprt* locus, and at the doses studied, the two different enzymes produce a similar mutational response. Figure 4.2.5.6 shows the effect of various different concentrations of *Pvu II* and *EcoRI* upon the induction of mutations at the *tk* locus in CHO-K1 cells. The data suggests that in the CHO-K1 cell line, *Pvu II* is substantially more effective at producing mutations at the *tk* locus than *EcoRI*. Figure 4.2.5.7 compares the effects of various different concentrations of *Pvu II* on the mutation frequency at the *tk* locus in the CHO-K1 and the *xrs-5* cell lines. The frequency of induced mutations is higher per unit of *Pvu II* in CHO-K1 cells than in the *xrs-5* cells. Figure 4.2.5.8 (a) shows the relationship between the surviving fraction (linear scale) and the induced mutation frequency at the *tk* locus in the *xrs-5* and the CHO-K1 cell lines. The relationship between surviving fraction and the induced mutation frequency at the *hprt* locus in V79-4 cells as a result of *Pvu II* and *EcoRI* treatment is shown in Figure 4.2.5.8 (b).

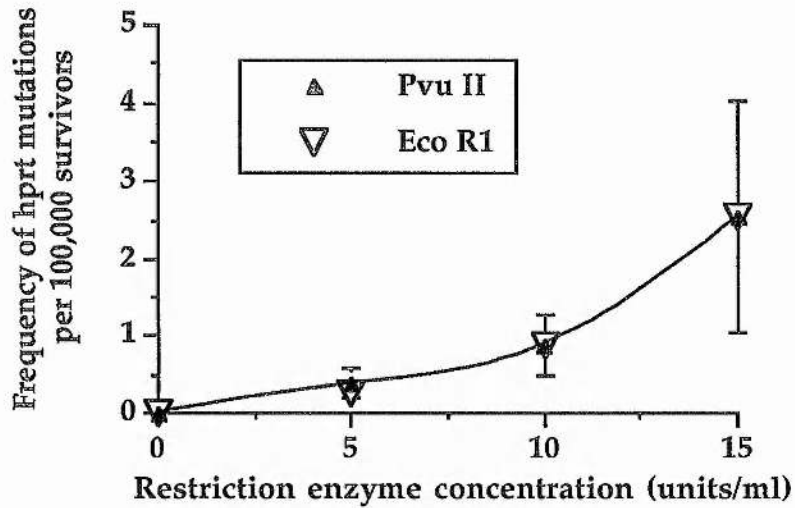
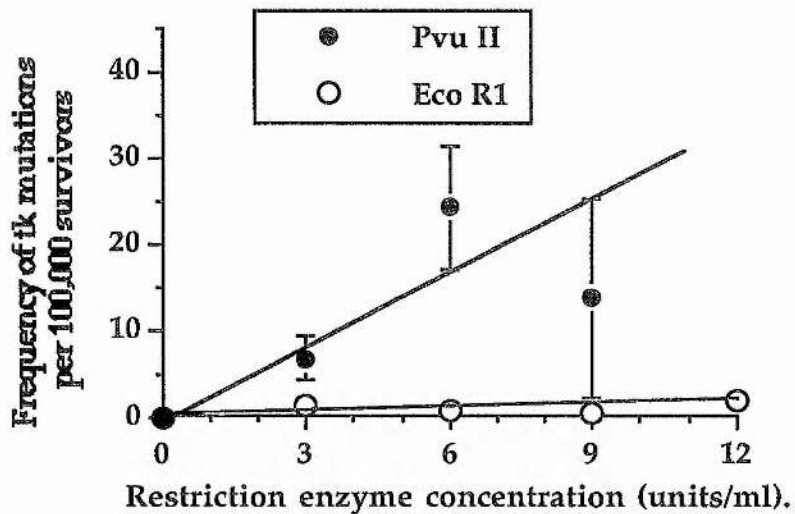


Figure 4.2.5.5: The effect of various concentrations of *Pvu II* and *EcoR1* on the induced mutation frequency at the *hprt* locus in the V79-4 cell line. Each point on the graph represents the mean of three independent experiments and the vertical lines represent the standard error of the mean.



4.2.5.6: The effect of different concentrations of the restriction enzyme *Pvu II* and *EcoR1* on the mean induced mutation frequency at the *tk* locus in the CHO-K1 cell line. Each point represents the mean of three independent experiments and the vertical lines represent the standard error of the mean. Where error bars are not seen the standard error is too small to be observed.

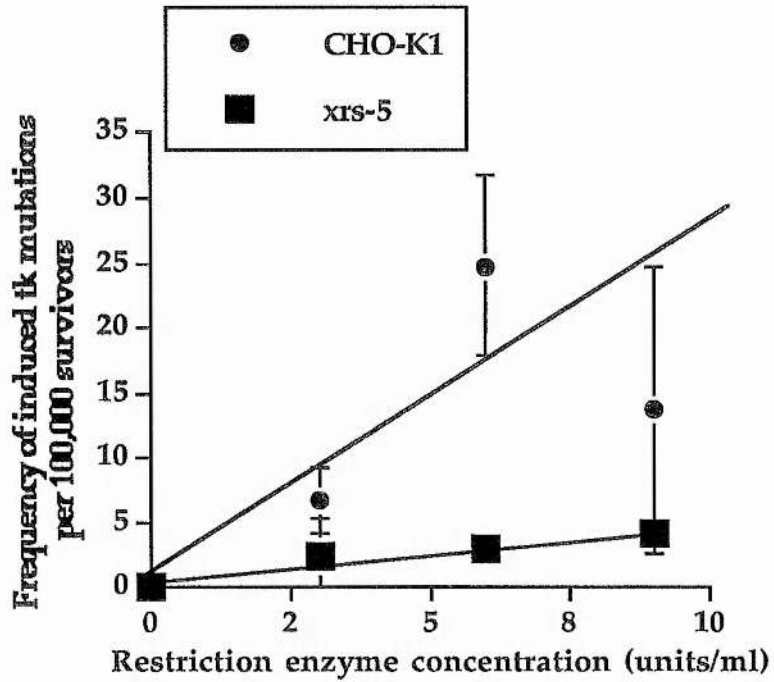


Figure 4.2.5.7: The effect of various concentrations of *Pvu II* on the mean induced mutation frequency at the *tk* locus in the *xrs-5* cell line and the parental CHO-K1 cell line. The results represent the mean of three independent experiments. The vertical lines represent the standard error of the mean. Where standard error bars can not be seen, the standard error is too small to be observed.

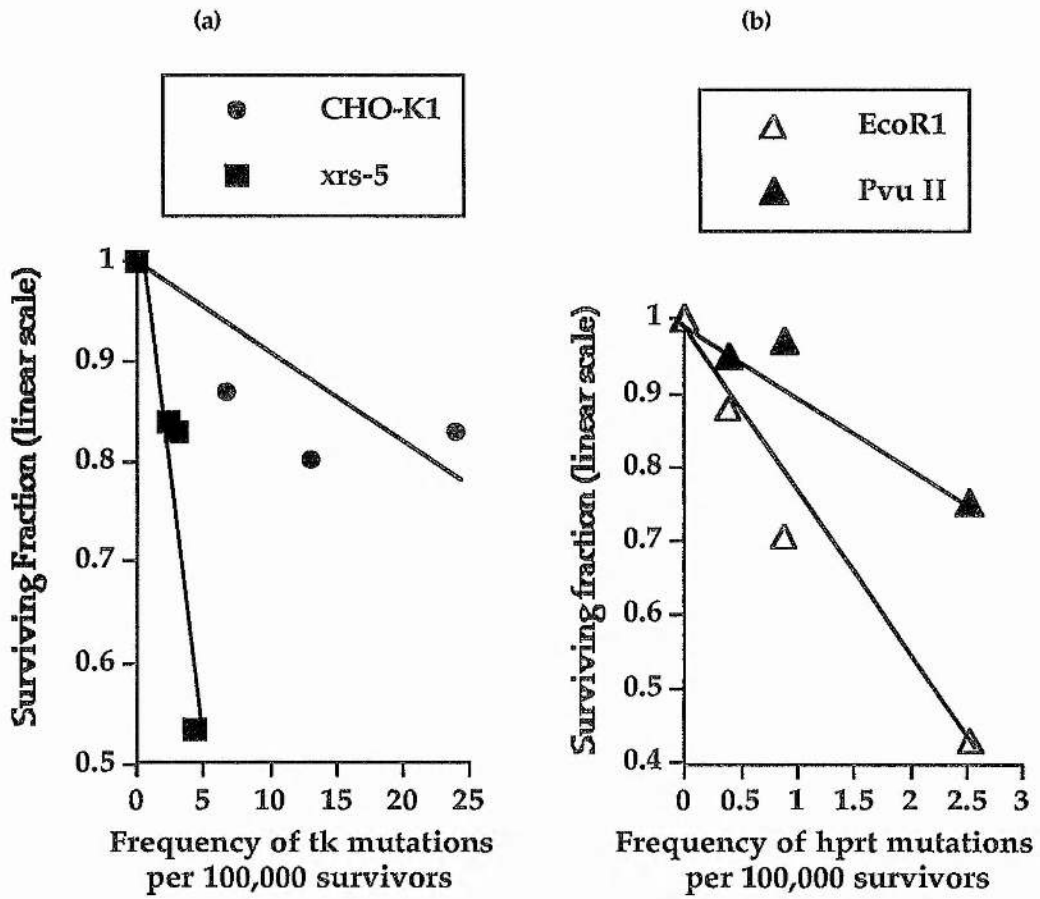


Figure 4.2.5.8 (a): The relationship between the mean surviving fraction and the mean induced mutation frequency at the *tk* locus in *Pvu II* treated CHO-K1 cells and *xrs-5* cells. (b) Shows the relationship between the mean surviving fraction and the mean induced mutation frequency in *Pvu II* and *EcoR1* treated V79-4 cells. Note that in both Figures the surviving fraction is drawn on a linear scale. (This is because on a log scale the difference between the relationships would not be observed so clearly).

At the *tk* locus in CHO-K1 cells *Pvu II* was shown to be more effective at inducing mutations than *EcoR1*, which produced little increase in the mutation frequency above the background (Figure 4.2.5.6). Similar results were also reported by Singh (1991) and Singh and Bryant (1991) who used electroporation as the method of introducing RE into the cell.

Figure 4.2.5.2 suggests that *EcoR1* causes more cell death per unit dose in V79-4 cells than *Pvu II*. *Pvu II* and *EcoR1* produced similar frequencies of induced mutations at the *hprt* locus per

unit dose (Figure 4.2.5.5) but, at equitoxic doses *Pvu II* appeared to be more effective (Figure 4.2.5.8(b)).

Bryant (1984) and Costa *et al.*, (1993) suggested that there are many different factors which effect the response of cells to RE treatment. These include the frequency and the locations at which the RE cleaves the DNA. Nuclear organisation may also play a role exposing specific parts of the genome, making them susceptible to the endonucleolytic activities of the RE while other regions of the genome remain protected. The locus of interest may also be important and the selective constraints upon that particular locus may also effect the final observations. For example, *Alu I* was shown to be an effective inducer of mutations at the *tk* locus in CHO-K1 cells (Singh, 1991), at the *hprt* locus in V79-4 cells (Obe *et al.*, 1986) and at the *gtp* locus in the Chinese hamster cell line PL61/1 (Costa *et al.*, 1993). However, *Alu I* was shown to be ineffective in the induction of mutations at the Na^+/K^+ *ATPase* locus in V79-4 cells (Obe *et al.*, 1986). This provides evidence that the locus of interest is also a determining factor when one considers the effects of RE. The *ATPase* locus does not tolerate deletion mutations. It is possible that aberrant DNA repair sometimes leads to the formation of deletion mutations.

Evidence suggests that isoschizomers sometimes produce different survival and mutation induction response (Kinashi *et al.*, 1992; Costa *et al.*, 1993). These response appeared to be dependent upon the longevity of the enzyme (Costa *et al.*, 1993). Enzymes whose activity is lost after approximately one hour do not cause a large amount of cell death but, are more efficient inducers of mutations than enzymes with high longevity which produce a greater decrease in the cell viability.

Perhaps the most surprising result of all was that the induced *tk* mutation frequency per unit *Pvu II* concentration was less in the *xrs-5* cell line than in the parental CHO-K1 cell line. This is especially surprising since *xrs-5* cells were shown to be hypermutable at the *tk* locus when treated with X-rays. The data concerning the surviving fraction of *xrs-5* per unit dose however suggests that *xrs-5* cells are more sensitive to the lethal effects of *Pvu II* than the parental CHO-K1 cell line. Costa and Bryant (1990) showed using non denaturing filter elution, that *Pvu II* was effective at inducing dsb in CHO-K1 cells. The observation that the *xrs-5* cell line is not hypermutable at the *tk* locus following treatment with *Pvu II* may be related to the type of dsb produced. Radiation produces a mixture of 'clean' and 'dirty' dsb, the majority of the breaks however are 'dirty'. *Pvu II* treatment produces only clean breaks. It is possible that the *xrs-5* is deficient in the 'enzymatic cleaning' of the dirty dsb. Recent evidence (Taccioli *et al.*, 1994) suggests that the *xrs-6* cell line, another X-ray sensitive daughter cell line of the CHO-K1, which is in the same complementation group as *xrs-5*, is deficient in a protein which is homologous to the ku protein in humans. This protein is a

heterodimer which is made up of the *ku70* and *ku80* gene products. These then join together to produce a DNA dependent protein kinase, which binds to the free ends of double stranded DNA and is believed also to play a part in genetic recombination. Mateos *et al.*, (1994) reported that the *xrs-5* cells were not deficient in DNA repair when treated with gamma radiation at the G₂ phase of the cell cycle, however during G₁ the neutral filter elution data suggested that breaks were not as efficiently repaired. It is possible that there is more than one mechanism of DNA repair, one which is responsible for DNA repair in G₁ and another which is active in G₂. Recombination in the G₁ stage of the cell cycle would involve homologous chromosomes where as recombination at the G₂ stage of the cell cycle may involve sister chromatids on the same chromosome. Therefore, for homologous recombination to occur in G₁ the homologous segments of the chromosomes must be in close proximity. The nuclear organisation of the *xrs-5* cells is believed to be different to that of the parental CHO-K1 cells (Yasui *et al.*, 1991). In *xrs-5* cells the topographical distribution of the homologous regions of the chromosomes within the nucleus may make recombination between homologous segments of DNA unlikely.

