Molecular and Cellular Damage Induced by Radiation and Restriction Endonucleases on C3H10T1/2 Cells

J. Ian Drummond, B.Sc.

Master of Science
School of Biological and Medical Sciences
The University of St. Andrews
May, 1995

Declaration

(i) I, John Ian Drummond, hereby certify that this thesis, which is approximately 15,000 words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree

date 17/5/95 signature of candidate

(ii) I was admitted as a research student under Ordinance No. 12 in October 1990 and as a candidate for the degree of Master of Science in October 1990; the higher study for which this is a record was carried out in the University of St. Andrews between 1990 and 1992.

date 17/5/95 signature of candidate

(iii) I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate for the degree of Master of Science in the University of St. Andrews and that the candidate is qualified to submit this thesis in application for that degree.

date 17/5/95 signature of supervisor

In submitting this thesis to the University of St. Andrews I understand that I am giving permission for it to be made available for use in accordance with the regulations of the University Library for the time being in force, subject to any copyright vested in the work not being affected thereby. I also understand that the title and abstract will be published, and that a copy of the work may be made and supplied to any bona fide library or research worker.

date 17/5/95 signature of candidate

INDEX

Acknowledgements

I would like to express my thanks and gratitude to the following people for their help and advice during the course of this work; Dr A. C. Riches, Dr P. E. Bryant, Tina Briscoe, Jean Melville, John Macintyre and Dr Simon Robinson.

ABSTRACT

A more comprehensive understanding of the initial events in radiation induced carcinogenesis may be obtained by studying the action of restriction enzymes used to mimic double strand break (dsb) damage caused to DNA by radiation. Comparative studies of ionising radiation and enzyme induced chromosomal lesions were carried out on C3H10T1/2 cells using micronuclei and cell survival techniques. Results indicate that certain restriction enzymes induce damage comparable to that produced by ionising radiation damage. One of these is the enzyme Pvu II. A dose effect curve was obtained following treatment of cells with 2.5,10,20 and 30 units of Pvu II. This enzyme cuts DNA leaving dsb with blunt ends. An increase in the number of micronuclei was observed following treatment with Pvu II and a decrease in clonogenic cell survival with increasing dose. The restriction enzyme EcoR I cuts the DNA leaving cohesive ended dsb which are thought to be more readily repaired. In this case there was no

effect of treating cells with 10,20,30 and 40 units EcoR I and micronuclei formation and cell survival were comparable with controls.

Consistent results were obtained at a molecular level using the filter elution technique to assess the damage induced by the two restriction enzymes.

The cellular and molecular study was a preliminary to an investigation into the effect of these two enzymes on cell transformation in the C3H10T1/2 system. Comparative studies with Pvu II and EcoR I indicated that damage caused by Pvu II is more effective at producing a transformed phenotype. Thus it seems that blunt-ended double strand breaks induced by Pvu II may be a key event in carcinogenesis.

INTRODUCTION

It has been known for many years that cellular transformation can be produced in mammals by a variety of physical and chemical agents. The investigation of transformation at a cellular level has been made possible by the developement of cell culture systems which are used to assay for transforming agents. These cell culture systems make it possible to study cellular and molecular mechanisms involved in transformation under defined conditions.

At a molecular level it is known that there is a relationship between the DNA double strand break (dsb) and the end point of chromosomal aberrations. It is also known that radiation induced dsb consists of two types of dsb: cohesive ended dsb's and blunt ended dsb's. Restriction endonucleases are enzymes which specifically cut DNA leaving either cohesive ended dsb's or blunt ended dsb's. Restriction enzymes may be used to identify the primary lesion which ultimately leads to oncogenic transformation.

The aim of this project is to compare radiation induced damage

to that caused by restriction enzymes and to elucidate whether the cohesive ended dsb or blunt ended dsb is the primary lesion involved in producing molecular and cellular damage and ultimately the transformed phenotype.

Ionising Radiation

Ionising radiation has been known to cause a variety of biological damage in cells. This damage is expressed in a number of measurable end points; cell killing, mutation, chromosomal aberrations and oncogenic transformation.

There is evidence that the damage to a cell is due to the absorption of energy within or close to the cell's DNA. Two types of formation of DNA damage are predominant; direct and indirect. The direct effect is due to ionisation or electronic excitation of the DNA, and the indirect effect is caused by reactive species, free radicals, which are produced in the vicinity of the DNA. These radicals may be primarily produced by energy absorption in water (the solvated electron, the H-atom and the OH radical) or organic radicals produced from organic material other than DNA either by interaction with radiation or by reaction with the primary radicals generated from water. This absorption of energy

leads to the occurrence of several types of DNA lesions. At a molecular level these lesions arise from different types of DNA damage; single strand breaks (ssb), double strand breaks (dsb), base damage, DNA-DNA crosslinks and DNA-protein crosslinks. It has been proposed that the biological effects of this molecular damage is a failure of the DNA repair systems or a misrepair of the damage (Elkind, Hill & Han, 1985). This repair or misrepair may lead to cell death or transformation of the cell.

Transformation of mammalian cells is the phenomenon whereby normal growth regulated cells are separated from their regulation and grow without normal cellular growth control mechanisms and produce tumours in suitable animals when inoculated. It is understood that oncogenic transformation is a multistage event arising from the accumulation of several genetic mutations in a single cell (Barrett and & Fletcher 1987) involving at least an initiation event such as ionising radiation and a promotion event such as genomic change. Molecular models for transformation of mammalian cells have been proposed; it is considered that an oncogene is activated and the cell is switched from its normal phenotype which is carefully regulated into the transformed phenotype which shows little regulatory control

(Bishop, 1987). The Tumour Suppressor model indicates that a tumour suppressor gene (a gene involved in the negative control of the proliferation of cells) is signalled to tell the cell to move from proliferation to differentiation. Subsequent loss of these genes would allow the activity of the oncogene to predominate and again cause a transformed phenotype. Several groups found that the fusion of malignant cells with normal cells led to the suppression of malignancy (Klein, 1987; Stanbridge et al., 1982). The retinoblastoma susceptibility gene RB is the prototype tumour suppressor gene and has been shown to suppress the transformed phenotype for several different cancers (Levine, 1990)

It has been possible to obtain dose-effect curves for the induction of transformed foci against radiation dose. The mechanisms of induction are unclear because of the variety of lesions caused by radiation. The DNA dsb has been implicated as the causative lesion of cell transformation (Frankenberg et al., 1984; Natararajan et al., 1980). Also it has been shown that treatment of mammalian cells with restriction enzymes induce dsb in DNA at specific recognition sequences which leads to chromosomal abberations and cellular transformation (Bryant,

1984; Bryant & Riches, 1989).

DNA Strand Breaks

A single strand break (ssb) is a lesion whereby one strand of the DNA duplex is broken leaving fragments of the disrupted deoxyribose. These have to be removed by exonucleases before the ssb can be repaired. Known mechanisms for induction of ssb's are free radical and enzyme mechanisms.

A double strand break (dsb) is a lesion that allows a double helical DNA strand to divide into two double helical DNA strands. This may be two ssb's directly opposite or separated by several bases and staggered. It may be possible that dsb's can be repaired by mechanisms which repair ssb's suggested by the fact that X-ray induced dsb and ssb were repaired at similar rates (Bradley & Kohn 1979). However dsb and ssb assays measure different things as shown by the fact that the ratio of dsb's to ssb's varied for different agents (Kohn, 1986). The most damaging of these lesions is the dsb since if it is not correctly repaired, it can lead to extensive loss or rearrangement of genetic material. Experiments have shown that the rad 52 mutant of Saccharomyces cerevisae (dsb repair negative) is extremely

sensitive such that 1-2 dsb's produce a lethal event (Ho, 1975).

Restriction Endonucleases.

Experiments in which restriction enzymes were used to generate dsb's showed that the dsb is the lesion leading to chromosomal aberrations in irradiated cells (Bryant, 1984; Natarajan & Obe, 1984; Bryant, 1988).

Type II restriction endonucleases are bacterial enzymes which serve to degrade foreign DNA entering the bacterial cell. The host DNA is not degraded as the DNA recognition sequences are methylated and are protected from excision by the restriction enzymes. Restriction enzymes recognise specific base sequences in double helical DNA and cleave both strands of the duplex at the phosphodiester bonds generating double strand breaks (dsb). Restriction enzymes usually recognise sites at a specific 4-8 base sequence which have an axis of rotational symmetry. Cleavage of the DNA may induce blunt ended dsb which have no base overlap or cohesive ended dsb which have overlapping bases. The type of dsb end structures, whether blunt or cohesive ended, is a property of the restriction enzyme and the end structures produced by these restriction enzymes are said to be "clean"

unlike radiation. Radiation produces a variety of end structures that require excision and repair mechanisms to prepare the DNA to be successfully rejoined. This phenomena can be exploited to study the variety of dsb induced by radiation. X-rays induce a mixture of blunt and cohesive end structures, however there is a preponderance of cohesive ended dsb. Also X-ray induced dsb will have end structures which have not been specifically excised at the phosphodiester bond ("dirty" end structures) and will require enzymatic cleaning before rejoining can take place.

Within the DNA there are potentially a very large number of recognition sites where an enzyme could cut the DNA. The cutting frequency of a restriction enzyme is a function of that particular enzyme and varies considerably. Figure (1) shows the cutting frequencies of several enzymes which have been calculated (Bishop et al. 1983; Bryant, 1988). The theoretical number, assuming a random distribution of the 4 bases, would show a cutting site every 256 bases for a four-base recognition site and a cutting site of once every 4096 base pairs for a six-base recognition site (Bishop et al., 1983). Frequencies may be modified by: non-random distribution of bases, methylation pattern of the bases, and packaging of the DNA.

The restriction enzyme Pvu II was initially chosen in this study since previous studies (Bryant, 1984; Bryant, 1985) have shown that this enzyme causes high levels of chromosomal aberrations and cell killing in Chinese Hamster Ovary CHO cells. The aberrations were found to increase linearly with increasing dose of Pvu II. However this result was not obtained for EcoR I which gave few chromosomal aberrations in comparison. This result was explained by the differences in dsb end structures. Pvu II produced 'blunt end' dsb's and was less readily repaired whereas EcoR I produced 'cohesive ended' dsb's which were more readily repaired.

Figure 1

Restriction enzymes producing different types of dsb

Blunt ended dsb

Pvu II			Cutting Frequency
CAGCTG	CAG	CTG	1/3200 base pairs
GTCGAC	GTC	GAC	

EcoR V			
GATATC	GAT	ATC	1/5000 base pairs
CTATAG	CTA	TAG	

Cohesive ended dsb

EcoR I			
GAATTC	G	AATTC	1/3000 base pairs
CTTAAG	CTTAA	G	

BamH I			
GGATCC	G	GATCC	1/5500 base pairs
CCTAGG	CCTAG	G	

Micronucleus Assay

DNA damage can be monitored at a molecular level using techniques such as the micronucleus assay and the filter elution assay. At a cellular level this damage can be identified using cell survival and transformation assays.

The micronucleus technique is regarded as a quick and easy screening method for the presence of chromosomal damage in mammalian cells (Fenech and Morely, 1985). Originally this technique was used by Fenech & Morely as a method of measuring chromosomal damage in human lymphocytes. A requirement of this technique was that it was necessary for a cell to undergo a mitosis in order for a micronucleus to be expressed. However those cells that had only undergone one division could not be distinguished from the total population of lymphocytes. This problem was investigated and two methods were used. A radiographic method in which the lymphocytes were pulse labelled with tritiated Thymidine: only the cells that had undergone mitosis would be labelled and scored. In the second method cytokinesis was blocked using Cytochalasin B and

micronuclei were scored in cytokinesis blocked cells which were recognisable by their binucleate appearance. A linear relationship between dose of radiation and number of induced micronuclei was similar for both techniques (Fenech & Morely, 1985).

There have been discussions concerning the origin of micronuclei. It is now generally accepted that micronuclei arise in most cases from acentric fragments but there may also be contributions from lagging whole chromosomes which have not successfully divided and from different forms of nuclear disintegration (Savage, 1988a). However the observed frequency of micronuclei is often much lower than the frequency for acentric fragments seen at first post-treatment metaphase (Savage, 1988b).

Ionising radiation causes numerous aberrations within cells. Restriction enzymes only make one type of lesion in DNA; a double strand break (dsb) with either blunt or cohesive ends. Double strand breaks contain intact 5' phosphate and 3' hydroxyl and should be readily repaired by ligation. Yet high frequencies of both exchange type and deletions are observed. Restriction enzyme treated cells are known to generate chromosomal aberrations (Winegar 1988). The scoring of chromosomal

aberrations and micronuclei for measuring the extent of chromosomal damage by mutagens indicates that a simple qualitative or quantitive relationship does not exist between micronuclei and chromosomal aberrations and although the micronucleus assay is a rapid screening method for mutagens it is not a substitute for scoring chromosomal aberrations.

Exchange type aberrations are believed to result from the misjoining of two dsb's either within or between chromosomes. Deletion type aberrations may be un-rejoined single chromosome fragments or composites of several acentric fragments. It is clear that DNA repair processes either through misrepair or incomplete repair play a role in the formation of chromosome aberrations hence micronuclei. This mis-repair or incomplete repair is thought to lead to oncogenic transformation (Klein & Klein, 1984)

A limitation in quantifying the results of damage caused by restriction enzymes has been previously a problem due to the methods used to introduce the restriction enzyme into a high proportion of the sample population without causing immediate cell death.

Cell Permeabilisation

Various methods have been used for the permeabilisation of the cell membrane to allow the entry of restriction enzymes. Originally the introduction of the T4 Endonuclease into cells was performed by treating the cells with Sendai virus (Tanaka, 1975). Other methods have included hypertonic shock induced by storage buffers (Obe & Winkel, 1985; Bryant & Christie, 1989), and lysis of pinocytic vesicles (Winegar & Preston, 1988). Poration of cells by electrical charge has been successfully used to introduce restriction enzymes into cells (Winegar & Philips, 1989). This poration technique has been characterised for CHO cells (Moses, 1990). The optimum Pvu II concentration was used to assay micronuclei in the CHO cell line. The number of micronuclei in Pvu II treated cells were found to increase with increasing enzyme dose whereas EcoR I treated cells showed little response to increasing enzyme dose. However with this method of introduction there was a high proportion of cell killing by the electroporation treatment itself (Moses, 1990).

Streptolysin-O-Tetanus toxin, produced by the bacterium Streptococcus pyogenes, was shown to be effective in permeabilising the plasma membrane. Pores were of a sufficient

size to allow macromolecules to enter Bovine Medullary Chromaffin cells (Gudrun et al. 1989). Recent work has shown that Streptolysin-O is efficient in allowing the entry of restriction enzymes into Chinese Hamster Ovary cells (Bryant, 1992).

Filter Elution Assay

It has been shown that DNA dsb's can be detected by the neutral filter elution technique (Bradley & Kohn, 1979) and has been used widely to measure the induction and repair in irradiated cells (Weibezahn & Coquerelle, 1981; Radford, 1985). This technique works on the principle that DNA from treated cells is released onto filters and cut DNA is eluted from the filters. The relative difference between the DNA collected and the DNA remaining on the filters gives an indication of the extent of DNA dsb's.