4.3.3 Summary

SLO poration treatment caused a slight reduction in the mean plating efficiency however, this decrease was not shown to be significant. SLO also caused an increase in the mean mutation frequency at the *tk* locus in CHO-K1 cells and *xrs-5* cells and at the *hprt* locus in V79-4 cells. In mutation assays it is advisable to have the minimum background mutation frequency. This becomes especially important when mutant cells are isolated for molecular analysis as in Chapter 5. Lowering the background mutation frequency increases the probability of the isolated mutation having been the result of the mutagen treatment rather than the result of spontaneous changes in the DNA or induced by the poration treatment. Cells were porated while suspended in different buffers in an attempt to find a buffer which reduced the mutagenic effect of SLO poration. The buffers used did little to reduce the mean background mutation frequency, indeed in some cases it actually increased it. Hence, during the RE poration treatment the standard HBSS buffer was used to suspend the cells. *EcoRI* was more effective at inducing cell death than *Pvu II* in V79-4 cells. This was surprising as evidence suggests (Bryant, 1984; 1988; 1989) that it is the blunt-ended dsb which are responsible for many of the cellular effects of radiation. In CHO-K1 cells a decrease in the surviving fraction of cells was seen with increasing *Pvu II* concentration, but in cells treated with *EcoRI*, there appeared to be little correlation between the RE dose and the surviving fraction. The mean surviving fraction per unit *Pvu II* was slightly less for *xrs-5* cells than for CHO-K1 cells, but the difference was only slight. More experiments need to be performed before any firm conclusions can be drawn. *Pvu II* and *EcoRI* were equally effective at inducing mutations at the *hprt* locus in V79-4 cells. However, at the *tk* locus in CHO-K1 cells, *EcoRI*

produced little effect on the mutation frequency above background. *Pvu II* in contrast was shown to be highly mutagenic at the *tk* locus in CHO-K1 cells. This observation cannot be attributed to the frequency of enzyme cutting, as *EcoRI* has been shown to have more cutting sites within the Chinese hamster *tk* gene than *Pvu II* (Lewis, 1986). When treated with *Pvu II* *xrs-5* cells were found to hypomutable at the *tk* locus as compared to CHO-K1 cells, when treated with equal amounts of *Pvu II*. This is a surprising result as *xrs-5* have been shown to be deficient in the repair of dsb. This observation has yet to be explained.

Chapter 5

Molecular analysis of *hprt*- mutants using the Polymerase Chain Reaction

An explorer entering new territory does not start by making a plan of the first village and extending it in excruciating detail until the whole country is covered. Instead he picks out the major landmarks and leaves the detailed map until later, when he knows what is likely to be interesting.

- Steven Jones, The Language of the Genes.

5.1.1 Introduction

Dsb are believed to be the major cause of chromosome aberrations and mutational deletions. Restriction enzymes which induce blunt-ended DNA dsb have been shown to increase the frequency of chromosome and chromatid aberrations (Bryant, 1988; 1989; Bryant and Christie, 1989; Moses *et al.*, 1990). Therefore blunt-ended dsb may be expected to be the major cause of large mutational DNA deletions. Over 70% of mutations induced by radiation treatment are the result of gross DNA changes, such as gene deletions and rearrangements which can be identified by Southern blot and PCR analysis. Evidence shows that treatment of cells with certain restriction enzymes also increases the mutation frequency (Obe *et al.*, 1986; Singh, 1991; Singh and Bryant, 1991; Kinashi *et al.*, 1992; Costa *et al.*, 1993; Tatsumi-Miyajima *et al.*, 1993; Ashraf *et al.*, 1993). However, the nature of these mutations has not, as yet been studied in great detail. It has been observed that radiation induced DNA deletion mutations are not randomly distributed throughout the mammalian genome (Thacker *et al.*, 1990; Aghamohammadi *et al.*, 1992). If it is the dsb which is responsible for the loss of large

segments of the DNA molecule, it might be expected that a large proportion of the mutations generated by restriction endonucleases in porated cells will be deletions. However, it has also been observed (see Chapter 4) that the SLO cell poration treatment itself induces mutations at both the *tk* locus and the *hprt* locus in Chinese hamster cells. It is therefore also necessary to ascertain the types of mutations caused as a result of the poration treatment in addition to those induced by radiation and restriction enzyme treatment.

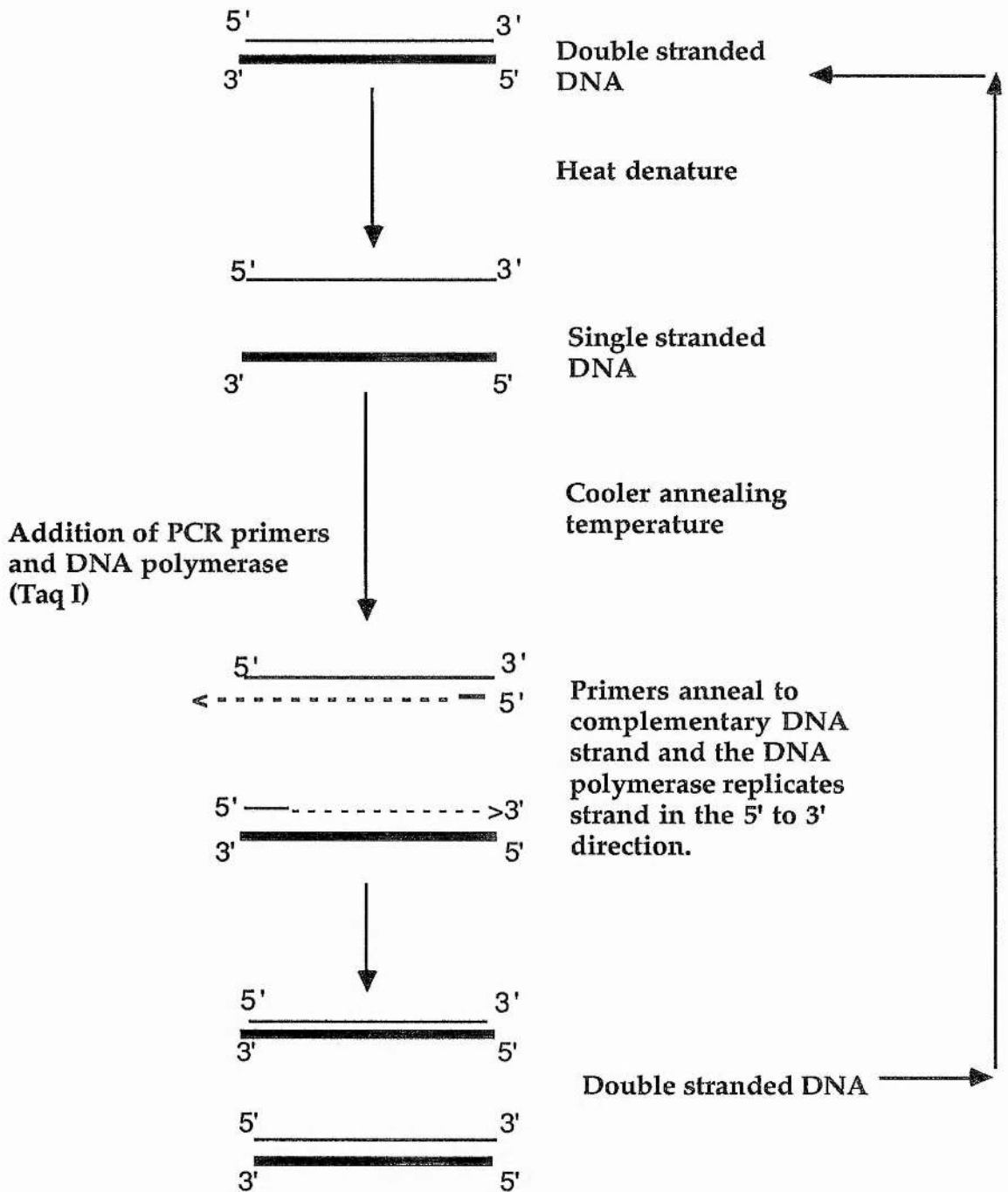
In this Chapter experiments are described in which V79-4 cells which were deficient in *hprt* enzyme activity were isolated. Some of the mutations were spontaneously induced, others were the result of treatment with gamma radiation, SLO, electroporation and *Pvu II* using either SLO or electroporation as the method of cell poration. The *Pvu II* induced *hprt* mutations (which used electroporation as the method of cell poration) were isolated by Singh (1991). Genomic DNA from these mutant cells was isolated and PCR was used to screen the *hprt* gene for deletions within the exon DNA sequences. The absence of a PCR product was used as an indication of the presence of a molecular deletion.

5.1.2 Molecular analysis of DNA by PCR deletion screening

a) The Principle of the Polymerase Chain Reaction

In 1983, Mullis struck upon an idea which involved the manipulation of the DNA replication machinery. Although the idea itself was conceptually simple it has come to revolutionise molecular biology. From a single DNA strand the "Polymerase Chain Reaction (PCR) can generate hundreds of thousands of similar molecules in the space of an afternoon" (Mullis, 1990). PCR is an *in vitro* DNA amplification technique allowing the selected sequences of interest to be amplified in large quantities in a relatively short period of time.

The principle of PCR is very simple and is described in Figure 5.1.2.1. The double stranded DNA molecule is heated to 94°C. At this point the hydrogen bonds holding the double helix together are broken and the DNA becomes single stranded. The mixture is rapidly cooled to the temperature which is optimum for the formation of hydrogen bonds between the single stranded primer DNA molecules and the DNA of interest. Following primer annealing the sample temperature is slightly raised, this is to allow the polymerase to copy the DNA molecule*. Once copied the new DNA is heat denatured and the PCR cycle begins again. Often up to 40 cycles are performed. This provides enough DNA for molecular analysis.



* The first replicated strand in the PCR cycle will be of unlimited length, but in the following cycles the primers will limit the lengths of the PCR products.

Figure 5.1.2.1: The principle of the PCR reaction (diagram redrawn from Read, 1990). The diagram illustrates how from a single DNA molecule thousands of copies of the DNA sequence of interest can be produced.

The early PCR reactions used the "Klenow" polymerase which is a heat sensitive enzyme, consequently new polymerase had to be added following each heat denaturation step. Since the isolation and the purification of a heat stable polymerase from the thermophilic bacteria *Thermus aquaticus* this expensive and monotonous step has become unnecessary. The primers are oligonucleotides of between 18 and 30 bases long and are homologous to the 3' flanking end of the target sequence on each of the homologous DNA strands. A minimum oligonucleotide length is necessary to ensure specificity of the DNA binding. It is also important that there is as little complementary overlap between the oligonucleotide primers as possible. This reduces the probability of primer-dimer artefact production. This is when one primer is extended over the other by the polymerase. The cycle numbers, the temperatures and the times of incubation are dependent upon the characteristics of the primers (eg) the length of the nucleotide sequence of the DNA being amplified. The denaturation and the annealing times are usually around 30 seconds and the extension time is approximately 1 minute per kilobase of target DNA. Usually there is an extended incubation time of 2 minutes per Kb at the end of the reaction to allow any partially amplified sequences to be completed. (McPherson, 1993). The reaction buffer provides the optimum conditions for the polymerase. It contains non ionic detergent which is necessary to obtain maximum processivity of the enzyme, that is the maximum length of sequence that a single DNA polymerase will copy at any one time. Also added is a solution containing magnesium ions. Magnesium is the cofactor required for the DNA polymerase to be active.

The polymerase chain reaction has many applications from archaeological to prenatal diagnosis. It allows simple and rapid DNA analysis, enabling the scanning of the DNA for molecular deletions to be performed with relative ease. It is however, limiting in the sense that it can only scan for large genetic deletion mutations in genes which are present in a single copy in the genome interest. Small changes of a few base pairs in length will go unnoticed. Providing the sequences flanking the exons of a particular gene are known the protein-coding exon DNA can be selectively amplified in large quantities. The PCR products are run on an agarose electrophoresis gel and the coding regions of the gene screened for deletions by absent or altered sizes of the PCR products.

b) The use of the Polymerase Chain Reaction to amplify the hprt exons

PCR primers, as designed by Rossiter *et al.* (1991) were synthesised by the department of Biochemistry, University of St. Andrews (primer DNA sequences given below). These were used for the PCR reactions described in this Chapter. Figure 5.1.2.2 shows a map of the Chinese hamster *hprt* gene showing the position of the exon sequences along the gene.

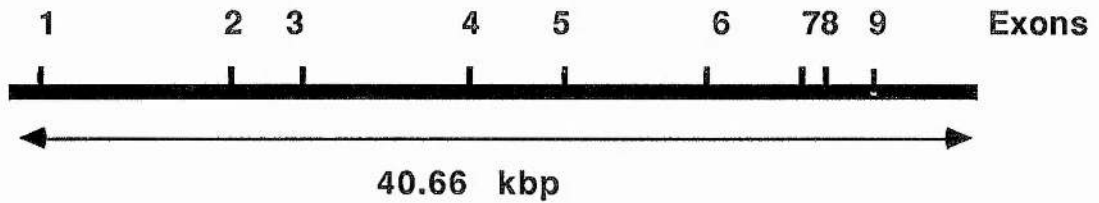


Figure 5.1.2.2: A map of the Chinese hamster *hprt* gene, showing the positions of the exons (the vertical bars) in relation to the intronic sequences. The distances between the exons are drawn approximately to scale (redrawn from Rossiter *et al.*, 1991)

Figure 5.1.2.3 shows the DNA sequences of the PCR primers used in the PCR amplification of the Chinese hamster *hprt* exon DNA. Preliminary experiments showed that the amplification of exon 1 was unreliable, therefore exon 1 was not routinely screened in the series of experiments to be described in this Chapter. The sizes of the PCR amplification products and the exons which they amplified are shown in Table 5.1.2.1.

- Primer 2a: 5' AGC TTA TGC TCT GAT TTG AAA TCA GCT G 3'
 Primer 2b: 5' ATT AAG ATC TTA CTT ACC TGT CCA TAA TC 3'
 Primer 3a: 5' CCG TGA TTT TAT TTT TGT AGG ACT GAA AG 3'
 Primer 3b: 5' AAT GAA TTA TAC TTA CAC AGT AGC TCT TC 3'
 Primer 4a: 5' GTG TAT TCA AGA ATA TGC ATG TAA ATG ATG 3'
 Primer 4b: 5' CAA GTG AGT GAT TGA AAG CAC AGT TAC 3'
 Primer 5a: 5' AAC ATA TGG GTC AAA TAT TCT TTC TAA TAG 3'
 Primer 5b: 5' GGC TTA CCT ATA GTA TAC ACT AAG CTG 3'
 Primer 6a: 5' TTA CCA CTT ACC ATT AAA TAC CTC TTT TC 3'
 Primer 6b: 5' CTA CTT TAA AAT GGC ATA CAT ACC TTG C 3'
 Primer 7a: 5' GTA ATA TTT TGT AAT TAA CAG CTT GCT GG 3'
 Primer 7b: 5' TCA GTC TGG TCA AAT GAC GAG GTG C 3'
 Primer 9a: 5' CAA TTC TCT AAT GTT GCT CTT ACC TCT C 3
 Primer 9b: 5' CAT GCA GAG TTC TAT AAG AGA CAG TCC 3'

Figure 5.1.2.3: The base sequences of the oligonucleotide primers used in the PCR amplification of the *hprt* exons in the Chinese hamster genome (sequences taken from Rossiter *et al.*, 1991).

Primers pairs	exon(s) amplified	Size of PCR product
2a & 2b	exon 2	166 bp
3a & 3b	exon 3	220 bp
4a & 4b	exon 4	191 bp
5a & 5b	exon 5	247 bp
6a & 6b	exon 6	145 bp
7a & 7b	exons 7 & 8	423 bp
9a & 9b	exon 9	734 bp.

Table 5.1.2.1 The sizes of the PCR products of the Chinese hamster *hprt* gene. Notice that exons 7 and 8 were amplified as one PCR product, that is exon 7 and 8 and the intervening intron were amplified together (data was taken from Rossiter *et al.*, 1991).

5.1.3 Chapter Aims

This Chapter explains the induction and isolation of V79-4 *hprt*⁻ mutations and the molecular analysis of these mutants by PCR deletion screening. These isolated mutants were either spontaneously generated, produced by 4Gy gamma irradiation, SLO poration, electroporation or by *Pvu II* treatment using SLO as the method of cell poration. Also analysed were a series of *hprt*⁻ mutants isolated by Singh (1991). These were induced by *Pvu II* using electroporation as the method of cell poration. The advantage of PCR is that it is rapid and does not use radioactive isotopes unlike more conventional techniques such as Southern blotting.

5.2 Methods and Results

5.2.1 Amplifying the *hprt* gene by PCR

The PCR reaction was carried out on DNA isolated from V79-4 *hprt*⁻ mutant cells. The method of mutation induction and isolation are described in the appropriate sections. The PCR reaction mix contained the following constituents:

- 5µl PCR reaction buffer (10 x concentrate, BRL)
- 0.5µl of 10mM stock of dATP (BRL)
- 0.5µl of 10mM stock of dGTP (BRL)
- 0.5µl of 10mM stock of dCTP (BRL).
- 0.5µl of 10mM stock of dTTP (BRL)
- 200ng of DNA sample

400nm of each of the required primers

0.1µl of 5units/µl stock of taq polymerase (BRL)

Sterile distilled water was added to produce a final volume of 50µl, then 50µl of mineral oil (Sigma) was layered over the surface. This was to prevent evaporation of the water at the high denaturation and amplification temperatures. Exons 3, 5 and 9 were amplified together in the same reaction. The reaction conditions are shown below:

(94°C, 4.5 minutes) 1 cycle

(60°C, 50 seconds; 72°C, 2 minutes; 94°C, 30 seconds) 29 cycles.

(60°C, 60 seconds; 72°C, 7 minutes; 4°C, 30 minutes) 1 cycle.

Exons 2, 4, 6, and 7&8 were amplified together in the same reaction. The reaction condition were as follows:

(94°C, 1 minute; 58°C, 1 minute; 70°C, 7 minute) 30 cycles

(94°C, 5 minutes; 70°C, 13 minutes) 1 cycle

The PCR reactions were carried out in a Techne Thermal Cycler (PCH-3) and the PCR products stored at 4°C until analysis. The PCR products were analysed by gel electrophoresis. The agarose gel was made by dissolving 3g of ultra pure agarose (BRL) in 100ml of 1 x tris-borate buffer (TBE) by microwave heating. The gel was allowed to set in the electrophoresis tray with the appropriate sized combs. TBE was made up as a 10 x concentrated solution and diluted just before use. The composition of the TBE buffer is as follows: 216g tris-base (BDH), 110g boric acid (BDH) and 18.6g EDTA (BDH) dissolved in two litres of sterile distilled water. 2µl of the bromophenol solution (50mM EDTA, 0.2% SDS, 50% glycerol, 0.05% bromophenol tracking dye) was added to 18µl of the PCR products and then loaded onto the gel. The gel was run at 40 volts for 6-10 hours in the TBE electrophoresis buffer. The gel was viewed under ultra violet light. Where absent PCR products were recorded, the PCR reaction was repeated at least two more times. This was to reduce the chances of getting false negative results.

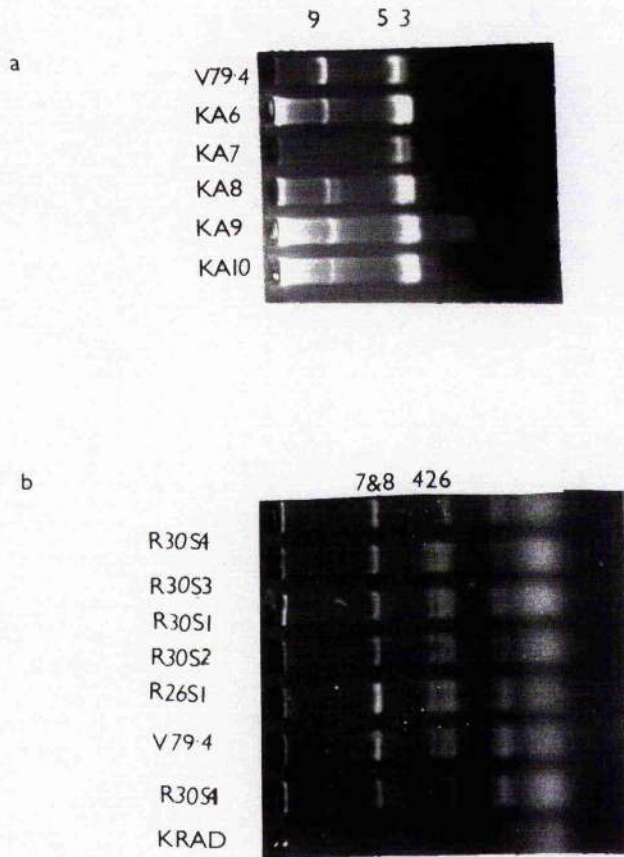


Figure 5.2.1.1: (a) Shows the results of agarose gel electrophoresis of the PCR amplification products of exons 3, 5 and 9 in selected *hprt*⁻ mutants and in (b) an agarose electrophoresis gel of the products of PCR amplification of exons 2, 4, 6 and 7&8 in selected *hprt*⁻ mutants.

5.2.2 Spontaneously induced *hprt*⁻ mutations

Passage cells of V79-4 which were maintained in non selective MEM/FCS were trypsinized and counted. Approximately 200 cells were placed in each 25cm² flask (20 flasks) and incubated (passaging when required) for approximately 10 days. The cells were transferred to 75cm² tissue culture flasks and cultured for approximately three weeks (passaging when required). The cells were then trypsinized, counted and plated at a concentration of 10⁵ per 90mm tissue culture plate in 6-TG selective medium.

A stainless steel pipe of internal diameter approximately 0.5cm was cut into segments of approximately 1cm thick to form many small rings. The upper surface of the rings were coated with silicon grease and the lubricated rings sterilised by autoclaving. Tweezers were sterilised by soaking in absolute alcohol for a minimum of 15 minutes. The tissue culture plates containing the mutant colonies were washed with sterile phosphate buffer pH 6.4 (Gurr) to remove any unattached cells. Using the sterile tweezers the steel 'trypsinization' rings were inverted and placed over the mutant colony, i.e. the silicon grease face down, sealing the ring to the plate. The mutants isolated were selected at random, as it is also believed that large colonies, would be more striking than the smaller colonies and that colonies of different sizes may be produced by cells containing different types of genetic mutations (Yandell *et al.*, 1986; Moore *et al.*, 1985a; 1985b). Only one mutant per independent treatment was isolated. This was to ensure that the mutations isolated had arisen independently. Each of the selected colonies were rinsed twice with 100µl of trypsin/EDTA solution and incubated at 37°C for 6 minutes in a humidified incubator. 100µl of MEM/FCS medium was transferred and pipetted up and down several times. The cell suspension was added to a 75cm² flask with 15ml of MEM/FCS/6-TG selective medium. The culturing in MEM/FCS/6TG medium was to prevent spontaneous reversion of the mutant cells while in culture. The DNA was isolated from the mutant cells and the DNA concentration and purity measured (as described in Chapter 2).

The PCR reactions were carried out as described in Section 5.2.1 and the PCR amplification products run on a 3% agarose electrophoresis gel. The PCR products of the *hprt*⁻ mutant DNA was compared with the products of the *hprt*⁺ V79-4 DNA and any differences recorded. The mutant cells and the isolated DNA were labelled with a laboratory code. The nomenclature of this code may to some seem somewhat bizarre however, from the code data, the person who isolated the mutant, the way in which the mutation was induced and the poration technique can be identified. In some, the experiment number is also indicated. The prefix K indicates that the mutants were isolated by myself (Kim) and the prefix B indicates that they were originally isolated by Dr Baldev Singh. SP tells that the mutations were spontaneously

induced and R (without the prefix K) that mutant cells were induced by 4Gy of gamma radiation. In the KS cells the mutations were induced by the SLO treatment and in the KE cells by electroporation. All *hprt*⁻ mutant cells isolated by Singh (1991) were *Pvu II* induced using electroporation as the method of cell poration. KA represents a code for *Pvu II* induced mutations where SLO was the method of cell poration.

Mutant code	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Exon 7&8	Exon 9
KSP 1	+	+	+	+	+	+	+
KSP 2	+	+	+	+	+	+	+
KSP 3	+	+	+	+	+	+	+
KSP 4	+	+	+	+	+	+	+
KSP 5	+	+	+	+	+	+	+
KSP 6	+	+	+	+	+	+	+
KSP 7	+	+	+	+	+	+	+
KSP 8	+	+	+	+	+	+	+

Table 5.2.2.1: The results of PCR deletion screening of *hprt*⁻ spontaneously induced V79-4 mutants. In all of the isolated mutants there was a full complement of exon PCR amplification products present.

The results of PCR deletion screening of the spontaneously induced *hprt*⁻ V79-4 mutants (KSP1-8) are shown in Table 5.2.2.1. None of the eight spontaneously generated *hprt*⁻ mutants isolated and analysed had absent PCR products. It was therefore assumed that there was no large scale DNA loss within the *hprt* coding regions of the mutant cells. This is consistent with the results obtained by other workers. Mutations occurring spontaneously at the *hprt* locus in the V79-4 cell line have been shown to be predominantly small scale changes that are not usually detected by Southern analysis or PCR (Xu *et al.*, 1989; Thacker and Ganesh, 1989; Aghamohammadi *et al.*, 1992). Similar results were also obtained with human cell lines at the *hprt* locus (Monnat, 1989; Papadopoulo *et al.*, 1990; Hou *et al.*, 1993; Morris *et al.*, 1993) and in *in vivo* derived *hprt*⁻ lymphocytes (Nicklas *et al.*, 1987). These results support the idea that a large proportion of the spontaneous mutations are the result of base substitutions or small deletions. However, the possibility that the isolated mutants analysed as part of this research, have large scale deletions outside the genetic region studied, for example upstream of exon 2 or in the intronic regions cannot be ignored.

5.2.3 The isolation of independently derived radiation induced *hprt*⁻ V79-4 mutants.

The V79-4 cells were diluted to a low numbers (1000 cells per 75cm² treatment flask) and grown for several days until confluent. This dilution procedure was designed to reduce the possibility of spontaneous mutations in the cell population (Morgan *et al.*, 1990). The cells were then trypsinized and diluted to 0.5×10^6 per flask and incubated for 24 hours prior to treatment with 4 Gy gamma radiation. Following irradiation the cells were incubated for 5 days and then plated at a concentration of 10^5 per 90mm tissue culture plate in selective MEM/FCS/6-TG medium and placed in a humidified incubator at 37°C for 10-14 days. The mutant colonies were removed from the plate and cultured as described in Section 5.2.2. The DNA was extracted from the mutant cells as described in Chapter 2. The results of the PCR deletion screening experiments of the DNA of radiation induced *hprt*⁻ mutants are shown in Table 5.2.3.1.

The results shown in Table 5.2.3.1 show that at approximately one third of the gamma radiation induced *hprt*⁻ V79-4 mutants analysed were the result of DNA deletions. This is suggested by the absence of PCR amplification products in 5 out of the 16 isolated radiation induced mutants. Point mutations which disrupt the annealing of the PCR primers to the homologous sequence on the genomic DNA could also result in the absence of PCR products. Such events however, are likely to be exceptionally rare (Aghamohammadi *et al.*, 1990) and were considered to be unlikely. Hence, absence of PCR products was used to indicate a DNA deletion event. The KRAD mutant cell line had no PCR amplification products suggesting that the whole or the majority of the *hprt* gene had been deleted. The *hprt* mutant R30S4 had absent amplification products for exons 2, 3 and 4 and R11A had absent amplification products of exons 2, 3, 4, 6 and 7&8. Mutants R10C had lost exons 5 and 9 and mutant R26S1 had lost exon 3. Thacker (1986) suggested that 70% of the mutations induced by radiation were the result of DNA deletion events. Approximately one third of the mutants analysed in this series of experiments are likely to be as a result of DNA deletion events. It is however, possible that deletions occurred in the intronic regions of some of these mutants, but not in the exonic regions hence, the deletion mutants still produced a full complement of PCR amplification products. Loss of intronic DNA sequences may effect splice sites or other mRNA processing events. The DNA deletion frequency may be related to the type of radiation and the dose used to induce mutations. Evidence suggests that different radiation doses may produce different proportions of different types of mutations (Whaley and Little, 1990). For example high LET radiation may be more effective at inducing deletions than low LET radiation and high doses may be more effective than low doses. However, Morgan *et al.*, (1990) found that 69% of the *hprt* mutations induced by 4Gy of X-rays, and analysed by

Southern blots were whole gene deletions and a further 24% had altered banding patterns. Environmental conditions such as humidity and air quality, beyond the control of the experimenter will vary from laboratory to laboratory. These variations may be responsible for some of the variations observed between different laboratories.

Mutant code	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Exon 7&8	Exon 9
KRAD*	-	-	-	-	-	-	-
R10A	+	+	+	+	+	+	+
R10C*	+	+	+	-	+	+	-
R11A*	-	-	-	+	-	-	+
R11B	+	+	+	+	+	+	+
R11C	+	+	+	+	+	+	+
R11D	+	+	+	+	+	+	+
R26S1*	+	-	+	+	+	+	+
R26S2	+	+	+	+	+	+	+
R13S2	+	+	+	+	+	+	+
R13S3	+	+	+	+	+	+	+
R13S4	+	+	+	+	+	+	+
R30S1	+	+	+	+	+	+	+
R30S2	+	+	+	+	+	+	+
R30S3	+	+	+	+	+	+	+
R30S4*	-	-	-	+	+	+	+

Table 5.2.3.1: The results of PCR deletion screening experiments on Radiation induced V79-4 *hprt* - mutants. The * indicate mutant cells with deletions.