Restriction enzyme damage can also be detected by this method (Costa & Bryant, 1990, 1991). It was found that Pvu II which generates blunt ended dsb's was successful in producing a measurable amount of dsb's using this technique. The frequency of these dsb's increased over a 3-12 hours post treatment incubation period and continued to increase up to 24 hours where

it reached a plateau. From this it was evident that Pvu II was active within the cell for a considerable length of time. EcoR I and BamH I did not give measurable levels of dsb's.

It was postulated that restriction enzymes like EcoR I and BamH I caused dsb at a rate slower than the rate of repair of the DNA by cellular processes (Costa & Bryant 1990, 1991). Hence, few dsb's were observed, and Pvu II induced dsb due to its blunt end structure would be repaired at a slower rate causing the accumulation of dsb. This work highlighted an important difference between enzyme generated dsb and those induced by radiation in that the duration of enzymatic activity within the cell was far greater than the duration of radiation.

Cell Survival

The major cause of cell killing by ionising radiation is the formation of chromosomal fragments which correlates with the appearance of micronuclei post-mitosis (Joshi et al. 1982), and other types of chromosomal aberrations (i.e. exchanges) are also lethal for a cell (Carrano 1973). Cell killing after exposure to ionising radiation has been attributed to unrepaired or misrepaired DNA dsb's (Blocher & Pohlit, 1982; Frankenberg et al.

1984). It is believed that ionising radiation produces dsb's because of the clustering of radicals along the ionisation path which favours multiple radical damage in small regions. Restriction enzymes Pvu II and BamH I were used to produce dsb in inactivated Sendai virus permeabilised Chinese Hamster V79 cells. Pvu II which produces blunt ended dsb's caused a dose-dependant decrease in clonogenic survival whereas BamH I which produces 'cohesive ended' double strand breaks did not effect clonogenic survival (Bryant 1985).

Transformation

Radiation or chemically induced malignant transformation in culture is defined as the ability of cells transformed in cultures to induce tumours in suitable animals. This had been reported in cells derived from the hamster, mouse and rat. Quantification of a transformation system using primary and secondary hamster cells was described (Berwald & Sachs, 1965). Chen and Heidelberger established a cultured line of fibroblasts derived from ventral prostates of an inbred genetically standardised C3H mouse. The C3H10T1/2 cell line was established from C3H mice (Reznikoff et. al., 1973).

Chromosomal exchanges and deletions could be induced by X-rays and following treatment with restriction enzymes (Natarajan & Obe, 1984; Bryant, 1984). These visible chromosome alterations are often associated with cancers in man and other mammals (Klein & Klein, 1984). When comparing data obtained from other cell lines with C3H10T1/2 it is important that the plating (colony forming) efficiency in the C3H10T1/2 system should optimally be 30% and not less than 20% at a seeding density of 200 cells/dish after ten days of growth as a strong effect of cell density on expression of transformed phenotype has been demonstrated (Little, 1977. When designing experiments the high dose should be selected to give 80 to 90% toxicity and the low dose should show no or minimal toxicity. It is critically important to standardise cell transformation assays as they are technically more difficult than most other in vitro screening tests.

Transformation of the mouse embryo C3H10T1/2 cells from normal cells which form a confluent monolayer into colonies of cells with uncontrolled growth formed over the confluent monolayer, are called foci. Transformation of C3H10T1/2 cells gives a quantification of chromosome damaging (carcinogenic) agents. The experimental protocol for the transformation assay of

C3H10T1/2 cells has been standardised over years of research; exponentially growing cells are plated into plastic petri dishes 18-24 hours before treatment at a density such that 300-400 viable cells will survive the treatment. The cells are incubated for a total of six weeks, fixed, stained and scored for foci (Hall et. al., 1985). A focus is defined as an area of increased cell density and/or altered cell morphology in a confluent monolayer. Transformed cells can be scored quantitively, using morphological criteria outlined by Reznikoff (Reznikoff, 1973). Three types of foci have been identified: Type I is a focus of tightly packed cells; Type II shows piling up into opaque multilayers with minimal criss-crossing; type III foci are the most aggressive with highly multilayered criss-crossed arrays of densely stained cells. Type III and Type II foci have been shown to yield malignant tumours in syngeneic mice after subcutaneous inoculation of a sufficient number of cells (Reznikoff, 1973).

Types Of Physical Transformants

The C3H10T1/2 transformation system has been used by many researchers for monitoring the induction of transformation by ionising radiation. It was found that X-irradiation produced malignant transformation of the C3H10T1/2 cell line (Terzaghi & Little, 1976). Densely ionising radiation such as fission-spectrum neutrons is also a potent transforming agent (Han & Elkind, 1979). Non-ionising radiation like ultraviolet is also effective in transforming C3H10T1/2 cells (Paquette & Little 1992).

The dose response curves for a variety of physical and chemical carcinogens show a similar shape. They are characterised by a rapid increase of transformants with increasing dose in the lower dose regions. This is followed by a plateau at higher doses where the yield of transformants reaches a limiting value. The shapes of the dose response curves are similar for X-rays and fission spectrum neutrons but differ significantly at the transformation frequency at which the plateau is reached. The plateau for neutron exposure is 2-3 times greater than that for X-Rays. Transformation frequency with X-rays is generally found to be within a certain range from spontaneous level of 1.1×10^{-5} to a maximum of approximately

1.2 X 10^{-3} within a dose range of 0-6 Gy

Factors Affecting Transformation

A number of factors have been found to influence cell transformation:

Cell Cycle Kinetics

It has been suggested that transformation depends upon kinetic status of the cells. It was found that 1-2 days were required for cells to recover from perturbations induced by suspension, dilution and replating of cells (Han et al., 1984). S-phase was found to show a high frequency of transformation when treated with chemicals (Bertram & Heidelberger, 1974).

Seeding Density

Reznikoff (1973) suggested that the more opportunity that cells have to divide (lower the seeding density) before confluence the greater the chance of transformation and a minimum of approximately 12 cell divisions (inoculum of 300 cells) was required so that transformation frequency was independent of seeding density (Terzaghi & Little, 1976; Elkind & Hill, 1985).

However Little found that calculated transformation frequency was highly dependent on the initial number of viable cells seeded when the number of viable cells seeded were less than 100 (Little, 1977). Some researchers argue that this parameter is meaningless in mechanistic terms and misleading to represent transformation frequency dependent on the initial number of cells seeded (Kennedy et al., 1980).

These studies showed that transformation frequency decreases linearly from 10^{-1}-10^{-5} as seeding density increased from 1-10,000. When Hill & Elkind examined seeding density and added TPA (12-o-tetradecanoylphorbol-13-acetate) they obtained a result resembling that of Kennedy et.al., 1980. TPA was reported to interfere with cell-cell communication (Thomson et.al., 1981). Han & Elkind suggested that the result obtained by Kennedy was perhaps due to a TPA type substance in the medium used. They concluded that a minimum of an average of approximately 12 divisions (300 viable cells) was required to yield a transformation frequency independent of seeding density. Twelve divisions was also the number for the expression of a maximum transformation frequency in the studies of Mordan et. al. (1983). It is widely accepted that an inoculum of 300 viable

cells (expressed as cells/cm^2) is required to yield a transformation frequency independent of seeding density.

Repair of subeffective and sub-lethal transformation damage

Fractionating the dose of radiation into smaller doses separated by appropriate time intervals and reducing the dose rate were both found to decrease the transformation frequency and increase cell survival. It was suggested that intra-cellular repair processes were involved in reducing the frequency of transformation and increasing cell survival (Han et al., 1979). Kennedy et. al. (1980) proposed a two event process for induction of transformation by ionising radiation. The first due to radiation is frequent and is registered in a large fraction of the surviving cells. The second is very infrequent and occurs randomly between the time of exposure and the time it takes single cells to grow into a confluent population. This process was modified by Elkind et. al. 1985; the first event is dependent on the quantity and quality of the radiation and thus the radiation damage may only be subeffective and modifiable by cellular processes during overall exposure. Transformation may occur if this damage is insufficiently repaired giving rise to chromosomal

rearrangements and a loss of growth control.

The advantages of the C3H10T1/2 transformation system have been found to outweigh the disadvantages. The C310T1/2 cell line is widely studied and documented, however it is no substitute for studying actual human cells. The C3H10T1/2 cell Line is an established cell line and can be grown in large quantities. Cells from the same cloned population can be used in different laboratories enhancing reproducibility of results; and most importantly transformed cells are easily distinguished from the non-transformed. In common with other assays this transformation system only tests for approximately 30% of the treated cell population.

Traditional studies of X-irradiation with this system were started in the mid 1970's (Terzaghi & Little 1976; Little 1977) and later pursued in a number of laboratories (Kennedy et al., 1980; Elkind & Hill et al., 1985). The process of transformation in the C3H10T1/2 cells is described as a multistage process, including initiation and promotion.

Transformation work using less flasks for each dose point was carried out by Kolman (Kolman et al., 1989). This method used 10

x 25cm^2 flasks per dose which was considerably less than the traditional studies which used a greater number of 75cm^2 flasks (greater than fifty) for each dose point. The results obtained were compatible with those observed in other studies.

Initial transformation work shows that restriction enzymes also transform C3H10T1/2 cells (Bryant & Riches, 1989). The transformation frequency of Pvu II treated cells was found to increase as the dose of restriction enzyme was increased.

In vitro transformation with the C3H10T1/2 cell system has answered many quantitative and mechanistic questions in cellular transformation. There is evidence that transformation is a multiple step or at least a two step process. The first event is frequent, and involves a large fraction of the treated cells, causing the production of DNA damage. The second event is rare (10^{-6} or less among initiated cells) and is the response of the cells to the non-specific DNA damage caused, which results in some change in the pattern of gene expression (Kennedy et al., 1984). The first event increases the probability that the 2nd rare random event (malignant transformation) will occur during the growth of the descendents of the treated cells to confluence. This

event must be an unusual class of genetic event that is not driven by mutagens. The mechanism of cellular transformation has to some extent been elucidated in the rodent system. However because the C3H10T1/2 cell line is immortalised and has therefore undergone some of the changes associated with transformation it can still only be used as a model to mimic what actually happens within the Host system. Although the process of immortalisation occurs readily in rodent cells, it is rare in human diploid cells.

Human Transformation Systems

There has been a move to study the mechanisms of transformation in Man. Initial transformation studies of irradiated human fibroblasts have been difficult to establish (Borek, 1980. A human hybrid cell line (HeLa X skin fibroblast) designated CGLI, when treated with radiation, expressed a tumour associated antigen (Sutherland & Bicknell, 1986) which was detected by a monoclonal antibody (Bicknell & Sutherland, 1985). The foci of the transformed CGLI cells could then be detected by immunoperoxidase staining of the antigen-antibody complex. This system has been used to study transformation

from a preneoplastic to a neoplastic state by radiation (Redpath & Sun, 1987) and a dose response relationship using gamma radiation has been established. The dose response was similar to that obtained for C3H10T1/2 cells, however the transformation frequency was found to be a factor of 5-10 fold lower for CGL1 cells (Sun & Redpath, 1988). Later studies, using a different staining method, show that transformation frequency for both is now similar (Redpath et. al. 1995).

This study attempts to: (i) correlate the variety of lesions caused by ionising radiation with the dsb produced by restriction enzymes (ii) compare their relative ability in producing molecular damage (iii) elucidate how this molecular damage translates into cellular damage and oncogenic transformation and (iv) investigate whether the dsb is the primary cause of oncogenic transformation in cells. The mouse embryo C3H10T1/2 cell line is used in this study to bridge the molecular studies of dsb's with CHO cells and traditional transformation experiments with C3H10T1/2 cells to give a greater understanding of the transformation process at a molecular level.

MATERIALS AND METHODS.

Stock cultures of C3H10T1/2 passage number 10 were reconstituted from liquid nitrogen storage by quickly thawing the cryo-tubes in a water bath at 37°C and suspending in a 75cm^2 flask by slowly adding 10cm^3 Basal Medium Eagle (BME) supplemented with 10% heat inactivated foetal calf serum (FCS) The flask was then gassed with 5%CO_2 /95% air (British Oxygen Company) and incubated at 37°C.

.

Routine Maintenance of C3H10T1/2 Cells.

Cells were maintained as exponentially growing substrate attached monolayers in a 75cm^2 flask containing 10ml BMEFCS. These monolayers were maintained in a subconfluent state (<80% confluent) and not permitted to become confluent by passaging the cells at regular intervals.

Passaging C3H10T1\2 Cells.

Cells were detached from the flask by adding 3ml of trypsin (0.05% trypsin(Difco) in PBS) onto the monolayer. This was removed and another 3ml of trypsin added to the monolayer and again removed just leaving enough trypsin solution to form a film over the monolayer. The flask was then placed in a 37^0 C incubator for 6 minutes and examined under an inverted microscope to ensure that cells had detached sufficiently. Cells were counted and approximately $5x10^4$ cells were seeded in a fresh $75cm^2$ flask containing 10ml BMEFCS and gassed with 5% CO_2/95% air.

X-Irradiation of C3H10T1/2 Cells.

The cells were trypsinised and counted (Coulter Count ZM). A mean of three counts was taken for cell counts in all experiments. The cells were diluted in BMEFCS to give a cell density of $5x10^5$/ml this ensured that for each experiment the number and distribution of cells were identical. The cell suspension was aliquoted in 1ml amounts to conical based plastic centrifuge tubes (Sterilin) and irradiated at 0.75 Gy. min^{-1} in an X-ray set (Siemens) producing 250 kVp X-rays in air using a 0.5mm copper filter at room temperature.

Gamma Irradiation Of C3H10T1/2 Cells.

A similar procedure for X-irradiation was adopted for Gamma irradiation. Cells were irradiated 4.6 Gy. min^{-1} in a gamma irradiator (CIS IBL 437C Caesium 137) at room temperature.

Restriction Enzyme Purification and Treatment Of C3H10T1\2 Cells.

Restriction enzymes were purified using an Amicon 10 ultrafilter to remove storage buffer. The filter was prepared by adding 50µl calcium-free Hanks balanced salt solution (HBSS) containing 6mmol/l $MgCl_2$ and 1% bovine serum albumin (BSA). The restriction Enzyme was carefully layered onto the filter and 1ml of HBSS without BSA was added. The filter unit was centrifuged (Beckman) for 90min, 8000rpm, 4°C. At the end of the first run 1ml of HBSS without BSA was added to the filter and centrifuged 60min, 8000rpm, 4°C. After this step the filter unit was inverted and centrifuged for 10min, 1000rpm, 4°C to retrieve the enzyme. The enzyme was diluted to 1unit enzyme/µl HBSS containing 6 mM $MgCl_2$+1% Bovine Serum Albumin (BSA).

Cells were trypsinised from culture flasks, suspended with

10ml BMEFCS in a centrifuge tube and centrifuged for 10min, at 1000rpm. The cells were resuspended in HBSS. A cell suspension containing $5x10^5$ cells was added to the restriction enzyme, streptolysin-O (Wellcombe Diagnostics, UK made according to the manufacturers specification from dehydrated powder into a stock solution 1.81 Units/ml) and made up to a volume of 1ml with HBSS to form the reaction digest. The reaction was incubated for 5min at room temperature with continual agitation of the digest. The reaction was quenched by adding 10ml BMEFCS and centrifuged for 10min at 1000rpm. The medium was aspirated and the cell pellet resuspended and centrifuged as before. The cells were counted and diluted for plating.