Isolation of mutant colonies from tissue culture plates is not 100% efficient. Only about 60% of the isolated colonies grew and proliferated when transferred to flasks. The failure rate may vary between laboratories depending upon the makeup of the trypsin solution and the plastics manufacturer. It may be dependent upon the technique used to remove the mutant colony from the plates, trypsinization is perhaps more efficient than scraping the mutant colony off the plate with sterile tweezers. It is possible that the growth failure rate is linked to the presence of large genetic changes (Moore *et al.*, 1985a; 1985b). This would explain some of the differences in the proportions of mutant cells with DNA deletions observed. The sample size of the radiation induced mutants with deletions is too small for any pattern concerning deletion distribution along the gene to be observed. However, Xu *et al.*, (1989) and Thacker *et al.*, (1990) in Chinese hamster cells and Hou *et al.*, (1993) in human cells observed

that deletions were more likely to occur in the 3' end of the gene than at the 5' end. There have been several theories proposed to explain these observations. It has been suggested (Morris *et al.*, 1993) that there could be an essential gene upstream of the 5' end of the *hprt* gene. Large deletions extending along this gene would result in the cell becoming inviable hence, the mutation will be lost from the population. The non random selection of deletion mutations, that is only selection of deletion mutations which result in phenotypic change could result in the observed unequal distribution of deletion mutations along the *hprt* gene. Some deletions in certain parts of the *hprt* gene which do not result in phenotypic change, will not be identified, only deletions which reduce the efficiency or remove the *hprt* gene would be selected by plating in 6TG selective medium. There may therefore be a population of DNA deletions which go undetected. It is also possible that deletion mutations are generated as a result of non random events. DNA is precisely packaged within the nucleus of the cell and the nature of the packaging is dependent upon the position of the cell within the cell cycle. Some DNA may be packaged tightly within the matrix of other DNA and protein molecules, thus protected from the damage caused by radiation. Other DNA may be more exposed, making it more susceptible to both radiation and accidental enzymatic damage. The DNA packaged around the histone molecule may be more protected from the damage of free radicals than the interlinking strands, this is because histones have been shown to be free radical scavengers (Ljungman, 1990). The arrangement of the DNA into looped domains could mean that loss of some DNA sequences are more likely to be associated with the loss of other adjacent sequences (Sankaranarayanan, 1991). Recent evidence also suggests that some parts of the gene may be preferentially repaired over other parts (Gao *et al.*, 1994). Hence, it is also possible that non random repair is responsible for the observed unequal distribution of radiation induced deletion mutations along the *hprt* gene.

5.2.4 Analysis of independent SLO and electroporation induced mutations

The V79-4 cells were diluted to 1000 cells per 75cm² treatment flask and grown for several days until confluent. The cells were then trypsinized and diluted to 1-3 x 10⁶ per flask and incubated for 24 hours prior to treatment with SLO (as described in Chapter 2) or electroporated (as described in Chapter 4). The cells were incubated for 5 days and plated at a concentration of 10⁵ per 90mm tissue culture plate in selective MEM/FCS/6-TG medium. The mutant colonies were removed from the tissue culture plate and cultured as described in Section 5.2.2 and the DNA extracted from the mutant cells as described in Chapter 2. The results of PCR deletion screening experiments on *hprt*⁻ SLO induced mutant DNA is illustrated in Table 5.2.4.1.

Mutant code	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Exon 7&8	Exon 9
KS1A*	+	-	+	+	+	+	+
KS1B	+	+	+	+	+	+	+
KS1D*	+	+	+	+	+	-	+
KS1E	+	+	+	+	+	+	+
KS2A	+	+	+	+	+	+	+
KS3A	+	+	+	+	+	+	+
KS3B*	+	-	+	+	+	+	+
KS3C	+	+	+	+	+	+	+
KS4A*	+	+	+	+	+	-	+
KS4B*	+	+	+	+	+	-	+
KS4C*	+	+	+	+	+	-	+
KS5B*	+	+	+	+	+	+	-
KS5C	+	+	+	+	+	+	+
KS2D*	+	+	+	+	+	+	-
KS20S1*	-	+	-	+	-	-	+
KS20S2	+	+	+	+	+	+	+
KS20S3	+	+	+	+	+	+	+
KS20S6	+	+	+	+	+	+	+
KS21S1	+	+	+	+	+	+	+

Table 5.2.4.1: The results of PCR deletion screening on the DNA of *hprt*⁻ cells which were generated by SLO treatment. PCR analysis showed that 9 of the 19 mutant DNA samples had absent PCR amplification products. The stars indicate that the mutant contains a deletion.

9 of the 19 SLO induced mutations were shown (Table 5.3.4) to have the absence of one or more of the PCR amplification products. Despite the small sample size there does appear to be a specific trend. 4 out of the 9 SLO induced deletion mutations were shown to contain deletions resulting in the absence of the PCR amplification product of exons 7&8. Of the remaining 5, 2 had lost exon 9 alone and 2 had lost exon 3 alone. Only one of the mutants (KS20S1) had lost more than one PCR amplification product and this mutant, did not produce PCR products from exons 2, 4, 6 and 7&8.

Mutant	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Exon 7	Exon 8
KE1	+	+	+	+	+	+	+
KE2	+	+	+	+	+	+	+
KE3	+	+	+	+	+	+	+

Table 5.2.4.2: The results of PCR deletion screening experiments on *hprt*⁻ V79-4 cells which were induced by the electroporation treatment.

Cell poration produces holes within the cell membrane allowing material to enter into the cell by diffusion. It also allows intracellular material to diffuse out of the cell, perhaps producing perturbations of the intracellular dNTP pools. Mattano *et al.*, (1990) observed that an imbalance in the purine dNTP pools produced an elevated mutation frequency at the *hprt* locus in human T lymphoblasts. The induction of DNA deletion mutations perhaps suggests the involvement of DNA strand breaks. Alterations in the dNTP pools have been shown to result in DNA strand breaks (Mattano *et al.*, 1990 and references therein). None of the three electroporation induced mutations analysed had DNA deletions. The effects of the SLO solution used in cell poration on the induction of DNA strand breaks was studied further. Incubating 1µg of the circular pBR322 plasmid in a 50µl digest for 30 minutes with SLO solution to produce final concentrations of 0, 0.05, 0.01, 0.02, 0.04, 0.08, 0.16 and 0.32 units/ml produced increasing amounts of the linear version of the plasmid (Figure 5.2.4.2). This suggested that either the SLO itself nicks the DNA or that there are contaminating endonucleases in the SLO preparations provided by the manufacturer. Incubating 2µg of λ DNA with the above concentrations of SLO does not produce specific bands, the λ remains intact. However incubation of the λ DNA for 18 hours with SLO produces a smear of DNA along the electrophoresis tracts perhaps suggesting exonuclease digestion. This is shown in Figure 5.2.4.3. Incubation of pBR322 for prolonged periods of time also produces the gradual disintegration of the DNA. This suggests that the streptolysin O provided by the manufacturer and used in cell poration was impure and contaminated with small amounts of exonucleases and other cellular enzymes.

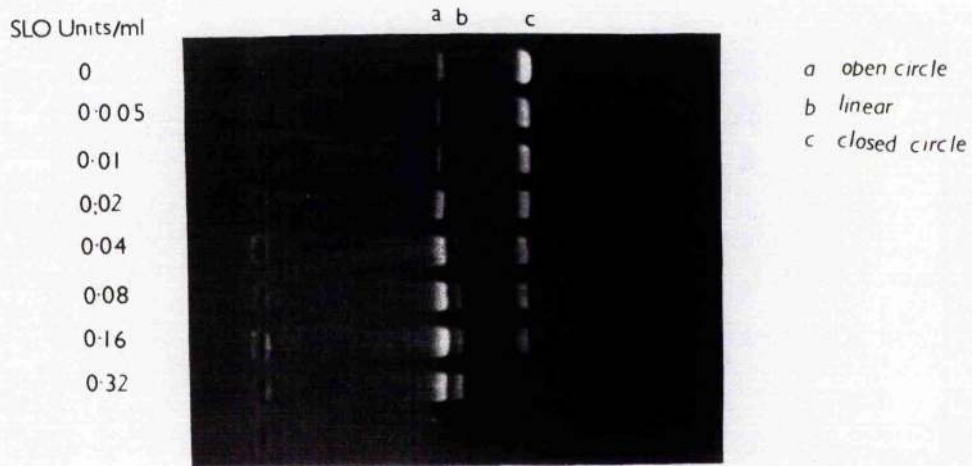


Figure 5.2.4.2: The effect of incubating pBR322 in different concentrations of SLO solution for 30 minutes. The photograph is of an agarose electrophoresis gel. Notice that as the SLO concentration increases the amount of the linear form of the pBR322 plasmid also increases.

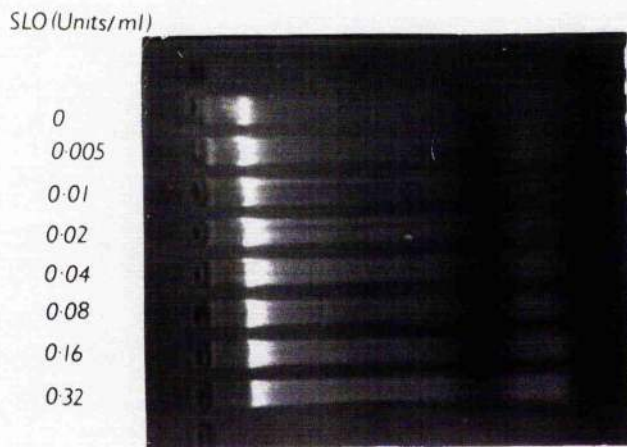


Figure 5.2.4.3: The effect of incubating λ DNA with SLO for 18 hours. The photograph is of an agarose electrophoresis gel. Note the amount of smearing in the lanes increases with increasing SLO concentration.

5.2.5 Analysis of *Pvu II* induced mutations using electroporation and SLO as the methods of cell poration

The V79-4 cells were diluted to 1000 cells per 75cm² treatment flask and grown for several days until confluent. The cells were then trypsinized and diluted to $1-3 \times 10^6$ per flask and incubated for 24 hours prior to treatment with purified *Pvu II* using SLO as the method of cell poration (as described in Chapter 2). 10^5 cells were plated on 90mm tissue culture plates (5 plates per treatment) and the mutant colonies removed from the plate and cultured as described in Section 5.2.2. The DNA was extracted from the mutant cells as described in Chapter 2. DNA extracted from the *Pvu II* induced mutants isolated by Singh (1991), using electroporation as the method of cell poration, were also analysed. The results of the PCR deletion screening experiments on *Pvu II* induced *hprt*⁻ mutants are shown in Figures 5.2.5.1 and 5.2.5.2

The distribution of *Pvu II* induced deletion mutations (both using electroporation and SLO poration) suggest that there could perhaps be a clustering of DNA breakpoints (Figure 5.3.1.1). 9 of the 14 deletion mutations did not produce the amplification product of exon 2 and of these 8, 7 had exon 3 present. This is interesting as the EMBL data base (which contains the exonic sequence data of the Chinese hamster *hprt* gene, data deposited by Rossiter, 1990, accession numbers of the exons X53073-X53080) indicates that *Pvu II* has a restriction site in the intronic region region between exon 1 and 2. This site is in the region bordering the DNA sequence of exon 2. Exon 2 was not lost with any noticeable frequency in the isolated SLO or the radiation induced *hprt*⁻ deletion mutations. The information obtained in the EMBL data base does not suggest that there are any other *Pvu II* restriction sites in or on the intron-exon border of any of the other 8 exons. However, the position of the *Pvu II* sites within the bulk of the intronic regions of the *hprt* gene are not known. With the frequent absence of particular PCR products and the suggested distribution of deletions it would not be surprising to find *Pvu II* sites between and around exons 3 and 4 and between and around exons 5 and 6.

Following enzyme purification (Chapter 2) the restriction enzymes were diluted in the buffer HBSS/BSA. The effects of HBSS/BSA on the DNA was studied using plasmid pBR322 and linear λ DNA. 1 μ g of pBR322 and 2 μ g of λ DNA were independently incubated in 50 μ l of HBSS/BSA solution for varying lengths of time. Even after an 18 hour incubation period the DNA remained intact (Figure 5.2.5.1) and there were no signs of endonuclease or exonuclease digestion.

Mutant code	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Exon7&8	Exon 9
B1	+	+	+	+	+	+	+
B2*	-	+	-	+	-	-	+
B4	+	+	+	+	+	+	+
B5	+	+	+	+	+	+	+
B6	+	+	+	+	+	+	+
B7*	-	-	-	-	+	+	-
B8*	-	+	-	+	-	+	+
B10*	-	-	-	-	-	-	-
B12*	+	-	+	-	+	+	-
B14	+	+	+	+	+	+	+
B15*	+	+	+	-	+	+	+
B16	+	+	+	+	+	+	+
B17	+	+	+	+	+	+	+
B18*	+	+	+	-	+	+	+
B19	+	+	+	+	+	+	+
B21*	-	+	-	+	+	-	+
B22	+	+	+	+	+	+	+
B23	+	+	+	+	+	+	+
B27*	+	-	-	-	+	+	+
B26*	-	+	-	+	+	-	+

Table 5.2.5.1: The results of PCR deletion screening experiments performed on the *hprt*⁻ V79-4 mutant DNA. The mutants were induced by *Pvu II* treatment using electroporation as the method of cell poration. The mutants in the above table were isolated by Singh (1991). 10 of the 19 mutant cell lines were found to contain deletions.

Mutant code	exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Exon 7&8	Exon 9
KA1*	-	+	-	+	+	+	+
KA2	+	+	+	+	+	+	+
KA3	+	+	+	+	+	+	+
KA4*	-	+	+	+	+	+	+
KA5	+	+	+	+	+	+	+
KA6	+	+	+	+	+	+	+
KA7*	+	+	+	+	+	+	-
KA8	+	+	+	+	+	+	+
KA9*	-	+	+	+	+	-	+
KA10	+	+	+	+	+	+	+

Table 5.2.5.2: The results of PCR deletion screening on *hprt*⁻ mutants generated from treatment of V79-4 cells with *Pvu II* using SLO as the method of cell poration. 4 out of the 10 mutants analysed were shown to have absent PCR amplification products.

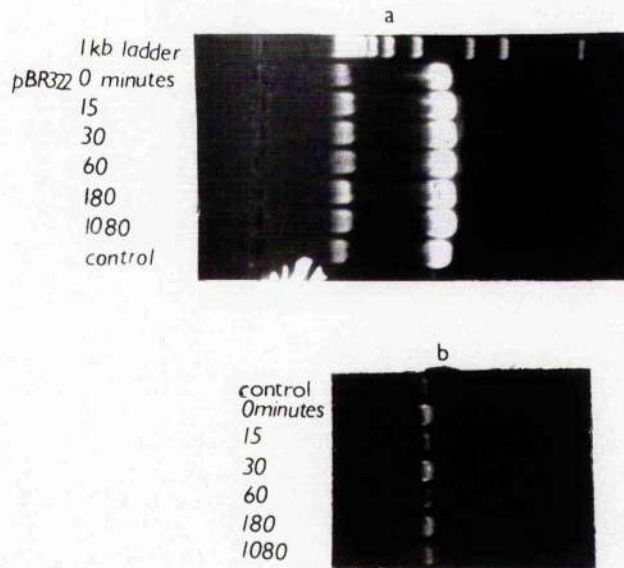


Figure 5.2.5.1: (a) Shows the effect of incubating pBR322 DNA in HBSS/BSA solution for varying lengths of time. (b) shows the effects of incubating λ DNA in HBSS/BSA for varying lengths of time. The DNA in all cases remains intact, there is no evidence to suggest that the HBSS/BSA contains any contaminating endonucleases or exonucleases.