Micronucleus Assay.

Treated cells were incubated at 37°C in the presence of 3µg/ml cytochalasin-B (Sigma). Following incubation cells were harvested by trypsinisation and approximately $2x10^4$ cells/0.5ml were spread onto a slide using a cytospin centrifuge (Shandon). The slide preparation was then fixed and stained (Merz+Dade Diff-Quik). The number of micronuclei per 100 binucleate cells was scored to give a reliable indication of cell damage.

Neutral Filter Elution(pH 9.6)

One day prior to experimental treatment, flasks containing exponentially growing cells were labelled with 3.7kBq/ml [methyl-3H]-thymidine. Even labelling of the DNA was obtained by adding 1μmole/l (final concentration) of cold thymidine.

The neutral filter elution apparatus was assembled prior to cell treatment and consisted of a Swinnex filter unit (Millipore Corporation) containing a polycarbonate filter (Nucleopore, 45mm diameter and 2μm pore size) premoistened with PBS. The Swinnex filter was connected to a 50ml syringe funnel and a peristaltic pump. Twenty millilitres of ice cold PBS was added to the syringe funnel and pumped through until flow could continue under gravity. The apparatus was clamped after 10ml flowed through. After treatment $5x10^5$ cells were loaded into 50ml syringe barrels containing the remaining 10ml PBS. The suspension was allowed to run through under gravity until the filter holders had emptied. They were then removed from the funnels and 1ml of lysis solution (0.025 mole/l Na_2 EDTA, 0.1 mole/l glycine, 0.068 mole/l N-laurylsarcosine Na salt) was pipetted into the Swinnex holders. The Swinnex holders were plugged and incubated at 60°C for 1 hour (Okayasu and Iliakis

1989).

After incubation the Swinnex filter holders were reconnected to the funnels and 40ml of eluting solution (0.02 mole/l EDTA and 0.06 mole/l tetrapropyl ammonium hydroxide) was poured into the funnels and the elution initiated. The pump flow rate was set at 4.2ml/hr. The first eluted fraction was collected over a period of one hour and not included in the results The fractions collected after this initial one hour were collected in pre-weighed flasks. A 5ml aliquot of this fraction was pipetted into scintillation vials and 10ml of Optiphase MP (LKB) added. The vials were thoroughly vortexed before counting. The polycarbonate filters were removed from the units and placed in plastic scintillation vials. Five millilitres of Filter Count (Packard) was added and the vial shaken ensuring that the filter was immersed in the Filter Count (Packard). Vials were counted in a scintillation counter using a quench correcting programme.

Survival Assay.

Exponentially growing cells (passage 11) were trypsinised, counted and diluted in BMEFCS to give a cell density of $5x10^5$/ml.

X-Irradiation

The cell suspension was aliquoted in 1ml amounts to conical based plastic centrifuge tubes (Sterilin) and irradiated at 0.75 Gy. min^{-1} in an X-ray set (Siemens) producing 250 KeV X-rays in air using a 0.5mm copper filter at room temperature. Treated cells were counted, diluted in BMEFCS and 150-3100 cells were plated into 100mm Petri dishes depending on the dose.

Restriction Enzymes

Cells were trypsinised from culture flasks, suspended with 10ml BMEFCS in a centrifuge tube and centrifuged for 10min, at 1000rpm. The cells were resuspended in HBSS. A cell suspension containing $5x10^5$ cells was added to the restriction enzyme, 0.271 units streptolysin-O and made up to a volume of 1ml with HBSS to form the reaction digest. The reaction was incubated for 5min at room temperature with continual agitation of the digest. The reaction was quenched by adding 10ml BMEFCS and centrifuged for 10min at 1000rpm. The medium was aspirated and the cell pellet resuspended and centrifuged as before. The cells were counted and diluted for plating and 925-2457 cells according to dose were plated in 100mm Petri dishes.

The dishes were incubated at 37°C in a CO_2 incubator for 10 days. After incubation the colonies were washed with PBS and fixed

with methanol. The colonies were then stained using a 10% Giemsa solution. Only colonies containing more than 50 cells were counted.

Transformation Assay

Exponentially growing cells (passage 11) were trypsinised, counted and diluted in BMEFCS to give a cell density of $5x10^5$/ml.

X-Irradiation

The cell suspension was aliquoted in 1ml amounts to conical based plastic centrifuge tubes (Sterilin) and irradiated at 0.75 Gy. min^{-1} in a X-ray set. Treated cells were counted, diluted in BMEFCS and 3000 cells in 10ml BMEFCS were plated into 25cm^2 flasks.

Restriction Enzymes

Cells (passage 11) were trypsinised from culture flasks, suspended with 10ml BMEFCS in a centrifuge tube and centrifuged for 10min, at 1000rpm. The cells were resuspended in HBSS. A cell suspension containing $5x10^5$ cells was added to the restriction enzyme, 0.271 units streptolysin-O, and made up to a volume of 1ml with HBSS to form the reaction digest. The reaction was incubated for 5min at room temperature with continual agitation of the digest. The reaction was quenched by adding 10ml BMEFCS and centrifuged for 10min at 1000rpm. The medium was aspirated and the cell pellet resuspended

and centrifuged as before. The cells were counted and 3600-4500 cells were diluted in 10ml BMEFCS and were plated in 25cm^2 flasks. The flasks were incubated at 37^{o}C in a CO_2 incubator and incubated for 6 weeks with weekly medium changes. After cells became confluent (approximately 3 weeks) the serum content in the BME was reduced to 5%. Medium was poured off and the cell layer rinsed with Sorenson's buffer and fixed in methanol for 10min. Cells were finally stained in 5% Giemsa for 3 hours, rinsed with buffer and allowed to dry.

Scoring of transformed foci was carried out according to the classification of Reznikoff (1973).

RESULTS

Optimisation of sampling time for micronuclei using X-rays (Graphs 1 & 2)

Experimental Procedure

Exponentially growing cells were trypsinised and exposed to X-irradiation over the range 0 - 6Gy. Samples for the micronucleus assay were taken after 24 and 48 hours of incubation with 3μg/ml Cytochalasin B and 100 binucleate cells were scored for micronuclei. A set of 3 experiments was conducted.

Results

This experiment shows that as the radiation dose is increased the number of micronuclei sampled at 24 hours increases linearly (Graph 1). There is also a similar linear increase when micronuclei were sampled at 48 hours, except that there were substantially more micronuclei at a dose of 6 Gy (Graph 2).

The effect of gamma irradiation on the formation of micronuclei (Graph 3)

Experimental Procedure

Exponentially growing cells were trypsinised and exposed to Gamma irradiation in the range 0 - 8Gy. Samples were taken after 48 hours of incubation with 3μg/ml Cytochalasin B and 100 binucleate cells were scored for micronuclei. A set of 3 experiments of was conducted.

Results

The number of micronuclei increases linearly with increasing gamma irradiation dose. This graph is similar to that of X-rays but the numbers of micronuclei produced for the corresponding dose were almost a factor of 2 lower.

The effect of X-irradiation on cell survival (Graph 4)

Experimental Procedure

Exponentially growing cells were trypsinised and diluted $5x10^5$ cells/ml of reaction digest. The cells were exposed to 0,2,4,6 Gy of X-irradiation. The cells were diluted accordingly and plated into 10cm dishes and incubated for 10 days at 37°C. The dishes were scored for clonogenic cell survival. The colonies were then stained. Only

colonies with more than 50 cells were counted and included in the data. Incubation conditions and colony criteria were constant for all survival experiments. A set of 2 experiments of 3 replicates was conducted.

Results

The graph shows a characteristic survival curve with a shoulder. As the dose is increased the survival decreases. This result agrees with survival data obtained by Kolman.

Optimisation of Streptolysin-O concentration for cell survival (Graph 5)

Experimental Procedure

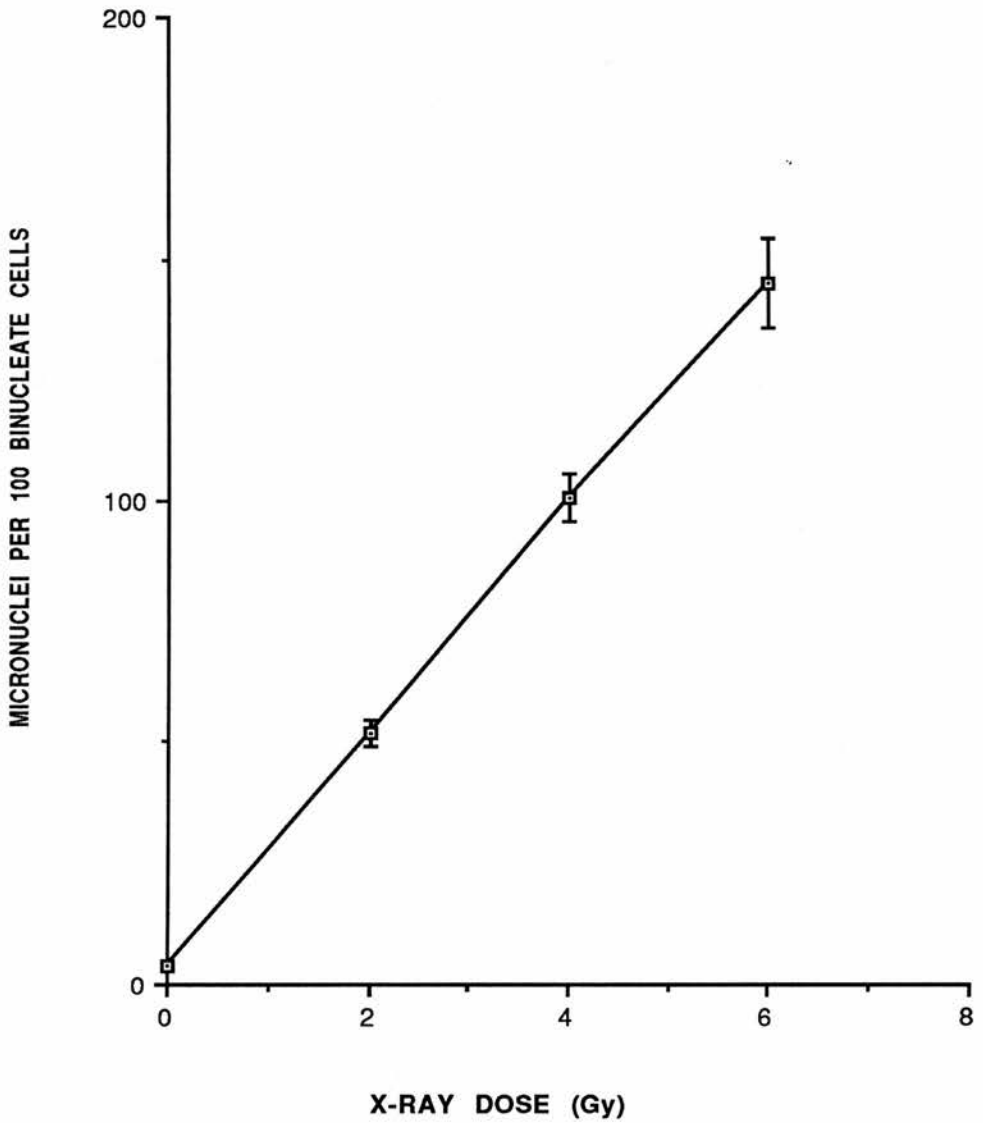
Exponentially growing cells were trypsinised and diluted $5x10^5$ cells/ml of reaction digest. The cells were exposed to 0, 0.090, 0.181, 0.271, 0.361, 0.452 units of Streptolysin-O. A set of 2 experiments of 3 replicates was conducted.

Results

A characteristic dose response curve with shoulder was obtained There was a decrease in cell survival with increasing Streptolysin-O concentration indicating the potency of the Streptolysin-O.

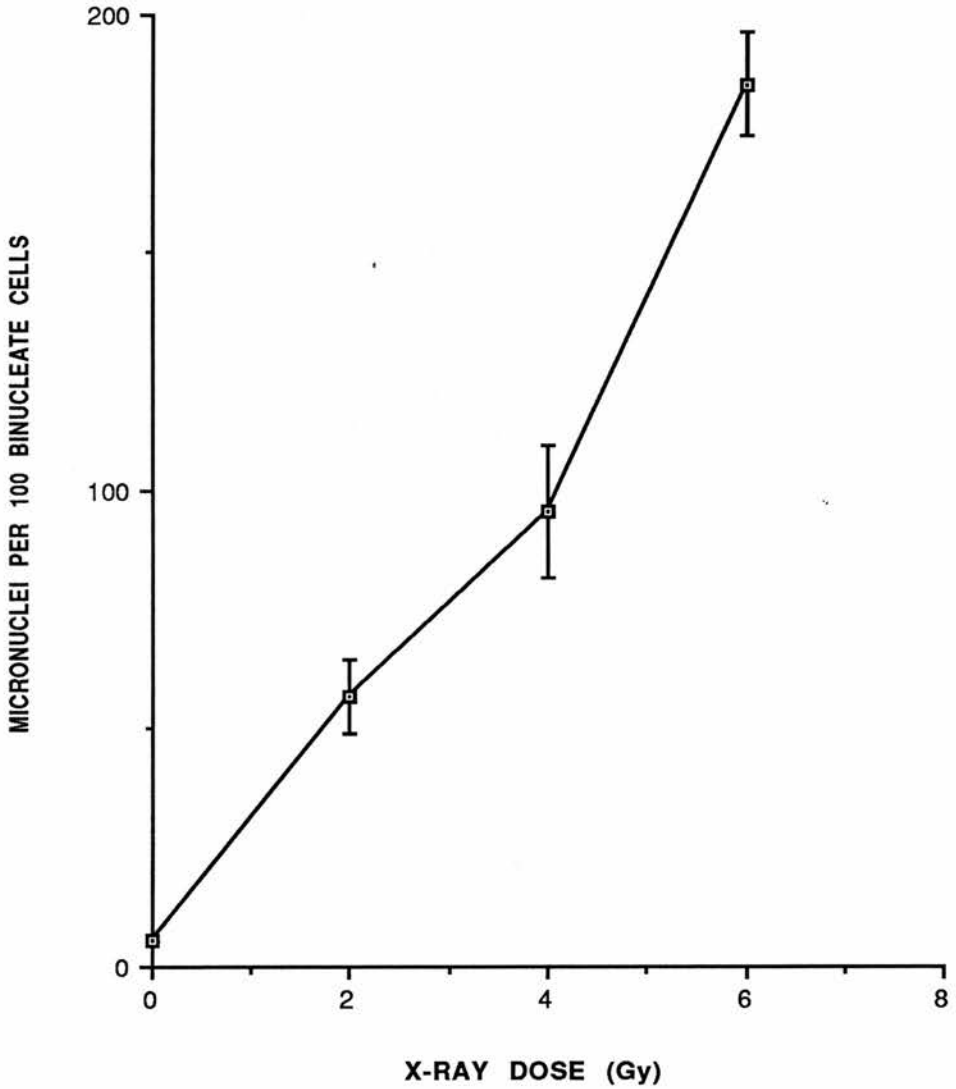
GRAPH 1

The Effects of X-irradiation on C3H10T1/2 Cells Measured by the formation of Micronuclei at 24 Hours.