5.2.6 Analysis of the *hprt* gene in the CHO-K1 cells and *xrs-5* cells

The *xrs-5* cell line maintained in MEM/FCS in this laboratory has been shown to be *hprt*⁻ in phenotype unlike the MEM/FCS maintained CHO-K1 and V79-4 cell populations. The *hprt* gene of the CHO-K1, V79-4 and *xrs-5* cell lines was also analysed by PCR deletion screening. Genomic DNA was isolated as described in Chapter 2 from passage line cells and the PCR reaction performed. Table 5.2.6.1 shows the results of the *hprt* PCR deletion screening experiment on DNA isolated from the wild type CHO-K1, V79-4 and *xrs-5* cells maintained in the St. Andrews laboratory.

Wild type Cell line	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Exon 7&8	Exon 9
V79-4	+	+	+	+	+	+	+
CHO-K1	+	+	+	+	+	+	+
<i>xrs-5</i>	+	+	+	+	+	-	+

Table 5.3.6.1: The results of PCR deletion screening experiments, amplifying the exons of the genomic DNA of the wild type cell lines maintained in the St. Andrews laboratory. Notice that the wild type CHO-K1 and the V79-4 cell lines possess all the PCR amplification products. However, the *xrs-5* cell line does not have the exon 7&8 amplification product.

PCR deletion screening of the DNA of the wild type V79-4 cells and the CHO-K1 cells produces a full complement of PCR products. In *xrs-5* however, the PCR amplification product of exons 7&8 was absent. Agarose gel electrophoresis photographs of the PCR products of the three different cell lines are shown in Figure 5.3.6.1. The results of Chapter 3 suggested that the *xrs-5* cell line maintained in this laboratory was *hprt*⁻ in phenotype and that the *hprt*⁻ phenotype was a result of a genetic rather than an epigenetic change. The results of the PCR deletion screening experiments suggest that there is a deletion in the *hprt* gene which spans at least one of the primer sites of either exon 7 or 8.

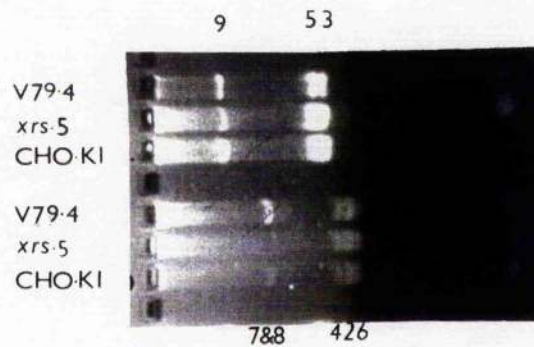


Figure 5.2.6.1: An agarose gel of the *hprt* PCR amplification products of exons 3, 5 and 9 and of the PCR amplification products of exons 2, 4, 6 and 7&8 of the CHO-K1, V79-4 and *xrs-5* passage line DNA.

5.3 Summary

None of the isolated spontaneous *hprt*⁻ mutants contained observable DNA deletions within the exon sequences screened. This supports the findings of Thacker (1986), Hou *et al.* (1993), Aghamohammadi *et al.* (1992) Morris *et al.* (1993) and Schwartz *et al.* (1994) that the majority of spontaneously induced *hprt* mutations are the result of base pair changes or small scale deletions which are not usually detected by PCR or Southern blot analysis.

Approximately one third of the radiation induced *hprt* mutations were shown not to have the full complement of PCR amplification products, thus suggesting that one third of the induced mutations were deletions. This frequency is much lower than the value suggested by Thacker (1986). It is possible that Southern blotting of restriction enzyme digested mutant DNA and hybridisation to a *hprt* cDNA probe may reveal deletions within the intronic regions or rearrangements that could not be detected by PCR analysis.

Evidence suggests (Chapter 4) that cell poration induces mutations at the *hprt* locus in V79-4 cells. 19 SLO induced V79-4 *hprt*⁻ mutations were analysed. Of the 19 analysed, 9 were shown (Table 5.2.4.1) to have absent PCR amplification products suggesting deletions spanning at least around the primer binding sites. Despite the small sample size it did not appear that the distribution of deletions was random (Figure 5.3.1.1). None of the three electroporation induced *hprt*⁻ mutant cells were shown to have absent exon sequences. The

effect of SLO on DNA *in vitro* was studied by incubating the plasmid pBR322 and phage λ DNA with different concentrations of the SLO solution. The results suggested that the SLO provided by the manufacturer contained contaminating exonucleases and endonucleases. These contaminating enzymes may be the reason for the high frequency of exon deletions observed in mutants induced by SLO poration. Whether the nick is in a random location or not is unknown. It would be interesting to know whether the site at which the contaminating enzymes nicks the DNA is in anyway related to the non random distribution of SLO induced *hprt*⁻ V79-4 mutations.

Pvu II was introduced into the cell by electroporation (mutants isolated by Singh, 1991) and by SLO poration (Chapter 4). Only a small fraction (~20%) of the mutants induced by this treatment can be predicted to be as a result of the poration treatment. 14 of the 30 *Pvu II* induced *hprt*⁻ mutants were shown to be the result of DNA deletion events. The distribution of the DNA deletions are shown in Tables 5.2.5.1 and 5.2.5.2 and summarised in Figure 5.3.1.1 below. 9 of the 14 deletion mutants were shown to have absent PCR amplification products from exon 2. This high proportion of exon 2 deletions was not observed in radiation or SLO induced mutant cell populations suggesting that deletion of exon 2 is a hotspot for *Pvu II* induced mutations. The EMBL DNA sequence library revealed that *Pvu II* has a single restriction site in the intronic region adjacent to the exon 2 sequence. No other exons were shown to have *Pvu II* sites within or adjacent to their sequence. Whether *Pvu II* cuts within the remaining intron sequences is not known. It would be interesting to know whether and where the *Pvu II* cuts within the remaining intron sequences of the *hprt* gene and whether the distribution of the remaining deletions are related to these sites. Sequencing the end points of the deletions would reveal whether the deletion was the result of the poration treatment or the result of an initial *Pvu II* cleavage. This however is left as a recommendation for future work.

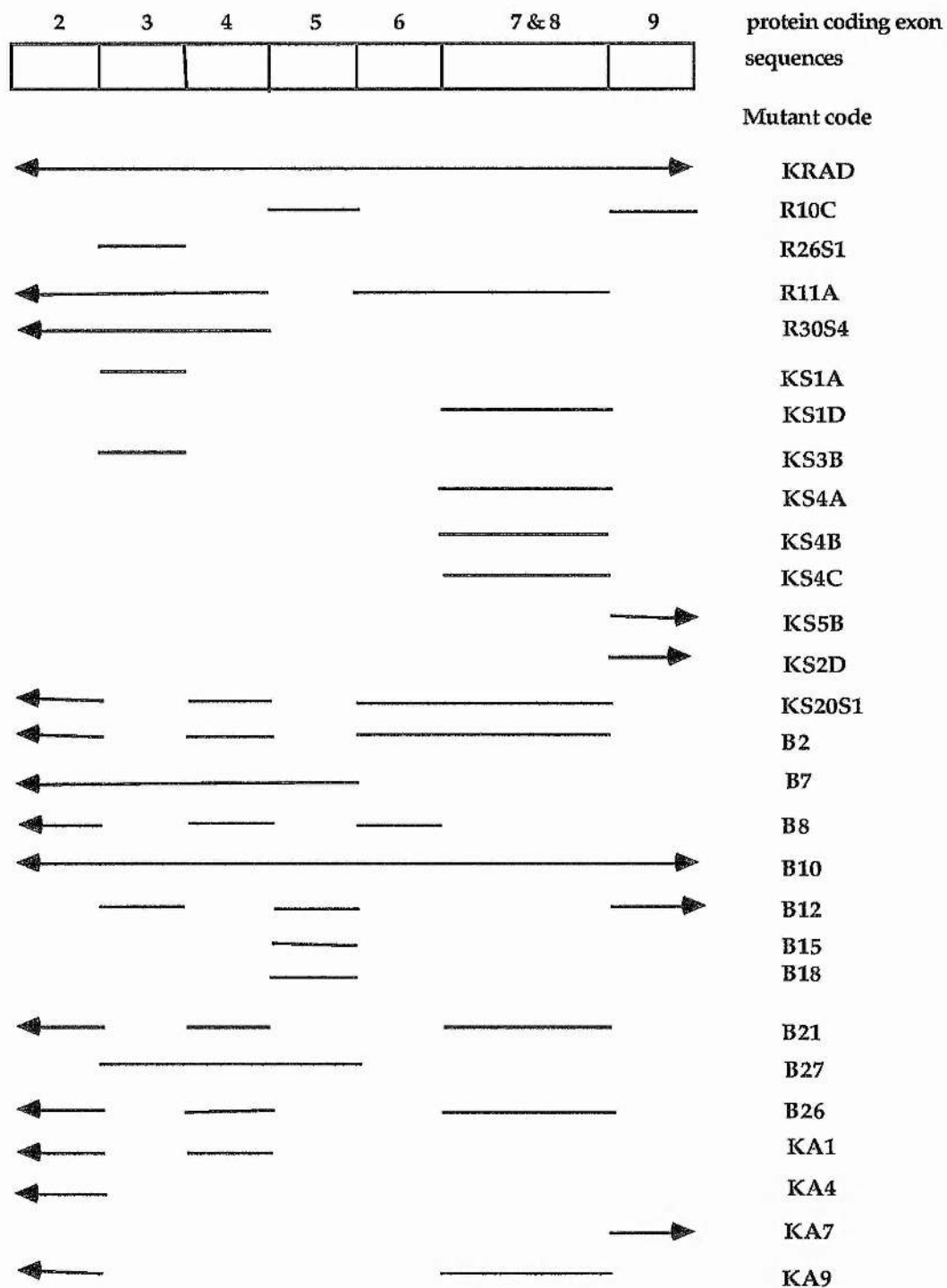


Figure 5.3.1.1: The diagram shows the exons where there was failure of PCR amplification in the isolated V79 *hprt*⁻ mutants. The lines (-----) indicate absent PCR products. The arrow at the end of the line indicates that it is possible that deletions may extend beyond the analysed region. Mutant KRAD and mutants with the prefix code R are mutations induced by 4Gy gamma radiation, mutants prefixed with KS were induced by streptolysin, B indicates that the mutants were induced by *Pvu II* using electroporation as the method of cell

poration and mutants prefixed with KA were induced by *Pvu II* using SLO as the method of cell poration.

Chapter 6

Attempts to locate the *thymidine kinase* gene in CHO-K1 cells using the method of Fluorescent *in situ* Hybridisation (FISH)

*As love breeds outward talk,
The hound some praise and some the hawk,
Some better pleased with private sport,
Use tennis some, a mistress court,
But these delights I neither wish,
Nor envy while I freely fish.*

-Izaak Walton (1932), *The Compleat Angler*

6.1.1: Introduction

The CHO-K1 cell line maintained in the St. Andrews laboratory has only one functional copy of the *tk* gene within its genome. This is suggested from the anomalously high background mutation frequency and the high induced mutation frequency following gamma irradiation (Singh, 1991; Singh and Bryant, 1991). In the normal diploid Chinese hamster genome the *tk* gene is located on Chromosome 7 (Stallings and Siciliano, 1981). In the aneuploid CHO-K1 cell line the chromosomes have been rearranged. Chromosomes have been lost and some fragments of chromosomes have been translocated to other non homologous chromosomes. The genetic linkage map of the CHO-K1 cell line would therefore be expected to be different to that of the normal diploid Chinese hamster cell. It is uncertain what has happened to the second copy of the *tk* gene. It is possible that the second gene copy has been completely deleted during the evolution of the cell line. It is also equally possible that the second *tk* copy is still present but, in an inactive form; perhaps an error in the gene promoter sequence exists or the gene has been relocated and placed adjacent to a promoter which is not constitutively expressed. The gene could also be present and

active but, its protein product mutated or may be the gene is broken in half and one half relocated else where in the genome. At present it is impossible to predict which of these hypotheses is the most likely.

When studying the molecular spectrum of gene mutations, it is useful to know the copy number of the gene of interest, especially if one is to obtain useful information from PCR and Southern blotting. These techniques are used to analyse the molecular characteristics of the DNA. Southern analysis without the gene frequency data is possible but, the information obtained from such data is limited.

Fluorescent *in situ* hybridisation (FISH) is a relatively new technique which allows specific nucleotide sequences to be detected on morphologically preserved chromosomes, cells or tissue sections (Viegas Pequignot *et al.*, 1989, 1991; Lichter *et al.*, 1991; Natarajan *et al.*, 1992; Viegas-Pequignot, 1993). The FISH technique is an adaptation of the radioactive technique developed by Pardue and Gall (1969) and independently by John (1969). This technique gives topological information concerning the nucleotide sequence of interest. FISH has many advantages over the radioactive technique, such as high sensitivity and specificity, probe stability and direct mapping without tedious statistical analysis (Viegas-Pequignot, 1993). Probes as small as 0.5-1 kb recognising single copy targets can be visualised on preserved chromosomes (Viegas-Pequignot *et al.*, 1991). FISH is a joining of the traditional *in situ* hybridisation techniques and immuno-cytochemistry.

The aim of this section of work was to locate the *tk* gene and discover the gene copy number. If a second *tk* copy was present and inactive it should be observed on the morphologically preserved chromosomes following FISH. A λ 10, full length Chinese hamster *tk* gene clone was produced at Cold Spring Harbour in 1986 (Lewis, 1986). However, this clone was unavailable. As an alternative, a commercial mouse *tk* cDNA cloned into a plasmid was obtained from the American Cell Culture Lab, Maryland, USA. This was initially cloned by Ruddle in 1985 (Lin *et al.*, 1985). Prior to the FISH experiment much preliminary work had to be performed. The bacteria containing the plasmid had to be cultured and the plasmid isolated and purified. Bacteria often contain many extra chromosomal DNA segments therefore, the plasmid identity was checked by restriction enzyme digestion and analysis of the size of the restriction products. Also the cloned DNA was not of hamster origin. The homology of the probe to the hamster DNA had to be checked prior to any FISH work. Sections 6.2.5-6.2.9 of the method were performed at the Western General Hospital, Edinburgh with the kind help of Dr. Judy Fantès. The basic principles of the technique used in this Chapter are described below. As a control for the FISH procedure a telomere probe,

biotinylated and synthesised by PCR was also used. This probe was also kindly provided by Dr Judy Fantes.

6.1.2 The principles behind Fluorescent *In situ* Hybridisation

It is now possible to modify DNA with "non-isotopes", that is non radioactive markers. These markers are often described as reporter molecules and include chemicals such as biotin, photo-biotin and 5-bromodeoxyuridine. The most commonly used reporter molecule is biotin-16-dUTP but, the use of the digoxigenin technique developed by Boehringer is increasing in popularity (J. Fantes, Personal communication). Biotin labelled probes were used for the work presented in this Chapter. Biotin is a vitamin B complex which has a specific binding affinity for avidin. The plasmid probe was labelled with a biotin-16-dUTP reporter molecule by the method of nick translation. During this process the biotin-16-dUTP was incorporated into the plasmid DNA instead of dTTP. DNase was added at a specific concentration so that the probe was cut into fragments of approximately 400 bases in length. The labelled probe was denatured and hybridised to denatured morphologically preserved metaphase chromosomes. Contaminating RNA material and cytoplasmic proteins were removed to reduce background and non specific probe hybridisation. Following probe hybridisation, layers of avidin-FITC and biotin labelled anti-avidin were added. This builds up a complex of fluorescent dye on top of the probe, the more layers of avidin-FITC and biotin labelled anti-avidin the greater the signal emitted. However, the more layers that are built up the greater the amount of background and non-specific hybridisation. Therefore, there is a point at which adding more layers is no longer useful. The chromosomes are stained red using a propidium iodide counter stain, hence the fluorescent green FITC signals can be seen against a red chromosomal background when the slides were viewed down a fluorescent or a confocal microscope with the appropriate filters.

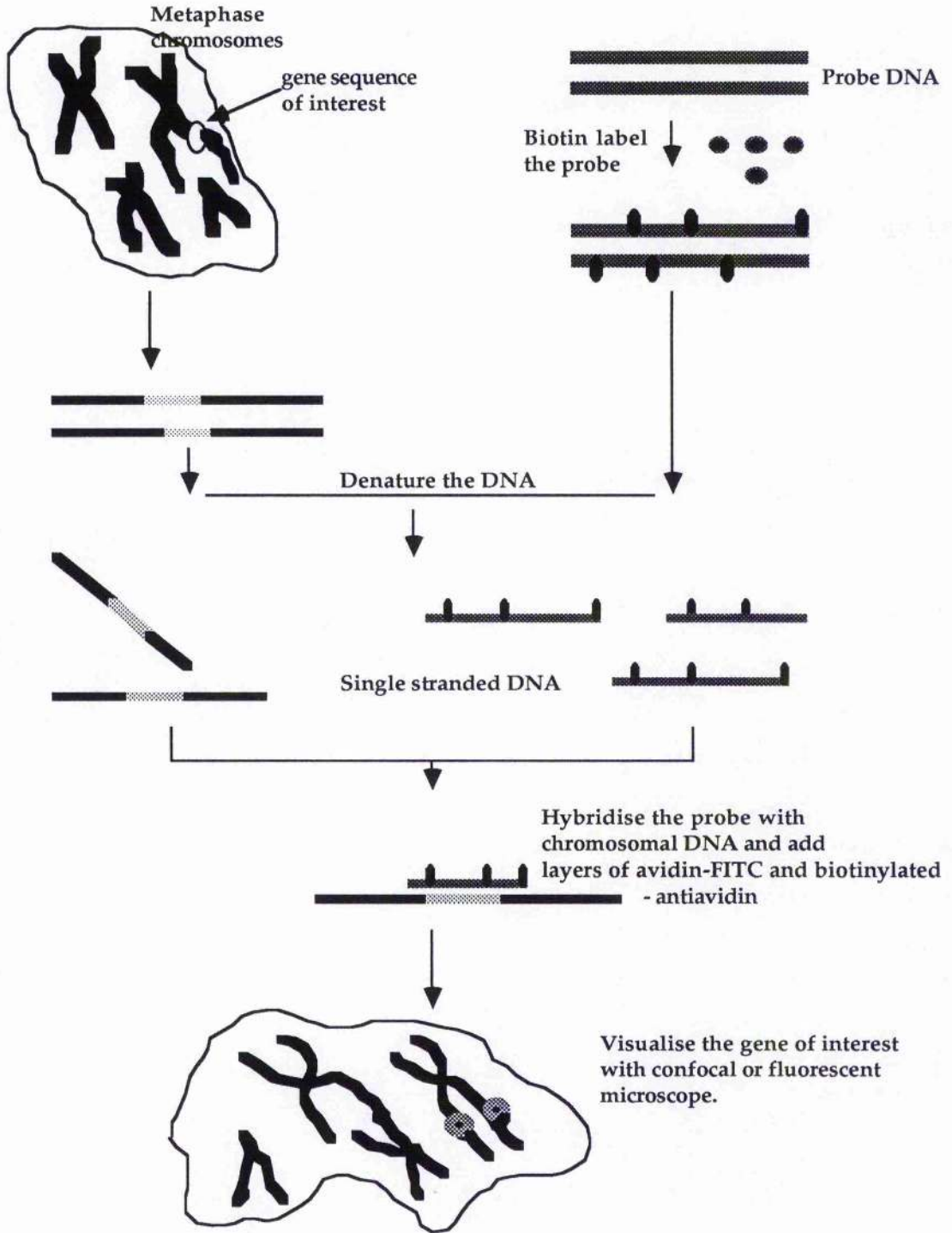


Figure 6.1.1: The principle of Fluorescent in situ hybridisation

6.2.1 Growing bacteria and plasmid isolation

Escherichia coli DH1 containing a pcD plasmid containing the mouse pMTK4 DNA clone insert (isolated from L cells) was obtained from the American Type Culture Collection, USA. The pMTK4 clone is the cDNA of the mouse thymidine kinase gene. It was isolated in 1985 at Yale University by Frank Ruddle (Lin *et al.*, 1985). The total size of the pMTK4 clone is 1.16kbp. The plasmid containing the insert has a total size of 4.7kbp. The plasmid containing the cDNA clone also confers a resistance to ampicillin to the host bacterium. The bacteria containing the plasmid of interest was therefore selected for by culturing in an ampicillin rich medium. The plasmid identity was checked by the characteristic banding patterns produced by agarose gel electrophoresis following restriction enzyme digestion and reference to the plasmid restriction map provided by supplier.

The bacteria were obtained in a freeze dried state. They were initially revived in 0.3ml of LB media. LB media was made up by dissolving 10g bactotryptone (Oxoid); 5g bacto yeast extract (Oxoid); 10g NaCl (BDH) in 1 litre of distilled water and adjusted to pH 7 with NaOH solution. Prior to use the LB medium was autoclaved. The bacteria were pipetted up and down until a homogeneous suspension was obtained, then a further 5ml of LB media was added. Ampicillin stock solution was made up by adding 50mg of ampicillin powder (Sigma) to sterile distilled water to produce a concentration of 50mg/ml. Ampicillin was added to the LB medium to produce a concentration of 5µg/ml. The bacterial broth was then incubated overnight at 37°C in a shaking water bath.

Agar plates were made by dissolving (by microwave heating) 1.5g of bacto-agar (Sigma) in 100ml of LB medium. When the solution had cooled to a temperature of less than 50°C, the ampicillin stock solution was added to produce a final concentration of 5µg/ml. Ampicillin is inactivated at temperatures of greater than 50°C. The solution was poured into non-tissue-culture grade petri dishes and allowed to cool in a sterile laminar flow hood. The bacteria were streaked onto the plates using a heat sterilised loop and incubated overnight at 37°C. The following morning colonies were observed. A "tooth picked" bacterial colony was added to 3ml of LB/ampicillin medium and incubated overnight at 37°C in a shaking water bath. The following day the suspension was added to 300ml of LB/ampicillin medium and incubated at 37°C for 15 hours, shaking continuously. Shaking was essential to maintain aeration of the medium and hence ensure optimum growth of the bacteria.

300ml of bacterial suspension was centrifuged at 13,000rpm for 10 minutes (RCF = 5645), the supernatant drained and the pellet redissolved in 100ml of hypotonic GTE solution. GTE solution was made up by dissolving 3.72g of ethylenediaminetetra-acetic acid (BDH); 3.0285g Tris (hydroxymethyl) methylamine (BDH); 9g glucose (BDH) in 1 litre of sterile distilled water. 200ml of freshly prepared NaOH/SDS solution, produced by adding 4ml of 5M sodium hydroxide stock solution and 10ml of 10% sodium dodecyl sulphate solution (BDH) to 84ml of sterile distilled water, was added and the suspension placed on ice for 5 minutes. This was to cause cell lysis and to denature the cell proteins. 160 μ l of 3M sodium acetate was added and the suspension was replaced on ice for a further 5 minutes. This causes the genomic DNA to precipitate. The mixture was centrifuged for 5 minutes at 13,000rpm (RCF = 5645). The supernatant containing the small circular plasmid DNA was then filtered through sterile gauze (to remove any contaminating genomic DNA) into a clean sterile container. 1 volume of ice cold absolute ethanol was added and the mixture centrifuged for 5 minutes. The supernatant was discarded and the plasmid pellet was washed with 100ml of 70% ethanol and centrifuged for 10 minutes at 13,000rpm (RCF = 5645). The pellet once dried under vacuum, was redissolved in sterile TE buffer which was made up by adding 10ml 1M tris-HCl (BDH) and 0.372g EDTA (BDH) to 990ml of sterile distilled water. The DNA was treated with RNase to remove any contaminating RNA molecules and further purified using Promega DNA clean up columns.

6.2.2 Checking the identity of the plasmid by restriction analysis

Bacteria often contain many different types of plasmids hence, it was essential to verify that the plasmid isolated was the one of interest. The plasmid of interest was reported to be 4.7kbp in size including the 1.16kbp insert. Digestion of the altered pcD plasmid with *Bam* HI was known to yield 2 fragments of 3.2kbp and 1.5kbp.

Plasmid digestion was performed by adding :

- 1 μ l plasmid solution (0.5 μ g/ μ l)
- 1 μ l restriction buffer (x 10 concentration)
- 1 μ l restriction enzyme (10 units/ μ l)

to 7 μ l of sterile distilled water. The reactions were incubated in 500 μ l microfuge tubes in a 37°C water bath for 3 hours. The reactions were stopped by the addition of 1 μ l of 20% SDS and 1 μ l of 50mM EDTA to each of the digestion reactions. 1 μ l of bromophenol blue solution was added as the electrophoresis tracking dye. The samples were run on a 100ml, 0.8% agarose electrophoresis gel (BRL, ultra pure grade) containing 100 μ l of ethidium bromide solution (stock, 0.5 μ g/ μ l) in tris borate electrophoresis buffer at 20 volts for 15 hours.

Markers were also run adjacent to the samples. Figure 6.2.1.1 shows a photograph of the agarose electrophoresis gel. The size and the identity of the isolated plasmid was checked against the plasmid pBR322 marker and a DNA ladder.

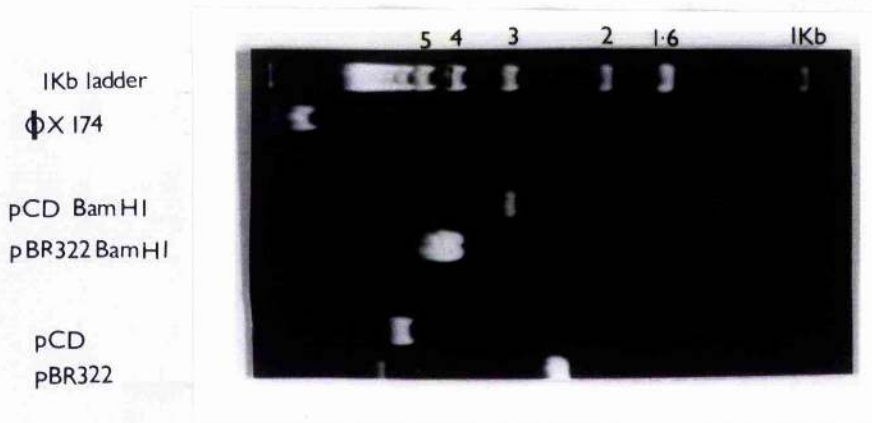


Figure 6.2.2.1: A photograph of an agarose gel following electrophoresis. The isolated and the *Bam* *H*1 digested plasmid was run as was pBR322, *Bam* *H*1 digested pBR322 and a 1kb DNA ladder.

6.2.3 Homology testing

Prehybridisation solution (10ml)

5ml of 100% deionised formamide (Sigma); 2ml of 25 x SSC; 1ml of 50% Denhardt's solution (BDH); 0.25ml of 2M phosphate buffer; 0.5ml of 10mg/ml stock of single stranded salmon sperm DNA (Sigma) and 0.75ml of distilled water were all mixed together immediately before use and preheated to 42°C.

Hybridisation solution (10.9ml)

5ml of 100% de-ionised formamide (Sigma); 2ml of 25 x SSC; 1ml of Denhardt's solution (BDH); 0.1ml of 2M sodium phosphate buffer solution; 2ml of 50% dextran sulphate (Sigma) solution; 0.3ml (10mg/ml) of single stranded salmon sperm DNA solution; 0.5ml of 20% SDS solution were all mixed together immediately before use and preheated to 42°C.

Wash buffer (1000ml)

100ml of 20 x SSC; 10ml of 20% SDS solution and 890ml of sterile distilled water were mixed together, autoclaved and stored at room temperature.

The thymidine kinase DNA isolated from the plasmid was derived from the mouse genome. It was therefore important prior to any further experiments, to elucidate whether the homology between the mouse and the hamster thymidine kinase DNA was sufficient to allow hybridisation.

DNA was isolated from CHO-K1 cells, mouse cells and human lymphoblastoid cells by the technique described in Chapter 2 and the amount of DNA measured on a spectrophotometer. A nylon H⁺ membrane (Amersham) was soaked in 10 x SSC for 10 minutes and placed on the BRL slot blot apparatus which had been assembled according to the manufacturers instructions. 1µg of each of the prepared DNA samples was heat denatured for 10 minutes at 90°C and the single stranded DNA samples immediately placed on ice. This was to prevent reannealing. The slots within the blotting apparatus were washed through with 180µl of 10 x SSC and the DNA samples loaded into individual slots and allowed to drain. The dried nylon membrane was baked for 1 hour at 80°C to fix the DNA to the membrane. The plasmid was labelled with ³²P using the random prime method, the kit and the instructions supplied by Pharmacia, and the unincorporated nucleotides removed using Pharmacia Sephadex-50 nick spun column. The nylon membrane was carefully placed in the hybridisation chamber, 10ml of prehybridisation buffer was added and the chamber and its contents incubated at 42°C for 30 minutes. The prehybridisation mix contained single stranded salmon sperm DNA which hybridises to the repeat sequences hence, reducing non specific hybridisation. The prehybridisation buffer was removed following incubation and replaced with 10ml of hybridisation buffer containing 20ng of the ³²P labelled plasmid probe. The temperature of incubation was maintained at 42°C. Following 15 hours of incubation the hybridisation buffer was removed and wash buffer was added and the temperature raised to 68°C. This was to remove any unhybridised probe or probe attached to the membrane with poor homology. The membrane was washed in the washing buffer for 20 minutes. Using forceps the filter was removed and rinsed in 2 x SSC. The membrane was

blotted on Whatman 3MM filter paper and placed on a photographic film (Amersham) for autoradiography. The autoradiographic film was left in contact with the membrane for 3 days then developed and fixed. Figure 6.2.3.1 shows a photograph of the autoradiographic film. From the intensity of the bands it can be seen that the probe has bound most strongly to the mouse DNA and has bound almost as strongly to the Chinese hamster DNA. The probe however did not bind to the human DNA.