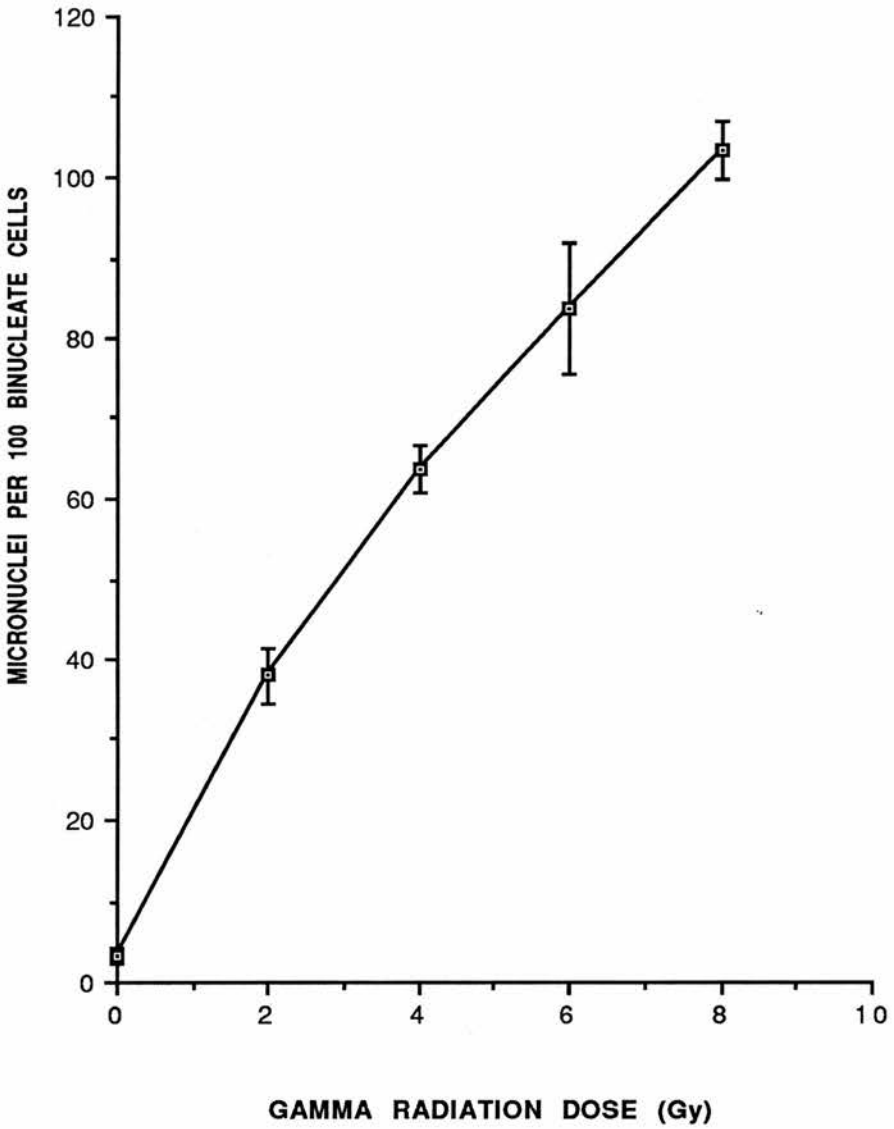
GRAPH 2

The Effect of X-irradiation on C3H10T1/2 Cells Measured by The Formation of Micronuclei at 48 Hours



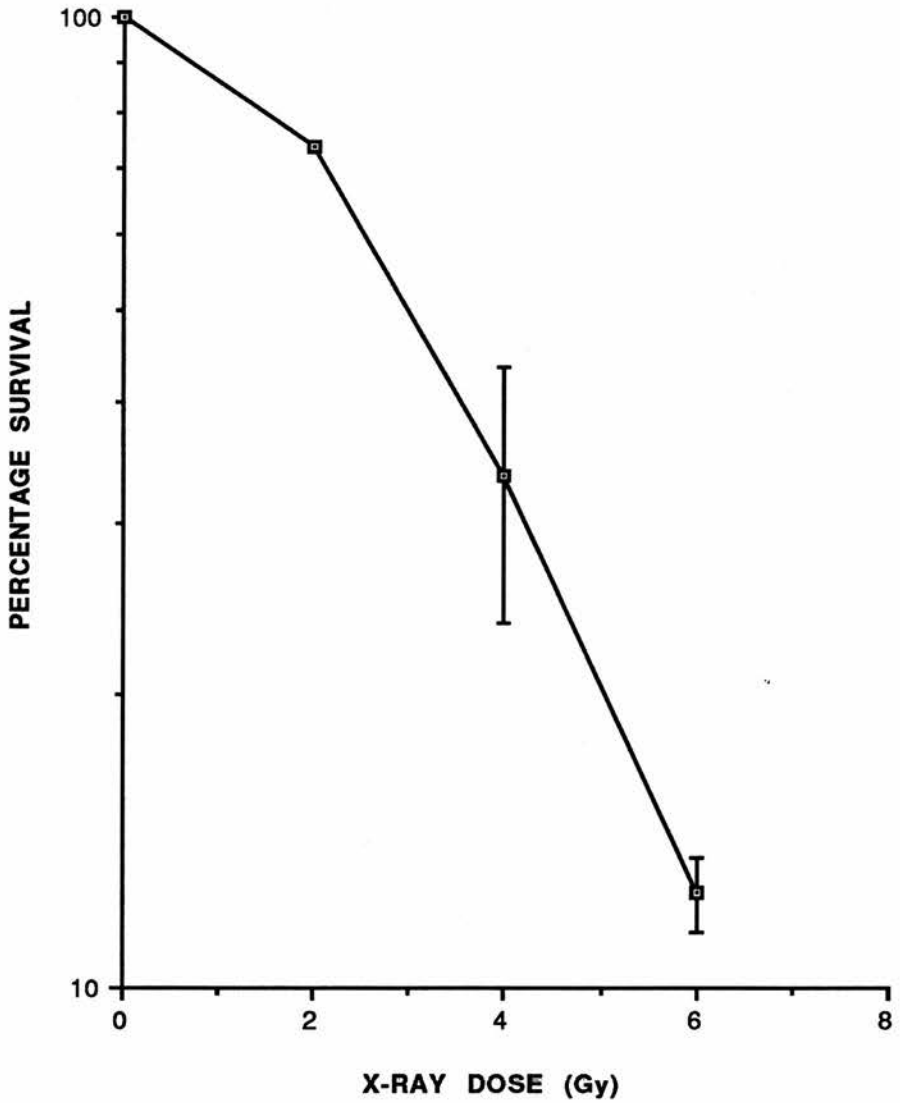
GRAPH 3

The Effect of Gamma irradiation on C3H10T1/2 Cells Measured by The Formation of Micronuclei at 48 Hours



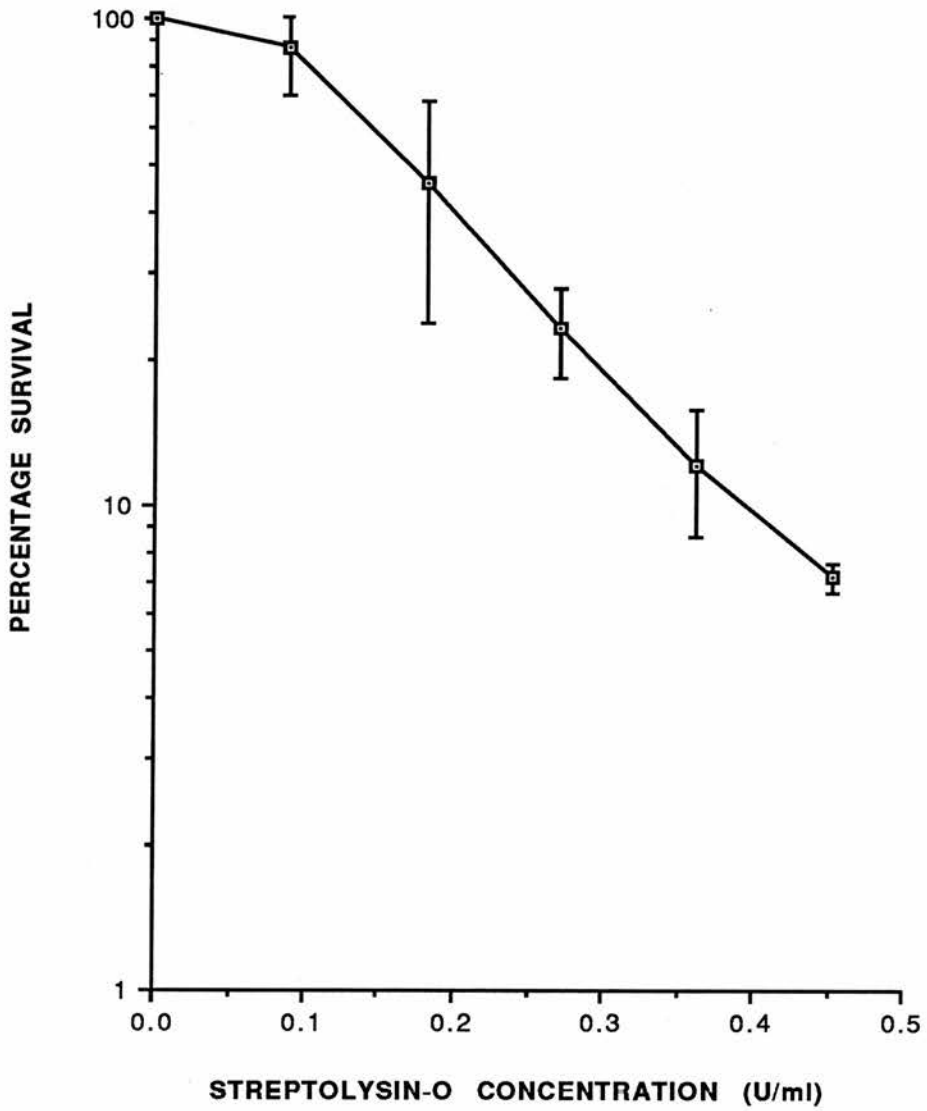
GRAPH 4

The Effect of X-irradiation on the Clonogenic Cell Survival of C3H10T1/2 Cells



GRAPH 5

The Effect of Streptolysin-O concentration on C3H10T1/2 Cell Survival



Optimisation of Streptolysin-O concentration for micronucleus production (Graph 6)

Experimental Procedure

Exponentially growing cells were trypsinised and suspended $5x10^5$ cells/ml reaction digest. The cells were treated within a range of 0 - 0.325 units Streptolysin-O. The concentration of Pvu II was constant at 30 units and the controls contained no Pvu II. The cells were incubated for 48 hours with 3µg/ml Cytochalasin B and 100 binucleate cells were scored for micronuclei. A set of 2 experiments was conducted.

Results

From this data there is a steady increase in micronuclei with increasing Streptolysin-O concentration up to approximately 0.25 units after which it starts to decrease. There is no significant micronucleus production when Pvu II is omitted .

Graph 5 shows the optimisation of Streptolysin-O concentration when cells were treated in the range 0-0.452 units. This data *in conjunction with* graph 6 (the equivalent experiment using micronuclei to assess the optimum Streptolysin-O concentration) showed that 0.271 units should be used for further experiments.

This dose was chosen to give maximum micronucleus production within a range of relatively low cell killing.

Optimisation of sampling time (Graph 7)

Experimental Procedure

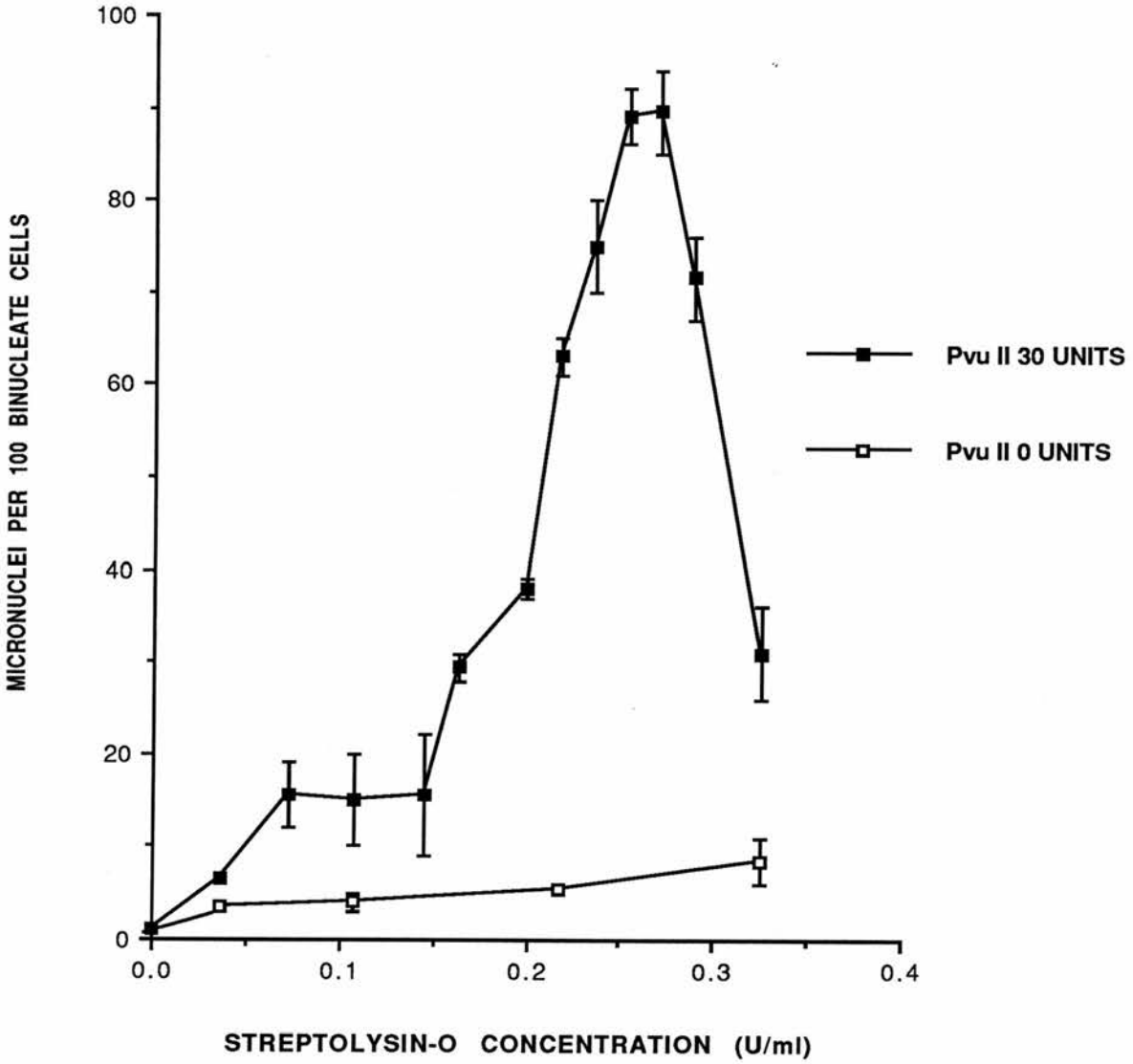
Exponentially growing cells were trypsinised and suspended $5x10^5$/ml reaction digest. The cells were treated with 20 units Pvu II and 0.271 units Streptolysin-O. Samples for micronucleus formation were taken over a period of 6-66 hours incubation with 3µg/ml Cytochalasin B and 100 binucleate cells were scored for micronuclei. A set of 2 experiments was conducted.

Results

As the sampling time is increased the number of micronuclei also increases reaching a peak at 24 hours and 40 hours. After 40 hours the graph shows a plateau effect. The 48 hours sampling time was within the plateau region of the graph and because of this it was chosen as the time for further experiments.

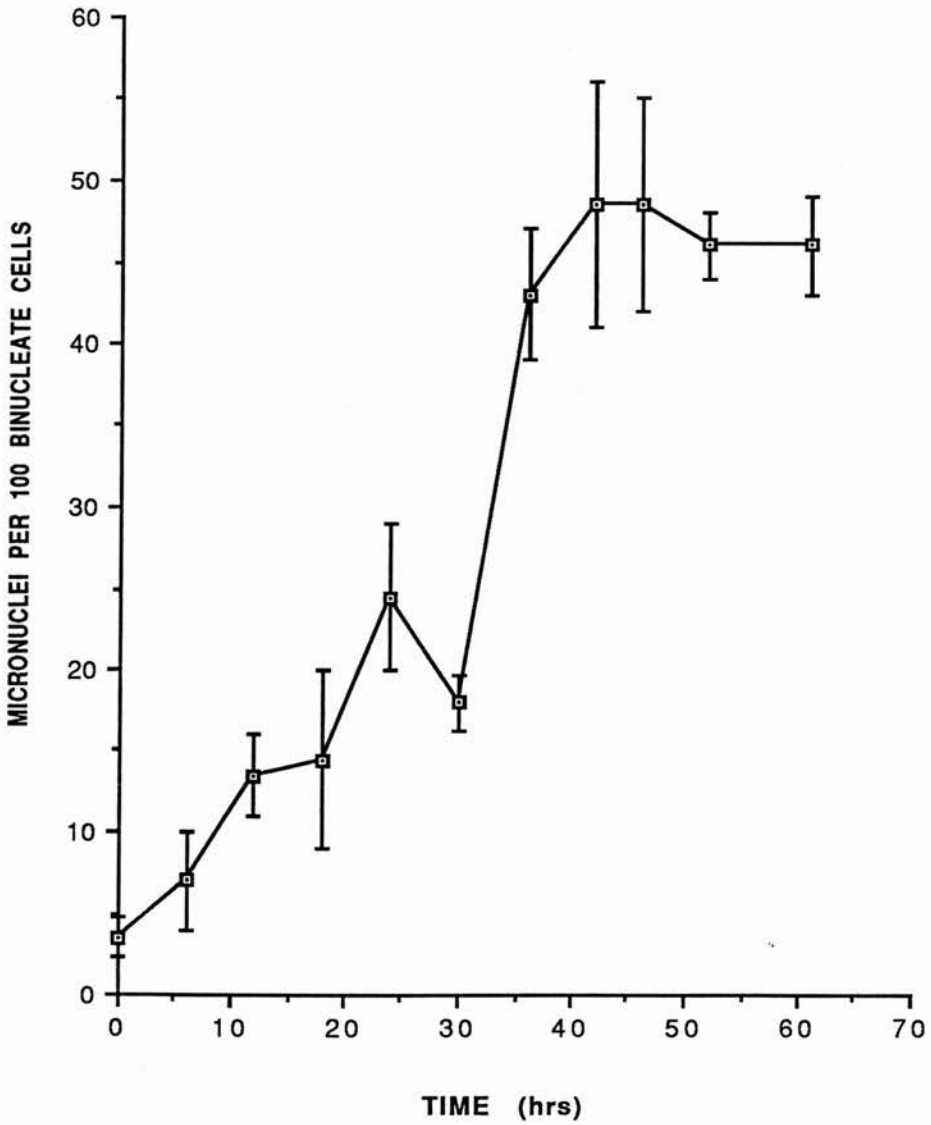
GRAPH 6

The Optimisation of Streptolysin-O Concentration for the Permeabilisation of C3H10T1/2 Cells



GRAPH 7

The Formation of Micronuclei in C310T1/2 Cells Permeabilised by 0.271 Units Streptolysin-O Treated with 20 Units Pvu II and Sampled after Incubation with 3μg/ml Cytochalasin B at Varying Times



The effect of Pvu II and EcoR I on micronucleus production (Graph 8)

Experimental Procedure

Exponentially growing cells were trypsinised and diluted $5x10^5$ cells/ml. The cells were treated with 0,1,3,6,10,20,30 units of restriction enzymes Pvu II and 3,10,20,30 units of EcoR I. The cells were permeabilised with 0.271 units Streptolysin-O in each experiment. Samples for micronuclei were taken after 48 hours incubation with 3µg/ml Cytochalasin B and 100 binucleate cells were scored for micronuclei. A set of 5 experiments of 3 replicates was conducted.

Results

As Pvu II dose is increased micronucleus production increases rapidly and almost linearly up to 10 units. After this point there is only a slight increase in comparison. Pvu II produced a significant number of micronuclei as opposed to EcoR I which showed no micronucleus production above control levels.

The effect of Pvu II and EcoR I on cell survival (Graph 9)

Experimental Procedure

Exponentially growing cells were trypsinised and diluted $5x10^5$ cells/ml of reaction digest containing 0.271 units Streptolysin-O and 0,3,10,20,30,40 units of Pvu II. Similarly cells were treated with 0,10,20,30 units of EcoR I. A set of 3 experiments of 3 replicates was conducted.

Results

Pvu II produced substantial cell killing as opposed to EcoR I which produced virtually no cell killing compared with controls.

Comparison of end type and cutting frequency on micronucleus production (Graph 10)

Experimental Procedure

Exponentially growing cells were trypsinised and diluted $5x10^5$/ml and treated with 20 units of Pvu II, EcoR I, EcoR V, Bam HI and permeabilised with 0.271 units of Streptolysin-O. Samples were incubated with 3µg/ml Cytochalasin B for 48 hours and 100 binucleate cells were scored for micronuclci. An experiment of 3 replicates was conducted.

Results

The graph shows the relative difference between the type of dsb's generated, whether blunt or cohesive, and the number of times that

the restriction enzyme was able to cleave the DNA. Pvu II produced the most micronuclei. EcoR V produced almost half the amount of micronuclei as Pvu II. BamH I and EcoR I produced significantly less than Pvu II.

Comparison of end type and cutting frequency on cell survival (Graph 11)

Experimental Procedure

Exponentially growing cells were trypsinised and diluted $5x10^5$ cells/ml of reaction digest containing 0.271 units Streptolysin-O and 20 units of Pvu II, EcoR I, EcoR V, BamH I respectively and plated for survival. An experiment of 4 replicates was conducted.

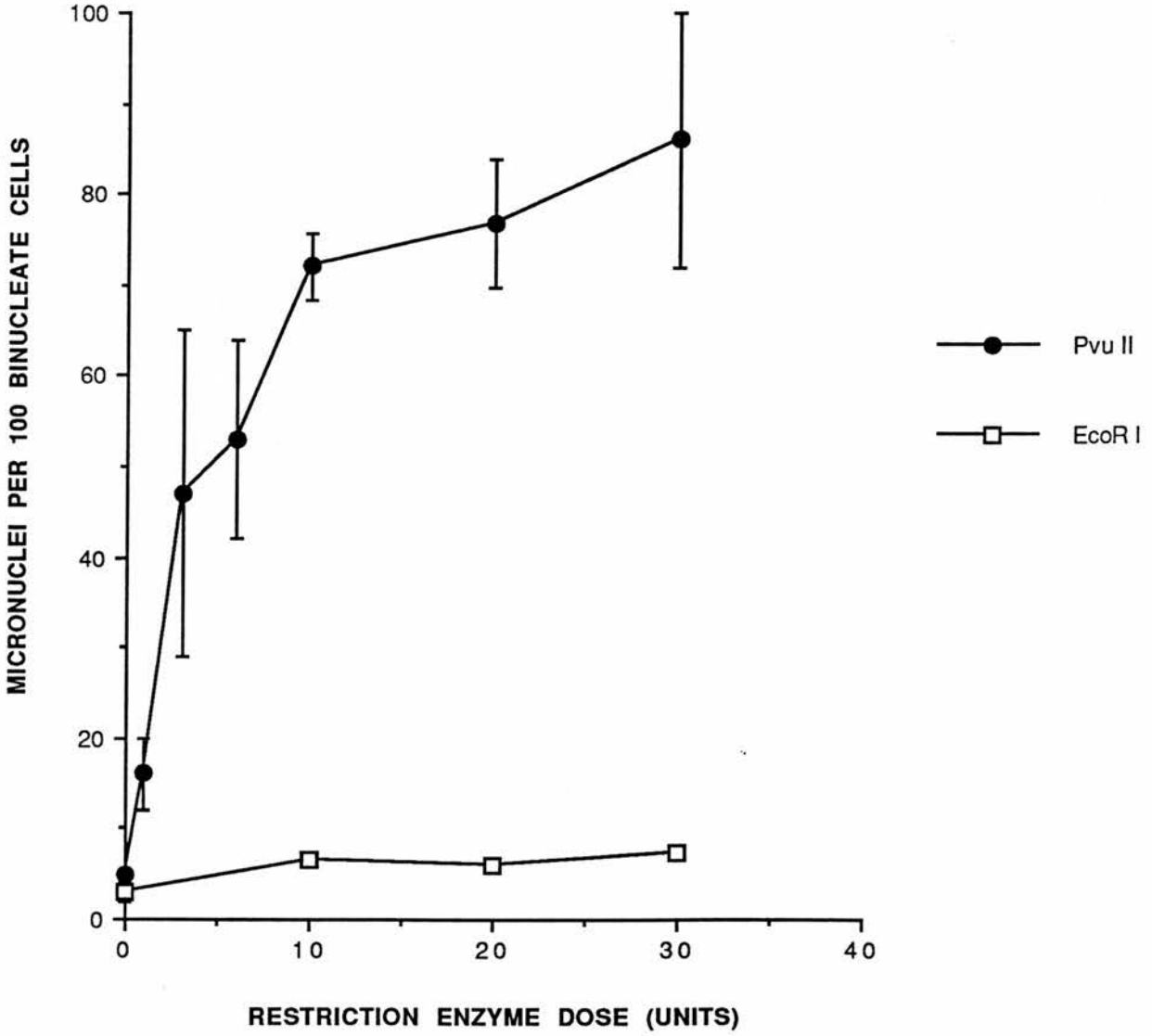
Results

The results are not related to a control and therefore only show a comparison of cell killing caused by the four restriction enzymes. Pvu II showed the lowest incidence of cell survival followed by EcoR V. Both EcoR I and BamH I showed similar amounts of cell survival which were significantly higher than the other two

Comparison of end type and cutting frequency on micronucleus production and cell survival (graph 12)

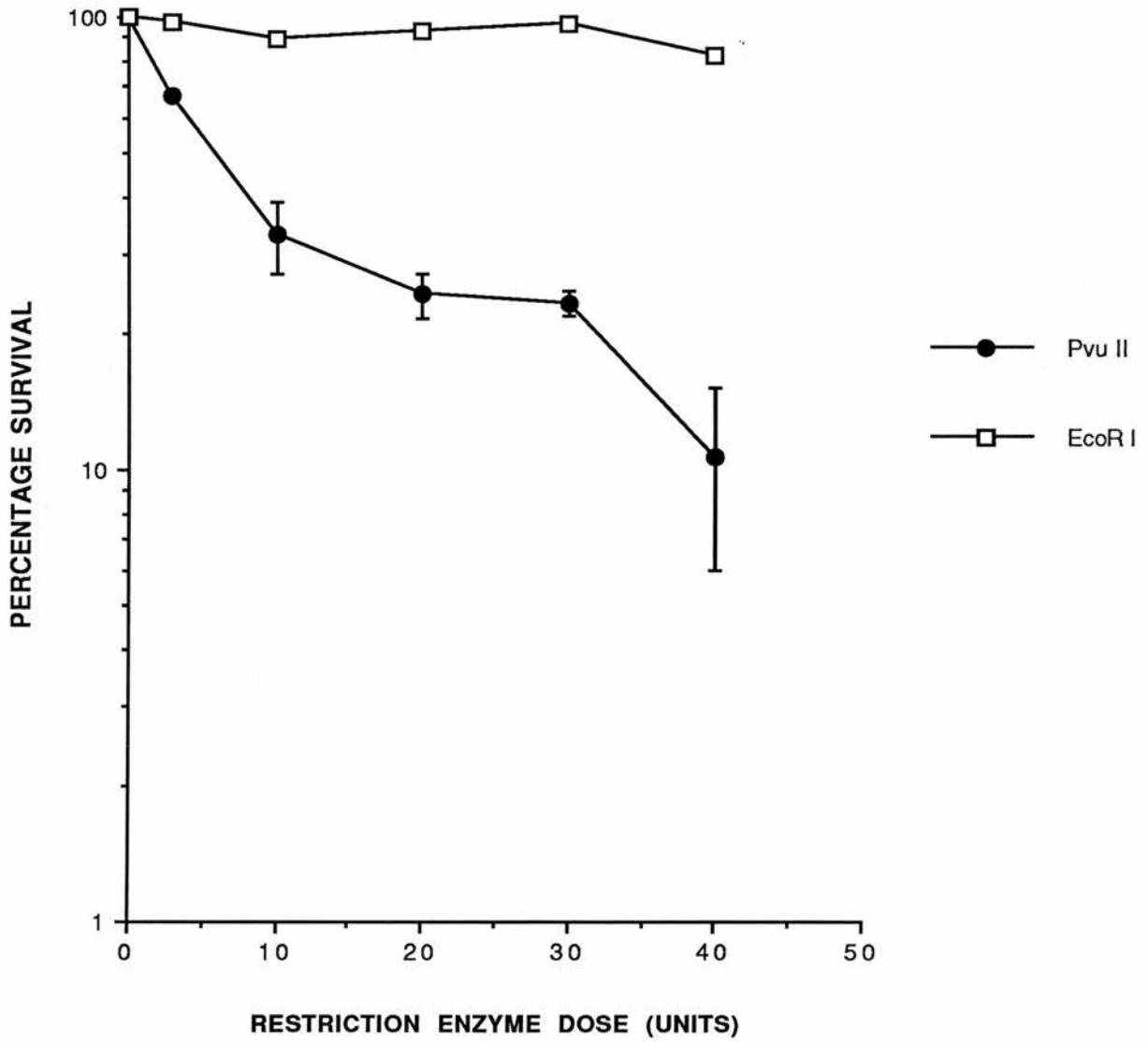
GRAPH 8

The Effect of the Formation of Micronuclei by the Type of End Structure Produced by Pvu II (Blunt) and EcoR I (Cohesive) Permeabilised with 0.271 Units Streptolysin-O Sampled after Incubation with 3μg/ml Cytochalasin B for 48 Hours