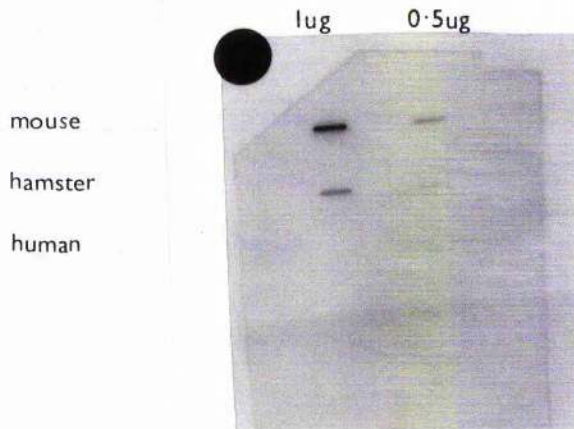


Figure 6.2.3.1: A photograph of an autoradiograph showing how the ^{32}P labelled plasmid probe containing the mouse *tk* gene hybridised to mouse, hamster and human DNA attached to a nylon membrane.

6.2.4 Plasmid biotinylation by nick translation and estimation of the concentration of biotin in the labelled probe

The nick translation buffer, the deoxyribonucleotide-triphosphate solution and the biotin-16-dUTP were made up as follows:

Nick translation buffer (10ml)

0.0475g (50mM) of magnesium chloride (BDH) and 5mg of nuclease-free bovine serum albumen (Sigma) were dissolved in 5ml of 1M tris-HCl and made up to 10ml with sterile distilled water and stored frozen at -20°C .

Deoxyribonucleotide triphosphate solution (100 μ l)

5 μ l of 10mM dATP (BRL); 5 μ l of 10mM dCTP (BRL); 5 μ l of 10mM dGTP (BRL) and 85 μ l of sterile distilled water were mixed immediately before use.

Biotin-16-dUTP (Vector labs):

The stock was obtained ready dissolved at 1mM concentration and was stored at -20°C.

The nick translation mix was made up as follows:

- 5 μ l nick translation buffer
- 5 μ l dithiothreitol (100mM)
- 5 μ l deoxyribonucleotide triphosphate solution
- 6 μ l biotin-16-triphosphate solution (0.4mM, BRL)
- 1 μ g plasmid DNA
- 5 μ l DNase solution (stock of 1 μ g/ml)
- 1 μ l DNA polymerase solution (10U/ μ l, BRL)

The final volume was made up to 50 μ l with sterile distilled water and was incubated for 90 minutes at 15°C. 5 μ l of EDTA solution (0.5mM) was added to stop the reaction. The probe was separated from the unincorporated nucleotides using Sephadex G-50 columns (Pharmacia).

The concentration of biotin in the labelled probe was checked in comparison to standard biotin labelled DNA supplied by BRL as part of the Blue Gene detection kit. The biotin was detected by incubating with streptavidin alkaline phosphatase and a colorimetric reaction was observed. The filter preparation and the biotin detection are described below.

Filter preparation.

The nitro-cellulose filters were washed in distilled water for 2 minutes followed by washing with 20 x SSC for 20 minutes and allowed to dry in the air. This helps to keep the dots small. The biotinylated probe was serially diluted in TE to make concentrations of 1 in 10², 1 in 10³ and 1 in 10⁴. 1 μ l and 2 μ l of each dilution were dotted on to the nitro-cellulose. In the case of the 2 μ l spot, 1 μ l was initially added, allowed to dry and then a second added on top of the first. Dilutions of 20, 10, 2 and 1 pg/ μ l were made from the supplied standards and 1 μ l of each dotted onto the nitro-cellulose. The DNA was fixed to the filter by exposure to UV.

Biotin Detection

The filters were washed for 5 minutes in buffer 1 (0.1M Tris; 0.15M NaCl; at a pH of 7.5) at room temperature and then incubated for 1 hour in buffer 2 (0.1M Tris; 0.15M NaCl; 3% BSA Fraction V; at a pH of 7.5) at 60°C. The filter was then transferred to 10ml of buffer 1 containing 10µl of streptavidin alkaline phosphatase and incubated at room temperature for 10 minutes. The streptavidin alkaline phosphatase binds to the biotin on the filter. The filter was then washed twice with 200ml of buffer 1 for 15 minutes and once with buffer 3 (0.1M Tris; at a pH of 9.5) for 5 minutes, both at room temperature. For the colour detection, the filter was placed in a polythene bag containing the ready prepared BCIP/NBT (from Blue Gene kit) and incubated in the dark for 2 hours. A blue colour was produced as the added chemicals reacted with the alkaline phosphatase. The sample colour was compared with those of the standards and an estimation of the biotin concentration made.

6.2.5 Slide preparation

Colcemid was added to exponentially growing mouse and hamster cells at a concentration of 0.04µg/µl (stock 4µg/µl). Colcemid blocks the cell cycle at the metaphase stage of mitosis. This treatment increases the proportion of the cell population in the metaphase stage of the cell cycle. The cells were incubated at 37°C for 1 hour and the medium removed and stored. The cells were then trypsinized and the tissue culture flask rinsed with the previously removed medium. The cell suspension was centrifuged at 1,600rpm and the pellet of cells resuspended in 1ml of 0.075M hypotonic potassium chloride solution and left at room temperature for 10 minutes. The solution was then recentrifuged. The supernatant was removed and the pellet resuspended in 5ml of freshly prepared fix (methanol: glacial acetic acid, 3:1) and recentrifuged. The fixing procedure was repeated 5 times. This maximises the removal of lipids from the cell and helps to remove the cytoplasm from around the chromosomes. Excess cytoplasm surrounding the chromosomes interferes with the hybridisation procedure. 100µl of the cell suspension was dropped from about a metre onto horizontally held alcohol soaked clean glass slides and left to dry. The slides were left at room temperature for up to 3 days in a desiccator .

A large series of slides were prepared in this way, also prepared were a small number of slides of human and mouse metaphase spreads. In addition to the *tk* plasmid probe, a telomere probe was also used. This was produced and biotinylated by PCR and was kindly provided by Dr J. Fantes, Western General Hospital, Edinburgh. A small number of hamster and human slides were treated with the telomere probe. The telomeric sequence is

relatively large and the results predictable. The telomere probe was therefore used as a control for the FISH procedure.

6.2.6 Slide pretreatment

PBS was made up by dissolving 8g, NaCl; 0.2g, KCl; 1.15g, Na₂HPO₄; 0.2g, KH₂PO₄ (BDH) in one litre of distilled water. Before use the PBS was autoclaved. The slides were washed for 5 minutes in PBS on a shaking water bath. The slides were then dehydrated in 70%, 90% and 100% ethanol, each concentration for 5 minutes, shaking continuously. RNase stock was made up by dissolving RNase A (Sigma) in 10mM tris-HCl; 0.15mM NaCl at a concentration of 100µg/ml. The solution was boiled for 10 minutes to destroy any contaminating DNase enzymes. The slides were then incubated with 100µl of RNase stock under a glass coverslip for 1 hour at 37°C. This removes any endogenous RNA which could also hybridise with the probe and cause background. The coverslip was removed and the slides were washed for 3 x 5 minutes with 2 x SSC, followed by a single wash with PBS solution. 100µl of proteinase K (1mg/ml) was added to 100ml of 10mM HCl at a temperature of 37°C. The slides were immersed in the solution for 10 minutes. The slides were then washed in PBS for 5 minutes, PBS/50mM MgCl₂ [5ml of 1M MgCl₂ (BDH) and 95ml of PBS] for 5 minutes and then fixed in 1% formaldehyde in PBS/50mM MgCl₂ for 10 minutes, followed by a final wash in PBS for 5 minutes. The slides were then dehydrated once again in the ethanol series. The slides were either used immediately or stored at -20°C.

6.2.7 Probe hybridisation

The hybridisation buffer was made up from 0.5ml of deionised formamide, 0.1ml of 20 x SSC, 0.5ml of salmon sperm DNA (Sigma-1mg/ml), 0.2ml of 50% dextran sulphate. These were all added together and made up to 1ml with sterile distilled water. The biotinylated plasmid probe was dissolved in the hybridisation buffer at a concentration of 20ng/µl. The hybridisation buffer used for the telomere however, did not contain the single stranded salmon sperm DNA. The telomere probe was diluted at a concentration of 5ng/µl. What is also to be noted is that the concentration given refers to that of the biotin labelled DNA.

40µl of the hybridisation mix was put onto an alcohol cleaned coverslip. The slide was inverted to pick up the coverslip rather than placing the coverslip on the slide. This helped to prevent bubble formation. The coverslip was sealed onto the slide with rubber solution (obtained from a bicycle store). The chromosomes and the probe were denatured together on the slide. This was achieved by floating the slides on a steel tray in an 80°C

water bath for 10 minutes. The slides were then transferred to a 37°C water bath and allowed to hybridise overnight. The following day the rubber solution was removed from the coverslip and the slide allowed to fall off in the subsequent washes. The slides were washed 4 times in 50% formamide/ 2 x SSC at 45°C and then in 2 x SSC for 3 minutes at 37°C.

6.2.8 Immunofluorescence detection

The immunological buffer was made up by dissolving 0.8g of non-fat dried milk (Nestle) in 10ml of 2 x SSC. The milk suspension was heated for several hours at 37°C until the majority of the powder had dissolved and then centrifuged. The supernatant was collected and the pellet of undissolved powder discarded. The slides were washed with 4 x SSC /0.1% Tween. The avidin-FITC stock (2mg/ml, Vector) was diluted 1 in 1000 in the immunological buffer and the biotinylated anti-avidin stock (0.5mg/ml, Vector) was diluted 1 in 100 in immunological buffer. The diluted reagents were spun in a microcentrifuge to remove any conjugates. The slides were incubated with 40µl of the immunological buffer under a 22 x 40mm coverslip for 5 minutes at room temperature. The coverslips were removed and 40µl of the diluted avidin-FITC was added to the coverslip and placed back on the slide. The slides were incubated at 37°C for 30 minutes. The coverslips were removed and the slides washed three times at 45°C for 2 minutes in 4 x SSC ; 0.1% Tween 20. The excess fluid was drained from the slides and 40µl of the diluted biotinylated anti-avidin added to a clean coverslip which was then picked up by the slide. The slides were incubated for 30 minutes at 37°C. Following incubation, the coverslips were removed and the slides washed at 45°C three times for two minutes in 4 x SSC/0.1% Tween 20. The slides were then incubated as before with 40µl of the diluted avidin-FITC solution. (More layers of the avidin-FITC and anti-avidin may be added alternately but although it will result in the hybridised probe producing a greater signal, it also results in an increase in the background). After the removal of the final layer the slides were washed 4 times for 2 minutes at 45°C in 4 x SSC /0.1% Tween 20. 40µl of vector shield (containing propidium iodide at a concentration of 1µg/ml) was added to the coverslip and the coverslip picked up by the slide. The coverslip was blotted to remove excess mountant and sealed with rubber solution. The slides were examined with a confocal microscope using Heitz filter blocks 12/3.

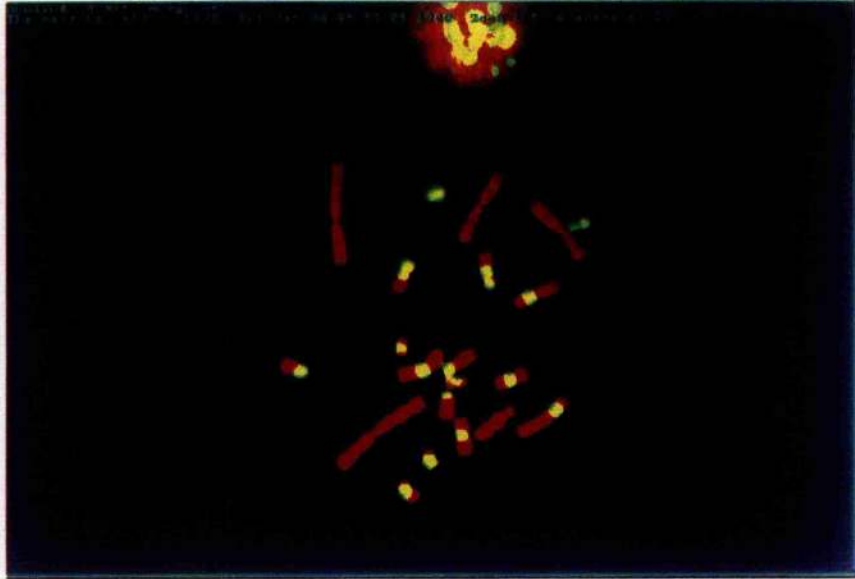


Figure 6.2.8.1: A photograph taken from a confocal microscope following immunofluorescence detection showing the biotin labelled telomere DNA sequences hybridised to CHO-K1 metaphase chromosomes. Notice that the CHO-K1 cells have internal telomere sequences. The terminal telomere sequences located at the end of the chromosomes are usually not visualised.

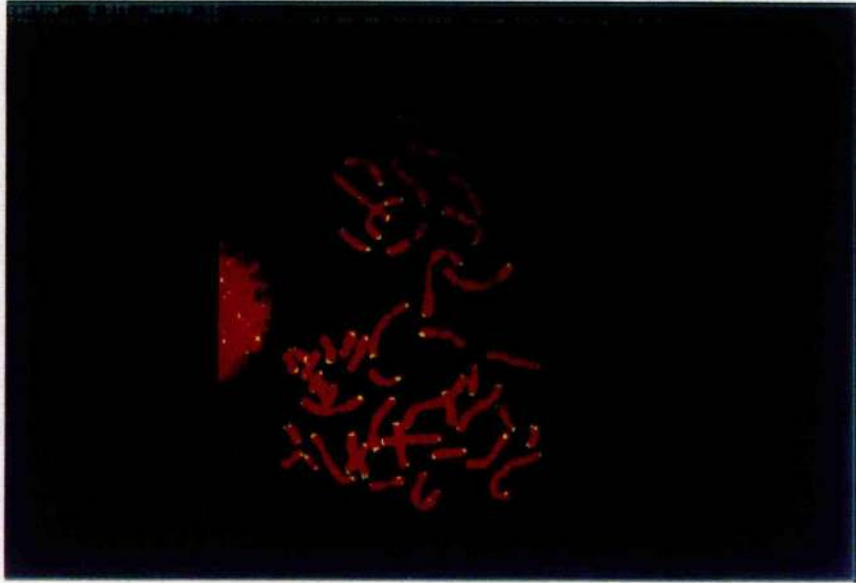


Figure 6.2.8.2: A photograph taken using a confocal microscope following immunofluorescence detection, showing the biotin labelled telomere probe hybridised to human metaphase chromosomes. Notice in this case that the telomere sequences are restricted to the ends of the chromosomes.