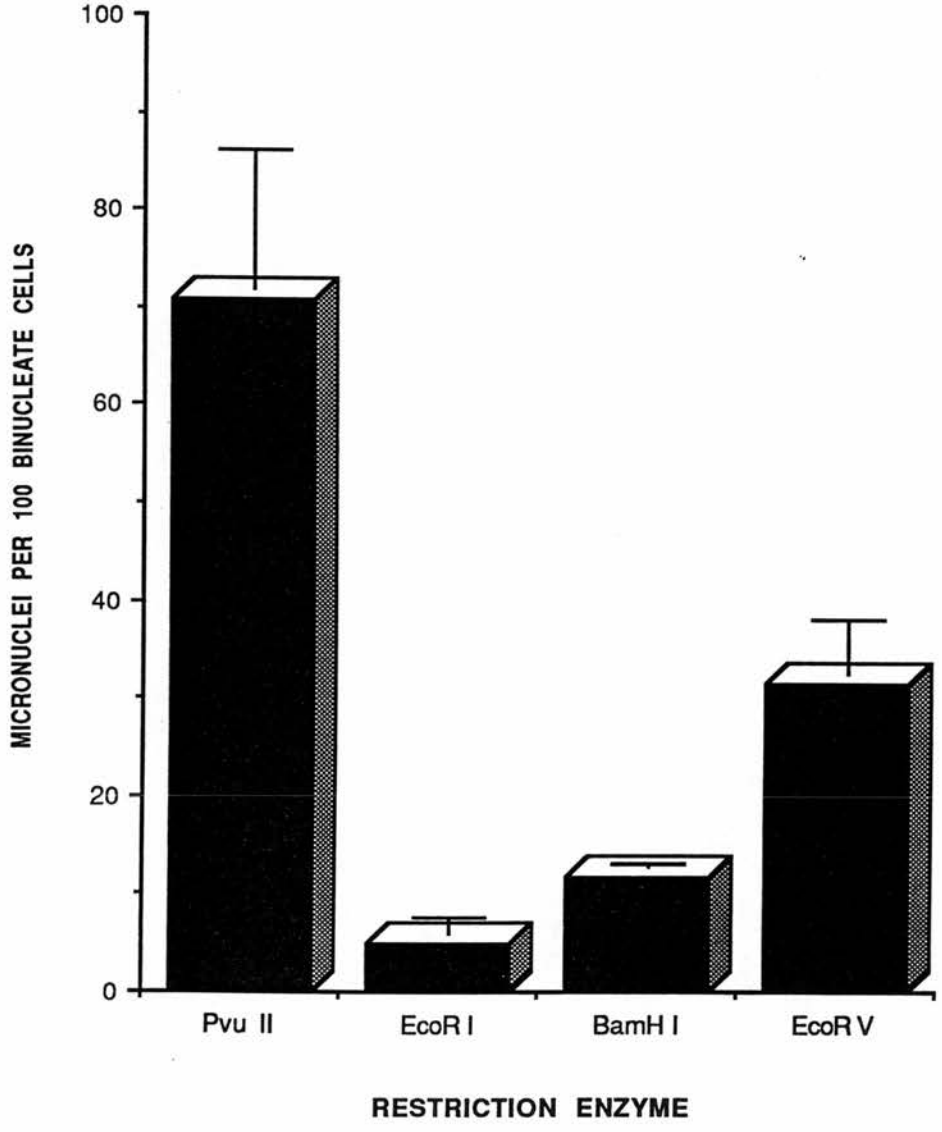
GRAPH 9

The Relative Effect on Survival of C3H10T1/2 Cells Treated with Pvu II (Blunt) and EcoR I (cohesive) and Permeabilised with 0.271 Units Streptolysin-O



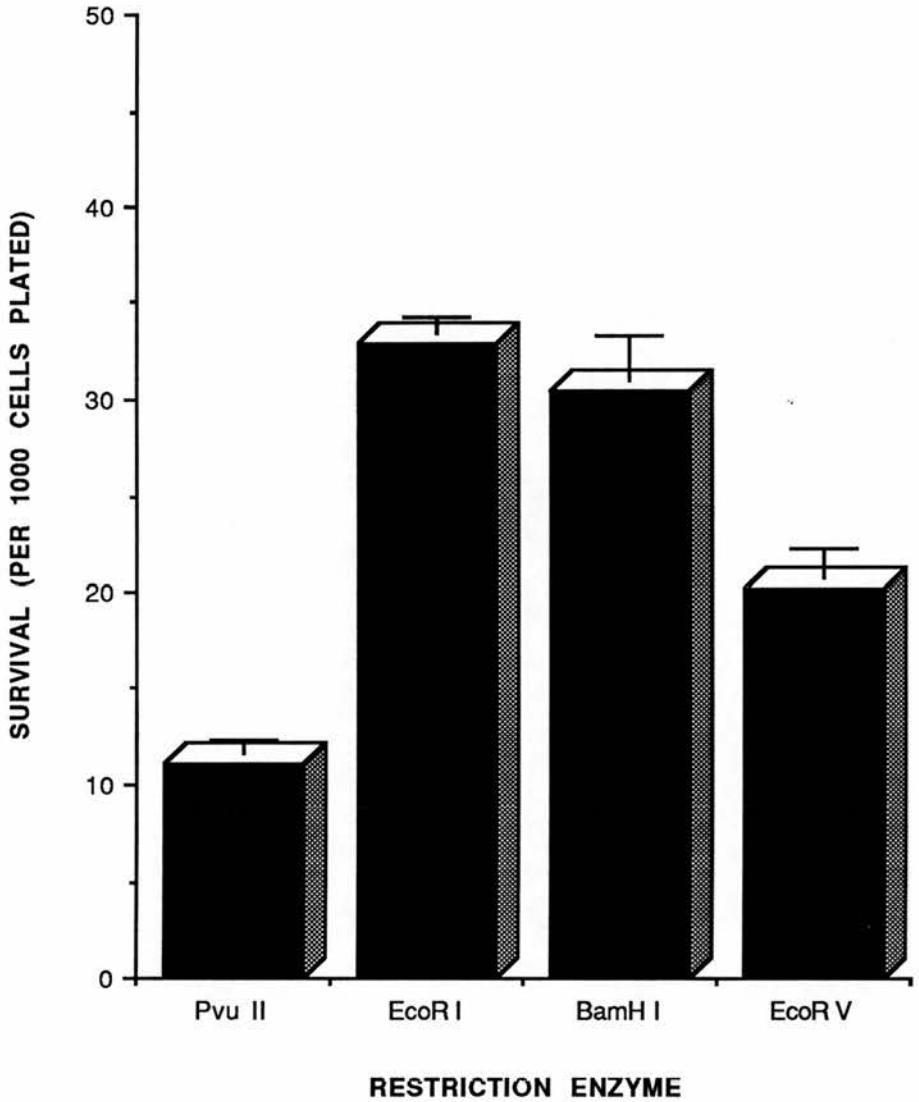
GRAPH 10

The Effect on the Formation of Micronuclei in C310T1/2 Cells Permeabilised by 0.271 Units Streptolysin-O Treated With 20 Units Restriction Enzyme Permeabilised with 0.271 Units Streptolysin-O and Sampled after Incubation with 3μg/ml Cytochalasin B for 48 Hours



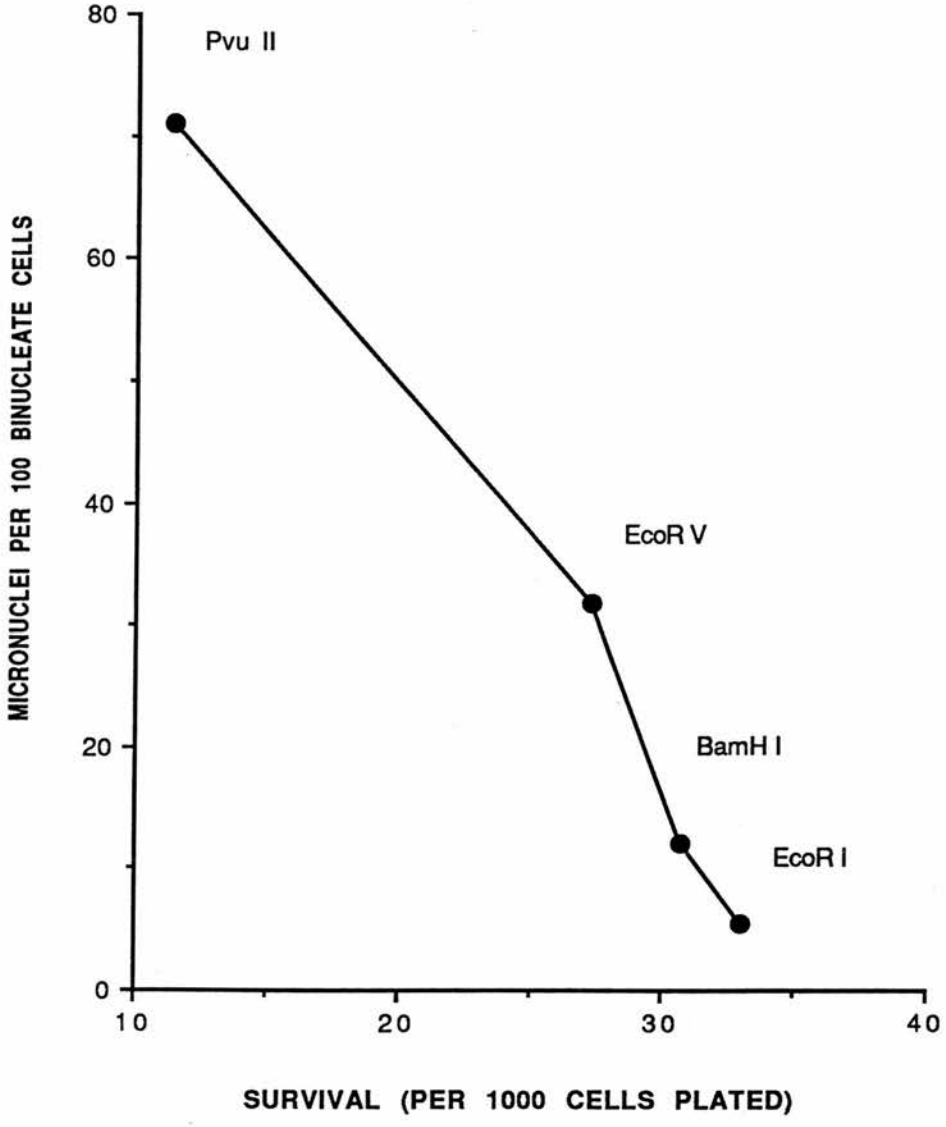
GRAPH 11

The Effect of the Type of dsb on C3H10T1/2 Cell Survival Treated with 20 Units Restriction Enzyme and Permeabilised with 0.271 Units Streptolysin-O



GRAPH 12

The Relative Effect of the Different Restriction Enzymes on Micronucleus Production as a Function of Survival in C310T1/2 Cells Permeabilised by 0.271 Units Streptolysin-O Treated With 20 Units Restriction Enzyme and Sampled after Incubation with 3μg/ml Cytochalasin B for 48 Hours



This graph shows the number of micronuclei produced against cell survival. Pvu II shows a high incidence of micronucleus production and a correspondingly high level of cell killing followed by EcoR V. Both BamH I and EcoR I showed very little micronucleus production and a high percentage cell survival.

Radiation damage measured by filter elution (Graphs 13 & 14)

Experimental Procedure

Exponentially growing cells were trypsinised and diluted $5x10^5$ cells/ml of reaction digest. The cells were exposed to 0,5,10,15,20 Gy of radiation. The cells were then filtered and assessed for DNA damage. Two experiments were conducted for X-irradiation and gamma irradiation (Graph 13 & 14).

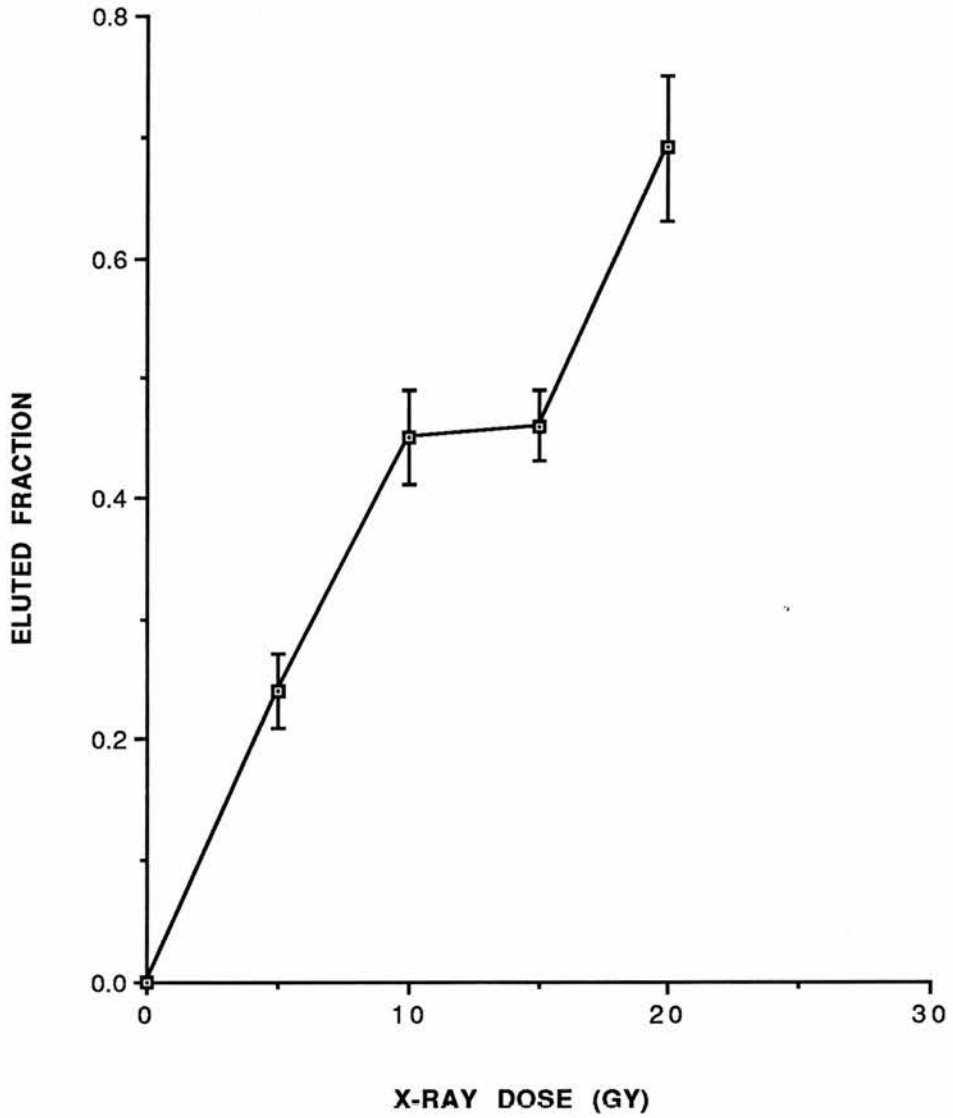
Results

X-irradiation shows an almost linear increase of DNA damage with increasing radiation dose. Gamma Irradiation (Graph 14) also gives an increasing linear dose response which is similar in shape and magnitude to the dose response obtained for X-rays (graph 13).

Comparison of Pvu II and EcoR I measured by filter elution (Graph 15)

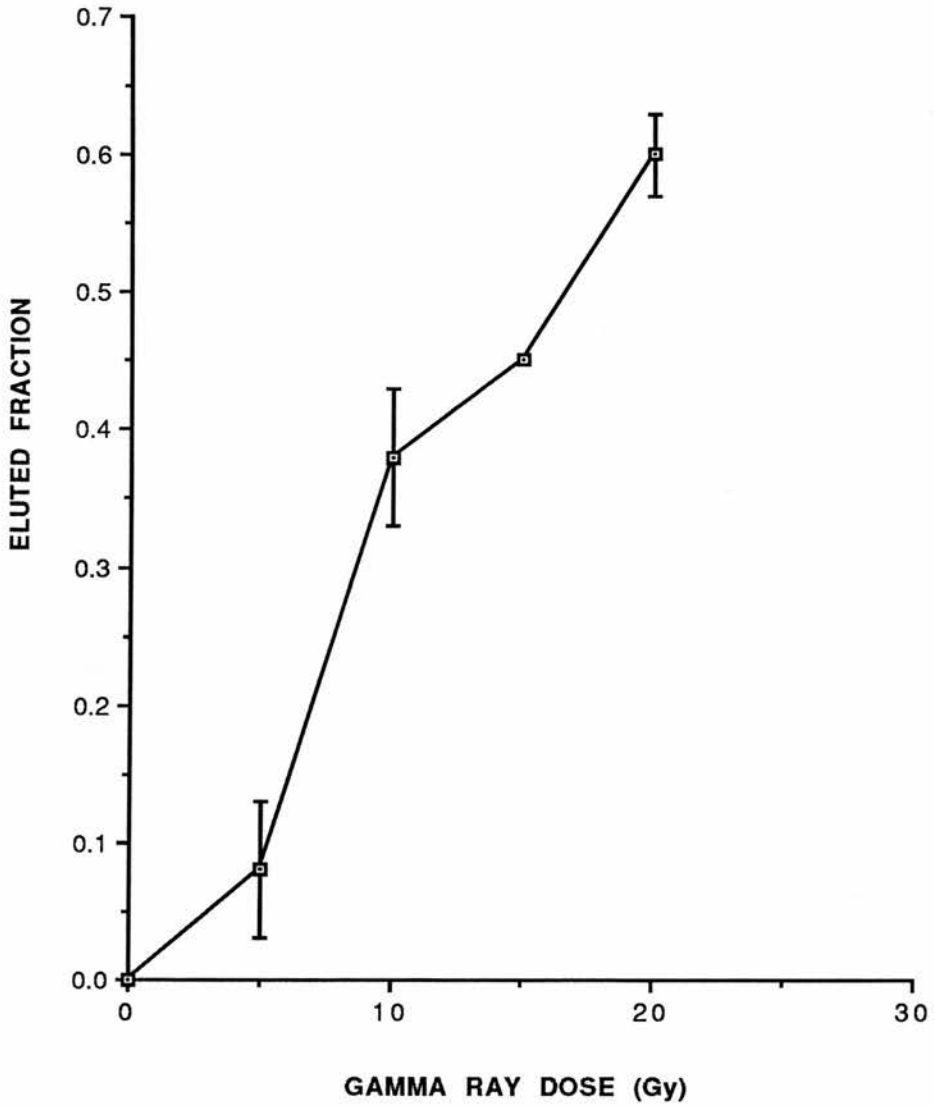
GRAPH 13

The effect of X-Irradiation on Producing DNA Damage Measured by the Filter Elution Technique



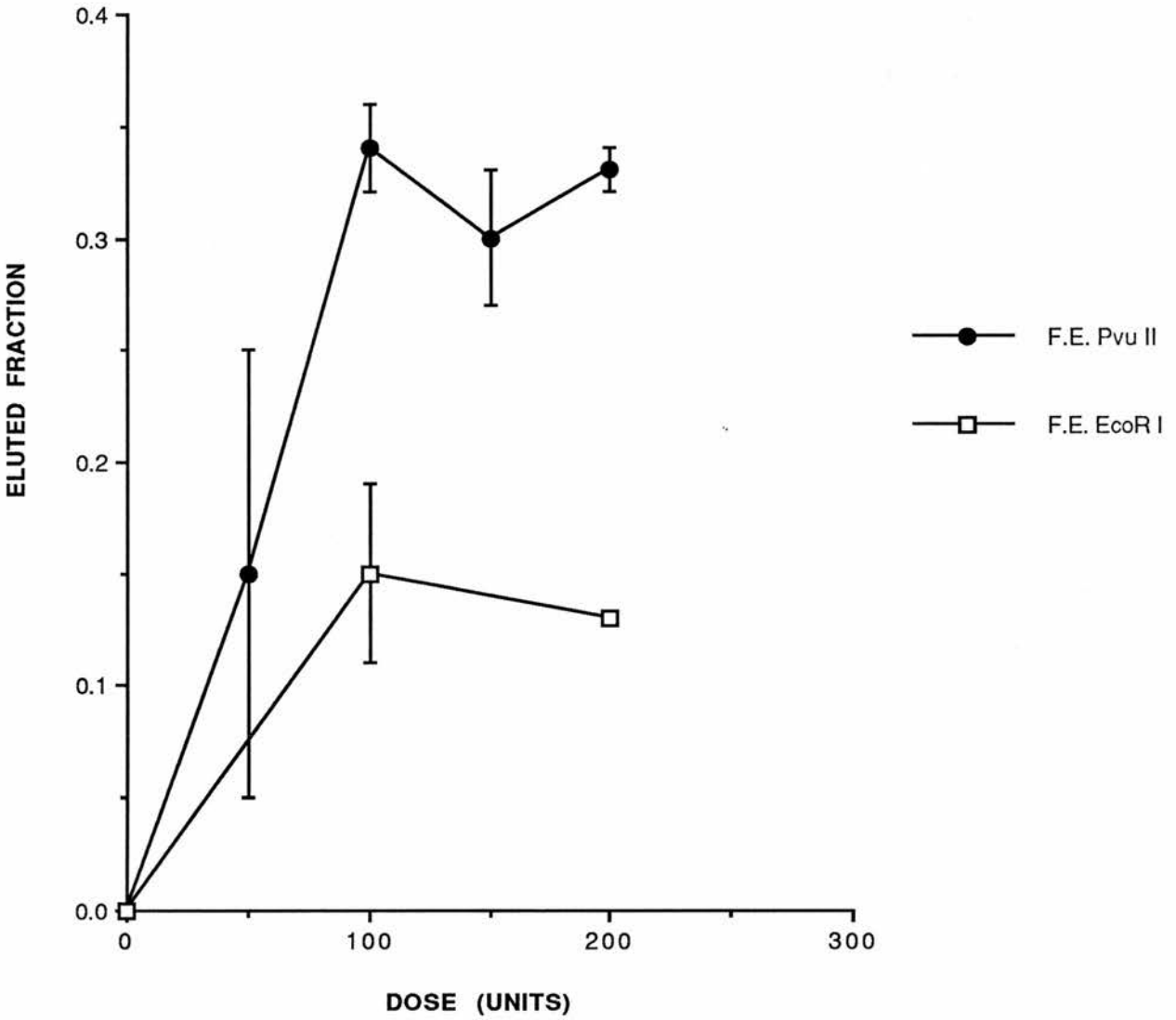
GRAPH 14

The Effect of Gamma Irradiation on Producing DNA Damage Measured by the Filter Elution Technique



GRAPH 15

The Effect of the Type of dsb on Production of DNA Damage in C3H10T1/2 cells Treated with Pvu II and EcoR I and Permeabilised with 0.271 Units Streptolysin-O



Experimental Procedure

Exponentially growing cells were trypsinised and diluted $5x10^5$ cells/ml of reaction digest containing 0.271 units of Streptolysin-O and treated with 0,50,100,150,200 units of Pvu II or 0,100,200 units of EcoR I respectively. The cells were then filtered and assessed for DNA damage. Two experiments were conducted.

Results

From the graph, Pvu II gives an increase in eluted fraction with increasing enzyme dose up to 100 units; beyond which the eluted fraction remains fairly constant with further dose increases. In comparison EcoR I does not show a large eluted fraction which is almost 4 times less.

Transformation using X-irradiation (Graph 16)

Experimental Procedure

Two experiments of 10 replicates were conducted.

Exponentially growing cells were trypsinised and diluted $5x10^5$ cells/ml and treated with 0,2,4,6 Gy of X-irradiation and incubated at 37°C for six weeks with routine medium changes. Two experiments of 10 replicates were conducted.

Results

As the dose is increased a sigmoid dose response curve is obtained. The number of transformants at 6Gy is almost double the background transformation frequency. The background transformation frequency was high in this experiment.

Transformation using Pvu II (Graph 17)

Experimental Procedure

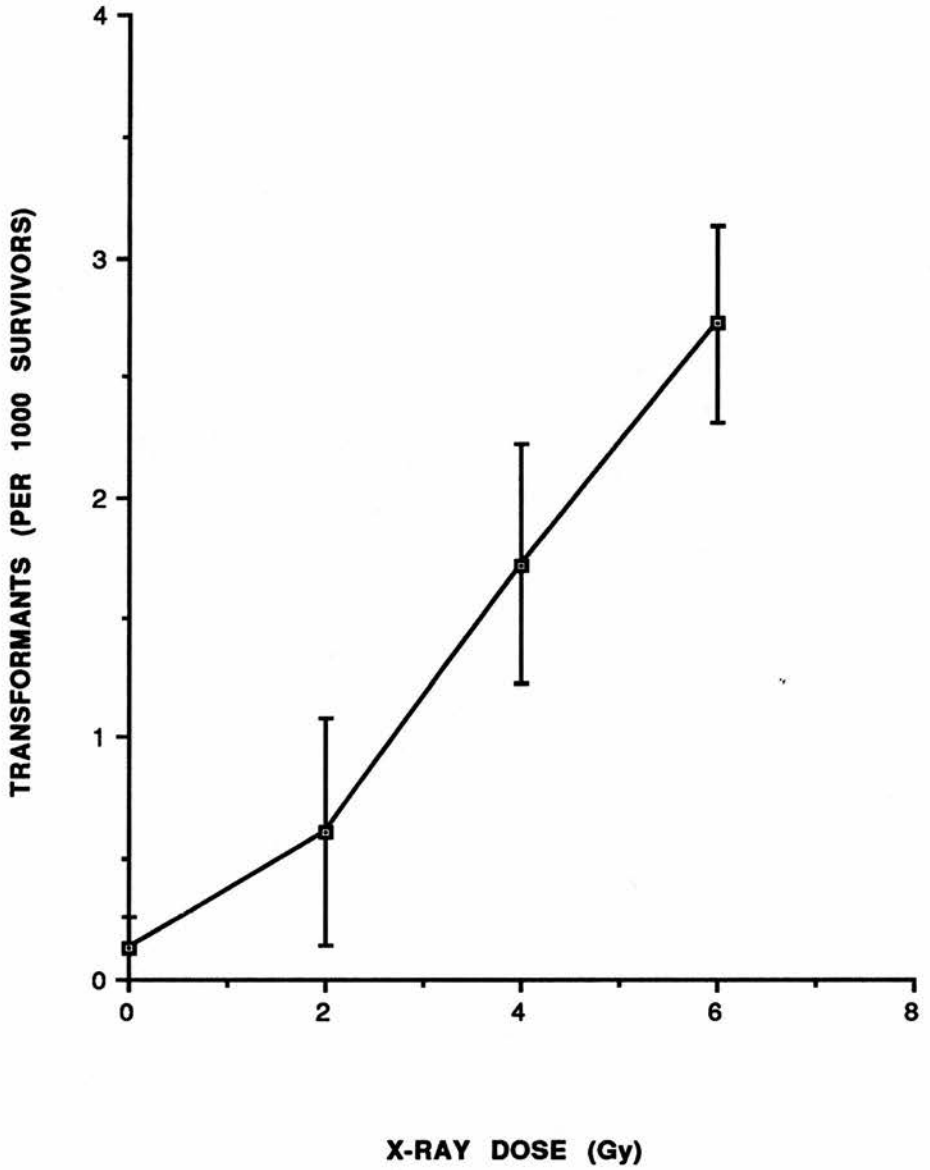
Exponentially growing cells were trypsinised and diluted $5x10^5$ cells/ml and treated with 0.271 units Streptolysin-O and 0,20,40 units Pvu II and incubated at 37°C for six weeks with routine medium changes. One experiment of 10 replicates was conducted.

Results

From the graph it can be seen that Pvu II gives an increasing transformation frequency with increasing dose. There was a fourfold increase in transformants when the enzyme dose was doubled. The background transformation frequency was again high in this experiment.

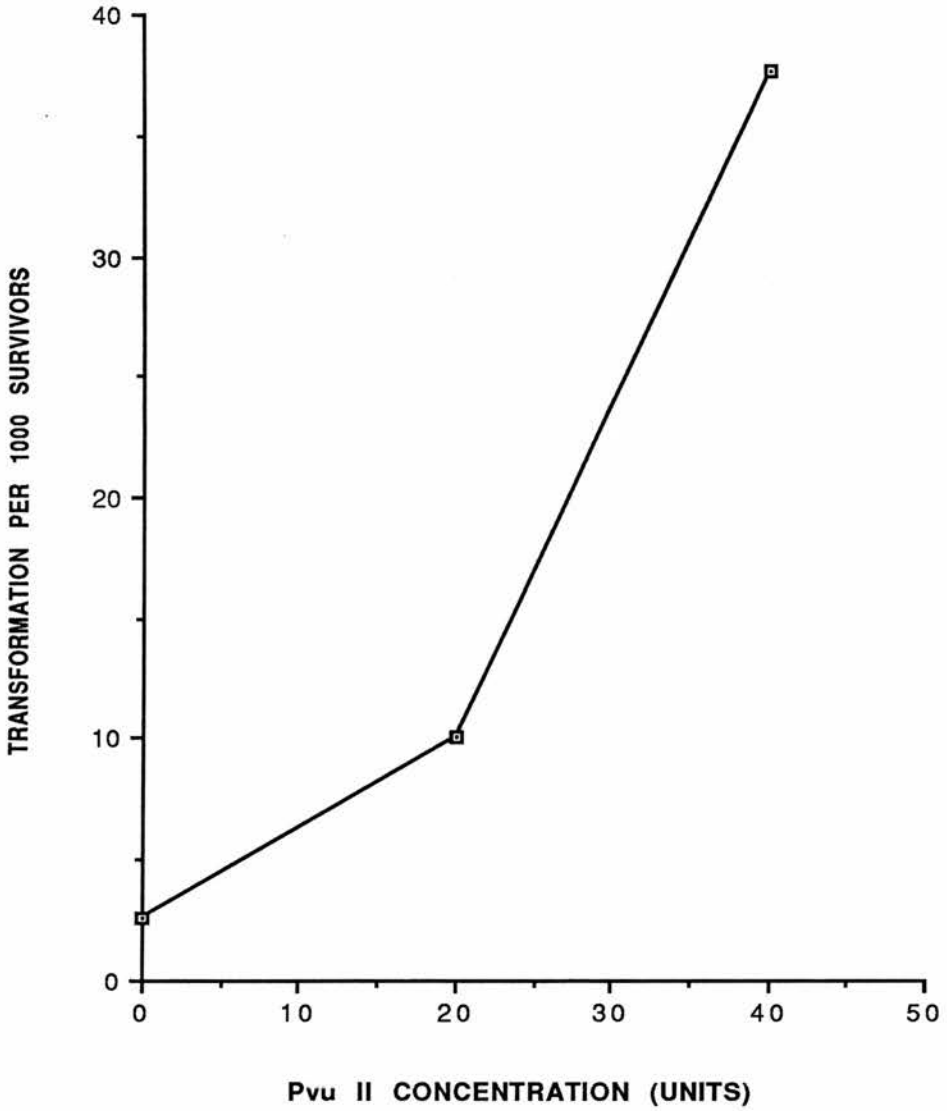
GRAPH 16

The Transformation of C3H10T1/2 Cells by X-Irradiation



GRAPH 17

The Transformation Frequency of C3H10T1/2 Cells Treated with Pvu II and Permeabilised with 0.271 Units Streptolysin-O



DISCUSSION

Irradiation

It is accepted that ionising radiation produces a variety of DNA lesions. An important question to be asked is which lesion is predominate in causing cell killing and cell transformation. This study aims to isolate the dsb and examine its consequences at a molecular and cellular level using restriction enzymes. Before any restriction enzyme work could be performed on the C3H10T1/2 cell line, assay conditions had to be optimised using Gamma and X-irradiation as a standard.