6.2.9 Slot blot analysis of the *tk* gene in *tk*⁻ CHO-K1 cells

DNA was isolated from CHO-K1 cells, *xrs-5* cells and from 2 CHO-K1 *tk*⁻ cell lines. TK4 was a 4Gy radiation induced *tk*⁻ cell line stock cell culture stored frozen in the St. Andrews laboratory and TK1 was a 4Gy radiation induced *tk*⁻ cell line isolated by myself. A slot blot of the DNA of the cells described above was prepared as described in Section 6.2.3 and the pMTK4 cloned plasmid was labelled and hybridised to the membrane, also as described in Section 6.2.3. A photograph of the developed autoradiograph is shown in Figure 6.2.9.1. The CHO-K1, *xrs-5*, and the TK1 cell lines all showed similar intensity, however the blot of TK4 was not so intense. As equal amounts of the DNA of the different cell lines were blotted on to the membrane, this perhaps suggests that there has been some *tk* sequence loss from the TK4 cell line.



Figure 6.2.9.1: Autoradiograph of the slot blot of DNA of the CHO-K1, *xrs-5*, TK4 and TK1 cell lines hybridised to the ³²P labelled mouse cDNA clone pMTK4.

6.3 Summary

The method of FISH has been used in the past to find the location of specific DNA sequences on chromosomes (Viegas-Pequignot *et al.*, 1989; 1991; Natarajan *et al.*, 1992; Lichter *et al.*, 1990) and has also been used to order genes along chromosomes (Korenberg *et al.*, 1992). It has been used in the form of chromosome painting to reveal small complex chromosomal translocations (Ledbetter, 1992). A high sensitivity has been reached which has enabled probes as small as 0.5-1kb to be visualised on fixed metaphase chromosomes (Viegas-Pequignot *et al.*, 1991) even when the sequence is in a single copy state. During this work,

telomere sequences, which are repeated thousands of times on chromosomes were observed on fixed metaphases of human and CHO-K1 cells. The large telomere sequences were easily seen at the terminal regions of the human chromosomes and internalised telomeres observed on CHO-K1 chromosomes. This was also reported by (Meyne *et al.*, 1990). The hybridised pMTK4 sequence however, was not observed. The total size of the cDNA insert was 1.16kb which is close to the limit of resolution for the FISH procedure. It is possible that the techniques employed to detect the hybridised pMTK4 probe were not precise enough to resolve such a small sequence. Dr Judy Fantes of the Western General Hospital, Edinburgh repeated the experiment using DAPI stain as an alternative to the propidium iodide counterstain. The hybridised pMtk4 clone was still not observed. It may therefore be necessary to obtain a whole gene probe of several kb to locate the *tk* gene and map it on to the CHO-K1 chromosomes.

Chapter 7

Discussion and Future Work

Education is an admirable thing, but it is well to remember from time to time that nothing worth knowing can actually be taught.

-Oscar Wilde

The aims of this thesis was to study the effects of ionising radiation and restriction endonucleases (RE) upon the mutation frequency and the surviving fraction of the different Chinese hamster cell lines and to isolate and analyse the DNA of isolated mutants using PCR deletion screening. The predominant radiation induced DNA lesion of interest was the double stranded DNA break (dsb). However, radiation produces numerous different types of DNA damage, this makes it difficult to attribute any particular primary DNA lesion to any particular lethal or mutagenic event. RE are bacterial endonucleases which bind to and cleave specific DNA sequences to generate dsbs. This therefore makes RE the ideal agent with which to study the mutagenic and lethal effects of the dsb. RE were introduced into the cells using SLO as the method of cell poration and the effects of the RE on the surviving fraction and the mutation frequency at the non essential *tk* and the *hprt* loci were measured. The dsb is believed to be responsible for the lethal effects of gamma radiation and is also believed to be involved in the formation of chromosomal and chromatid aberrations (Natarajan and Obe, 1984; Winegar *et al.*, 1989; Bryant 1984; 1988; 1992). It would therefore not be unreasonable to expect that the dsb had a role to play in the molecular events leading to the formation of deletion mutations.

Xrs-5, a dsb repair deficient cell line was shown to be substantially more sensitive to the lethal effects of gamma radiation than the parental CHO-K1 line from which it was initially derived (Jeggo and Kemp, 1983; Costa, 1990; Shadley *et al.*, 1991; Singh, 1991; Singh and Bryant, 1991). This observation therefore emphasises the importance of the dsb in the induction of lethal events in the cell. In work reported here, the male fibroblast cell line, V79-4 was more resistant to the lethal effects of gamma radiation than either the *xrs-5* cells or the CHO-K1 cells. The

effect of blunt- and cohesive- ended dsb on the cell surviving fraction was studied by introducing *Pvu II* and *EcoRI* into the cells. It is thought that it is the blunt ended dsb which is responsible for the lethal cellular effects of gamma radiation and the production of chromatid and chromosomal aberrations (Winegar *et al.*, 1989; Bryant, 1984; 1988; 1992). In the V79-4 cells however *EcoRI* (producing cohesive end dsb) was more effective at inducing cell death per unit concentration than *Pvu II* (blunt end producing). In CHO-K1 cells a decrease in the surviving fraction per unit concentration of *Pvu II* was observed however, when the cells were treated with *EcoRI* there appeared to be no correlation between the RE concentration and the surviving fraction. There was only a small difference in the surviving fractions between the CHO-K1 and the *xrs-5* cells when treated with equal amounts of *Pvu II*. This was initially a surprising result, especially when one considers that there was such a large difference in the surviving fractions observed between the two cell lines when they were treated with equal doses of gamma radiation. It must however, be remembered that there are many different types of dsbs. The dsb can either be described as clean or dirty. RE can only produce clean dsb breaks which require no modification prior to DNA ligation, in contrast radiation produces a mixture of clean and dirty dsb termini. Costa (1990) observed that the fraction of DNA eluted following neutral filter elution was greater in the *xrs-5* cells than in the CHO-K1 cells at incubation times of between 2 and 5 hours after treatment with *Pvu II*. This suggests that there were more unrejoined dsb in the *xrs-5* cells than in the CHO-K1 cells at the incubation times studied. Following such an observation one might expect a lower surviving fraction in the *xrs-5* cells treated with *Pvu II* than in the parental CHO-K1 cells when treated with equal concentrations. What however must be noted is that Costa (1990) treated cells with 200 units of *Pvu II* per ml where as in the series of experiments described in this thesis, the cells were treated with *Pvu II* concentrations of between 0 and 9 units/ml. At such low concentrations (which were necessary for the mutation assay) differences between the surviving fractions of the radiosensitive *xrs-5* cells and the parental CHO-K1 cells may not be apparent. It has been shown that the *xrs-5* cells have a different nuclear organisation than the CHO-K1 cells (Yasui *et al.*, 1991). The organisation of the chromatin within the nucleus may effect the sensitivity of the genomic DNA to radiation damage. It is possible that the chromatin of the *xrs-5* is more sensitive to dsb induction than the CHO-K1 cells when treated with radiation but, is less sensitive to the endonucleolytic activity of the RE. The DNA unwinding technique suggested that there was no difference in the frequency of induced dsb per unit dose of X rays between the *xrs-5* cells and the parental CHO-K1 cells (Costa and Bryant, 1988; Costa, 1990).

The radiation-induced mutation frequencies at the *tk* locus in CHO-K1 and *xrs-5* cells and at the *hprt* locus in CHO-K1 cells and V79-4 cells were studied. Radiation was shown to be effective at inducing *tk* mutations in both the CHO-K1 cells and in *xrs-5* cells. The data showed that the induced mutation frequency was greater per unit radiation dose in *xrs-5* cells than in the CHO-K1 cells. This supported the data obtained by Mussa *et al.* (1990) and Singh (1991). The

frequency of radiation induced *hprt*⁻ mutations in the V79-4 cells was approximately the same as the frequency of induced *tk*⁻ mutations observed in the CHO-K1 cells at the same radiation doses. Mutation induction studies were not performed at the *hprt* locus in the *xrs-5* cells. This was because the *xrs-5* cells maintained in this laboratory were shown to be *hprt*⁻ in phenotype. Further analyses showed that this phenotype was the result of a deletion mutation which spanned at least one of the PCR primer attachment sites of exons 7 & 8 of the *hprt* gene.

The effects of cohesive- and blunt- ended dsb on the induction of mutations was studied by SLO porating cells and introducing the restriction enzymes *Pvu II* and *EcoRI*. *Pvu II* was shown to be effective at inducing mutations at the *tk* locus in CHO-K1 cells and *xrs-5* cells. In contrast to the observations with radiation treatment the dsb repair deficient *xrs-5* cells were shown to be hypomutable at the *tk* locus when treated with *Pvu II* as compared with the parental CHO-K1 cells. The breaks induced by *Pvu II* are clean hence, although the repair kinetics suggest that repair of *Pvu II* induced dsb is slower in *xrs-5* than in parental CHO-K1 cells (Costa, 1990), the data here suggests that there is no reduction in the fidelity of the dsb rejoining.

EcoRI, unlike *Pvu II* was ineffective at inducing mutations at the *tk* locus in the CHO-K1 cells. This was also observed by Singh (1991). However, this is not believed to be due to the frequency of RE sites. *EcoRI* actually has more restriction sites within the *tk* gene than the *Pvu II* (Lewis, 1986). However the importance of the location of the sites at which the enzymes cut is not fully known. It is also possible that the internal environment of the CHO-K1 cells is not conducive for *EcoRI* activity, and that *EcoRI* is ineffective at producing dsbs *in vivo*. Costa (1990) measured the frequency of dsb in CHO-K1 cells between 2 and 12 hours following cell poration with *EcoRI* and *Pvu II*. The fraction of DNA eluted was significantly higher in the *Pvu II* treated cells than in the *EcoRI* treated cells. The amount DNA eluted increased with increasing incubation time in the *Pvu II* treated sample. The amount of DNA eluted remained at approximately the same low level with increasing time following *EcoRI* treatment. It is unclear whether the consistently low level observed with *EcoRI* was due to the rapid repair of cohesive-ended dsb or due to the ineffectiveness of *EcoRI* at inducing dsb in the CHO-K1 genome. Ara A is an analogue of dATP and is an inhibitor of dsb repair. It would be interesting to study the effects of *EcoRI* on the fraction of DNA eluted in Ara A treated CHO-K1 cells. This may provide more information on the activity of *EcoRI* *in vivo* in CHO-K1 cells; a suggestion for future work.

EcoRI was equally effective at inducing *hprt*⁻ mutations in V79-4 cells as *Pvu II*. This observation emphasises the fact that the relationship between the RE induced dsb and the mutation frequency is more complicated than was first believed. The effect of a RE on the mutation frequency at a particular locus is not wholly determined by the type of dsb it

produces. There may be many other factors, some of which may interact with each other, which determine the mutagenicity of RE. These factors may be for example, the longevity of the enzyme when introduced in to the cell (Costa *et al.*, 1993), the frequency at which the enzyme cuts (Bryant, 1984), the specific location within the gene of interest at which the RE cuts and also the internal environment of the cell, which may be intrinsically different for different cell lines may also be determining factors.

Hprt⁻ mutant cells were isolated from populations of V79-4 cells. The *hprt*⁻ mutants were induced either spontaneously, by 4Gy gamma irradiation, by SLO or induced by *Pvu II* using SLO as the method of cell poration and the DNA changes at the *hprt* locus analysed by PCR deletion screening. A number of *Pvu II* induced V79-4 mutant cells from the work of Singh (1991), using electroporation as the method of cell poration, were also analysed by PCR deletion screening.

The results of the PCR deletion screening of DNA of the spontaneously induced *hprt*⁻ mutants indicated that there were no observable deletions within the exon coding regions. This supports the hypothesis that spontaneous mutations are predominantly the result of small scale genetic changes which are not often observed by PCR or Southern blotting. Southern blotting experiments were not performed, therefore one cannot exclude the possibility that there were deletions within the intronic regions. PCR sequencing may reveal hot spots for base pair substitutions; this is a recommendation for future work.

Approximately one third of the 4Gy radiation induced *hprt*⁻ mutations were the result of deletions which could be identified by PCR deletion screening as loss of exon DNA sequence. This is in contrast to the 60-70% deletion frequency found by Thacker (1986). The number of radiation induced deletion mutations was too small for any non-random distribution of exon loss at the *hprt* gene to be observed.

SLO, the bacterial cytotoxin used in cell poration was shown to induce small numbers of mutations. Evidence provided in this thesis suggests that the SLO provided by the manufacturer contained contaminating endonucleases and exonucleases which were perhaps responsible for a large proportion of the SLO induced deletion mutations. Despite the fact that there was only a limited number of SLO induced deletion mutations isolated, the distribution of exon loss did not appear to be random. Exons 7 & 8 appeared to be hot spots for PCR amplification product loss. The nucleotide sequences at which the endonuclease which contaminated the SLO cuts the DNA are not known. It would be useful to isolate the endonuclease(s) and using DNA substrates of known nucleotide sequence identify the nucleotide sequence at which the enzyme(s) cleave the DNA. From this data it might be possible to see whether or not there is a relationship between exon loss and the nucleotide

sequence at which the contaminating enzyme cuts. The loss PCR amplification products from the *Pvu II* induced mutants did not appear to be random (in mutants where *Pvu II* was introduced into the cell by either SLO or electroporation). The PCR amplification products of exon 2 were more frequently lost than any other exons. The information obtained from the EMBL data base indicated that *Pvu II* cuts in the intron sequence adjacent to the nucleotide sequence of exon 2. There were no *Pvu II* sites in any of the other exons or in any of the intron-exon borders that were sequenced by Rossiter *et al.*, (1991). This therefore provides evidence for the importance of the blunt ended dsb in the production of mutations. It also supports the data of Philips and Morgan (1994) who found that the majority of the deletion and insertion mutations induced at the *aprt* locus following cell poration with RE were at the site at which the RE cleaved the DNA sequence. It is not known whether or where the *Pvu II* cuts within the bulk of the intronic *hprt* sequences but, it would be interesting to know whether the cleavage sites in the intronic regions of the *hprt* gene are related to the distribution of the remaining deletion mutations.

The *thymidine kinase* gene is located autosomally and it is known from mutation studies that in CHO-K1 cells the *tk* gene is either in a heterozygous or a hemizygous state. Attempts to locate the Chinese hamster *tk* gene(s) in the CHO-K1 cells using FISH and a mouse *tk* cDNA plasmid probe were unsuccessful. It was not possible to obtain a full length Chinese hamster probe. Perhaps when such a probe is commercially available these experiments may be more successfully performed. If the location and the number of the Chinese hamster *thymidine kinase* genes was determined, the reason for the anomalously high spontaneous mutation frequency would perhaps be better understood. It would also enable more accurate and useful information about the molecular nature of *tk* mutations to be obtained by Southern analysis and possibly PCR. It would be of interest to compare the mutational spectrum at the *tk* locus of the CHO-K1 and radiosensitive daughter cell line *xrs-5* to see if they have similar proportions of different classes of mutations. It would also be interesting to see the types of mutations which are generated at the *tk* locus by treatment with radiation and restriction enzymes and if there is a differences in the molecular spectrum of mutations in the CHO-K1 and the *xrs-5* cell lines in response to treatment with different mutagens. A comparison could also be made between the molecular spectrum of the *hprt* gene and the *tk* gene in CHO-K1 cells and this would perhaps give a better indication of whether the spectrum in autosomal and X linked genes are similar and perhaps facilitate the understanding of the constraints which govern mutation formation and their stability and maintenance in the cell population.

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