(i) X-irradiation (Graphs 1&2)

Cellular micronuclei showed an increasing linear dose response with increasing radiation dose at sampling times of both 24 and 48 hours. The magnitude of micronuclei at a sampling time of 48 hours was greater than that at 24 hours at a dose of 6 Gy. This is explained by the fact that the doubling time of of the C3H10T1/2 cell line is 15.5 hours (Reznikoff, 1973) and has a recovery time of approximately 6 hours after treatment before cell division

occurs. Therefore cells will double approximately every 24 hours. Cells which missed the Cytochalasin B block will be stopped during their next division. Hence the magnitude of micronuclei is greater at 48 hours for 6 Gy due to a dose dependent delay. This effect is shown in the Time Sampling experiment with restriction enzymes.

(ii) Gamma-irradiation (Graph 3)

The results from gamma-irradiation showed that a similar shaped dose-response curve was obtained to that of X-rays. However the gamma dose required to produce equivalent damage to that of X-rays was almost a factor of two higher. This may be explained by the possibility that the 0.5mm copper filter may have allowed some soft rays from the X-irradiation through which may account for the difference.

Optimisation of the micronucleus assay

Initially the optimum assay conditions for the use of restriction enzymes had to be determined. The micronucleus assay was used as a rapid screening method for later transformation experiments but in itself gave a good indication of DNA damage. The

restriction enzyme Pvu II was chosen as earlier studies on CHO cells showed that this enzyme produced significant numbers of micronuclei (Bryant, 1984).

The effect of sampling time on micronucleus production (Graph 7)

From graph 7 it can be seen that below 30 hours there is little micronucleus production whereas after 30 hours there is a considerable increase, which begins to plateau at 44 hours and maintains this level of micronuclei. A similar shape of graph was obtained by Hill et. al. who related DNA labelling index to transformation frequency on the same time scale. It was found that 1-2 days were required for the cells to recover from the perturbations introduced by the handling of the cells (suspension, dilution, replating). Cell division did not commence until 12 hours after replating (Han, Hill & Elkind, 1984). It was also suggested that transformation frequency depended upon cell age and that S-phase was a period of relatively high transformation. From the experiment it can be envisaged that as DNA damage (i.e. number of micronuclei) increases it would be likely that transformation frequency would increase. From this experiment a sampling time of 48 hours was chosen for restriction enzyme work due to the

consistency of micronuclei data within the range of the plateau and also to allow comparison with radiation results which were sampled at this time.

Introduction of restriction enzymes into cells .

As previously stated in the introduction a number of methods have been investigated to introduce restriction enzymes into cells and have been used with varying degrees of success. Streptolysin-O had been used in chromaffin medullary cells to introduce macromolecules. Restriction enzymes were also successfully introduced into CHO cells (Bryant, 1991). Initially the optimum streptolysin-O concentration had to be determined to (i) allow sufficient introduction of restriction enzymes and assess the dose effect using the micronucleus assay and (ii) minimise cell killing. Both the micronucleus assay and clonogenic cell survival assay were used to optimise the streptolysin-O concentration for further restriction enzyme work.

(i) Optimisation of Streptolysin-O concentration to measure restriction enzyme damage (Graph 6)

The optimisation of streptolysin-O concentration assessed using the micronucleus assay graph 6 gave a bell shaped curve. Concentrations below 0.145 units showed little enzyme action. The optimum streptolysin-O concentration was shown to be 0.271 units. At this concentration the cells showed maximum damage. At concentrations above 0.325 units a high majority of cells were killed. The control experiment showed that the cellular damage was in fact caused by the restriction enzyme entering the cell and acting on the DNA and not any effect by the streptolysin-O itself.

(ii) Optimisation of Streptolysin-O concentration to minimise toxicity (Graph 5)

The optimisation of streptolysin-O concentration assessed by clonogenic cell survival showed that cell killing occurred within a range of 0.145-0.452 units. Using the data from both these experiments the streptolysin-O concentration for further restriction enzyme work was set at 0.271 units to optimise micronucleus production and minimise the toxicity of the compound hence the cell killing. The results obtained here accord

with results obtained by Bryant (1992) with CHO cells; however more Streptolysin-O was needed in the C3H10T1/2 system. This was probably due to the difference in size of the cells. C3H10T1/2 cells are larger than CHO cells and thus a higher concentration of Streptolysin-O needed to produce a similar effect

The effect the type of dsb has on micronucleus production (Graph 8)

The Pvu II dose curve shows saturation effects. This process is determined by the rate of two enzymic reactions occurring with each individual cell; (i) introduction of sufficiently sized holes in the plasma membrane and (ii) the introduction of restriction enzymes through these holes. This process is occurring throughout the whole cell population and so is an average of the whole cell population. The initial part of the graph (0-10 units) appears to be linear and there are no saturation effects. This would be the ideal region for kinetic studies of the action of restriction enzymes. However this region is highly variable compared to the 20-30 units region which gives more consistent results.

Comparison of Pvu II and Radiation

From graph 8 the Pvu II dose of 20 units is approximately equivalent to a radiation dose of 3 Gy. And also from graph 9, which shows cell survival, a dose of 3 Gy is approximately equivalent to a Pvu II dose of 7 units, which is lower than the equivalent dose obtained for micronuclei at 3 Gy. The difference in dose requirements for micronuclei and cell survival may be due to the length of time that the cells are exposed to the restriction enzyme. Cells treated in the micronucleus assay only undergo one cell division whereas cells may undergo many divisions in the survival assay and this may accentuate the effect of the Pvu II. The shape of the curve obtained with enzymes is different to that obtained from radiation. Again this may be due to the fact that because the restriction enzyme remains within the cell it will still be excising the DNA, and there may be a threshold point where the cell dies, and hence over the total cell population this is seen as a plateau or saturation on the graph, unlike radiation which does not have the same exposure time.

Comparison of Pvu II and EcoR I

The restriction enzyme Pvu II (blunt end cutter) shows an increasing dose response curve with increasing enzyme dose. By contrast, the restriction enzyme EcoR I shows no increase in micronuclei with increasing enzyme dose and is comparable with controls. Thus this experiment shows that the type of end structure produced by the different enzymes is an important factor in the occurrence of micronuclei.

However it has been argued that both blunt and cohesive end producing enzymes are equally effective at producing chromosome aberrations (Obe et al., 1985) and that the aberration frequency is a function of the occurrence of the specific recognition sequences for the restriction enzyme in the DNA and not the end type structure. Winegar (1988) compared cutting frequency with end structure by using various restriction enzymes and found that cutting frequency rather than end structure is the determinant in aberration formation. Tuschy & Obe (1988) found that treatment of cells with either ammonium sulphate or sodium chloride solutions increased the frequency of chromosomal aberrations induced by restriction enzymes. They

postulated that high salt concentrations caused an opening up of the chromatin structure exposing otherwise blocked recognition sites; therefore both a high concentration of restriction enzyme coupled with a severe hypertonic shock could lead to an enhancement of the enzyme effectiveness by opening out the DNA. Also hypertonic conditions may influence the end structure of cohesive dsb by increasing the likelihood of the ends dissociating, leaving structures similar to those of blunt ends. Bryant & Christie (1989) disagreed with Obe et al. (1985) and Winegar & Preston (1988) and showed that cohesive ended dsb are less effective than blunt ended dsb in causing chromosomal abberations. Recent work with micronuclei has confirmed this (Moses et al., 1990).

The question could be posed as to whether micronucleus frequency is due to (i) actual cutting frequency or (ii) the end structure of the DNA.

<u>The effect the type and frequency of dsb have on micronucleus production (Graph 10)</u>

In order to examine further cutting frequency versus end

structure comparative experiments were carried out

These comparative experiments showed that Pvu II and EcoR V (blunt end) produced the greatest number of micronuclei whereas EcoR 1 and BamH 1 (cohesive ended) produced micronuclei only slightly above control levels. This experiment indicates that as the frequency of recognition is increased then the number of micronuclei also increases but only with restriction enzymes that produce blunt end dsb's. This result is consistant with Bryant et al. (1989) and hence micronuclusi damage is mainly dependent on the type of dsb which has been produced.

<u>The effect the type of dsb has on clonogenic cell survival (Graph 9)</u>

The clonogenic cell survival assay was performed on the cells using different restriction enzymes. It was also a preliminary to further transformation studies. The restriction enzyme EcoR I showed no marked effect on survival whereas Pvu ll gave a dose response of cell killing with increasing enzyme concentration. This fits in well with the micronucleus studies in that we would assume that the greater the number of micronuclei (hence DNA

damage) then the greater the incidence of cell killing. This was indeed found to be the case and the two experiments indicated that it is the cutting effect of the restriction enzymes (i.e. whether "blunt" or "cohesive" ended) which determined cellular damage and cell death.

The effect the type and frequency of dsb have on clonogenic cell survival (Graph 11)

Comparative experiments using Pvu II, EcoR I, BamH I and EcoR V were conducted to examine the effect of cutting frequency and end structure on cell survival. EcoR I and BamH I had less effect on cell survival whilst Pvu ll and EcoR V had a greater killing effect. Therefore for this comparative study the type of end structure and not cutting frequency determined cell survival. However in this experiment the results only show a comparison and cannot be related to other data as there were no controls due to contamination.

Compiling both micronuclei and survival results for the four different restriction enzymes (graph 12) shows that a high incidence of micronucleus production corresponds to a high level of cell killing.

There is more variation in the numbers of of micronuclei obtained for treatment with 20 units of Pvu II for the different experiments (graphs 7, 8 & 10) than for experiments using radiation to produce micronuclei (graphs 1, 2 & 3). This is probably due to the complexity of the preparation and treatment of cells with the restriction enzymes. This variation may be further compounded by the increased length of exposure to the mutagen.

Filter Elution (Graphs 13, 14 & 15)

The filter elution technique was used as an another method for determining dsb's at the molecular level. Both gamma and X-irradiated cells gave similar curves when assayed. This was not observed when the end point was measured by the production of micronuclei where X-rays produced results approximately two-fold higher than gamma irradiation. It was postulated that this inconsistency was due to the copper filter. However there may not be any considerable variation in results obtained in the filter elution assay due to the sensitivity of the technique. All the cells were sampled in the filter elution study, whereas in the

micronucleus assay only 100 binucleate cells were sampled and used as a representation of the total population treated. Hence it may be that the sample size is too small to give a more accurate representation of the total cell population between X-rays and gamma rays, and explains the two-fold variation. Therefore the filter elution technique is a suitable standard against which to assess restriction enzyme treated cells in order to measure the effect of the dsb. The results from this technique are used to supplement those from the micronucleus and survival assays.

Pvu II gives an increasing eluted fraction with increasing enzyme dose indicating greater DNA damage, while the EcoR I treated cells showed no marked increase of eluted fraction and remained constant with control levels. This is consistent with results obtained (Costa & Bryant, 1990 a,b.) which show that cohesive-ended dsb (because of their overhanging structure) were more readily repaired and were less likely to produce aberrations and micronuclei. However blunt ended damage caused by Pvu II showed a greater eluted fraction. This indicates that the induced damage is either not repaired or repaired at a slower rate compared to damage caused by EcoR I.

Transformation

It has been recognised that the end point of In Vitro transformation has a direct relevance to cell carcinogenesis. In this project a modified transformation system adopted by Kolman et. al. (1989) is used as a preliminary screening for transformed foci.

X-Rays (Graph 16)

The results obtained from this experiment were consistent with results obtained by Kolman (1989): as radiation dose increases there is an increasing transformation frequency and a dose effect curve is obtained. However the transformation per survivor is considerably higher in this experiment than that obtained by Hill & Elkind (1979). There is no apparent reason for this high transformation frequency except that the Kolman system uses 10 flasks for each dose point which is considerably less than that previously used. The variety of lesions obtained from irradiated cells complicates our understanding of what actually causes transformation. Ideally to be able to isolate one type of lesion and examine this lesion's effect on transformation would provide a greater insight into the cause of the transformed phenotype.

The effect of Pvu II on transformation (Graph 17)

Using the Kolman transformation method the restriction enzyme Pvu II has been able to induce morphological cell transformation. The transformation frequency increased with treatment dose and type I, type II, and type III foci were observed. At 20 units the number of transformants are well above control levels and at 40 units Pvu II there was a significant increase in the number of transformants. Enzyme induced transformation was considerably higher than that for radiation. Radiation induced damage is comprised of a variety of lesions whereas restriction enzymes only produce one type of lesion (the dsb) and it is known that the dsb is a major contributor to cellular damage and transformation. Also radiation produces a limited exposure whereas the activity of restriction enzymes within the cell is more prolonged. Streptolysin-O does not seem to have a significant effect on transformation above control levels. Therefore Sreptolysin-O is a potent tool for the introduction of restriction enzymes into the cell without itself causing cellular transformation above control levels. However there is a high background transformation frequency with cells

treated with Pvu II

Earlier transformation studies found Pvu II to cause transformation (Riches & Bryant, 1989). It is postulated that EcoR I induced dsb's are more readily recognised and repaired because of their overhanging nature, as indicated by the filter elution experiment, whilst the Pvu II induced dsb are not. It is this non-repair or mis-repair of the induced chromosomal damage which is thought to cause cellular transformation. From this study it can be concluded that restriction enzymes which initiate 'blunt ended' dsb produce greater chromosomal damage at both the molecular and cellular levels and as a consequence tend to cause cellular transformation. However this would need to be investigated to see if EcoR I can induce transformation.

Thus DNA cut end structure plays an important role in cellular transformation with blunt ended dsb being the major contributor. To validate this further more transformation studies (with a variety of both blunt-ended and cohesive-ended producing restriction enzymes) using traditional transformation systems (Little, 1977; Hall et al., 1985) would need to be conducted.

SUMMARY

1. Streptolysin-O is a suitable agent for porating C3H10T1/2 cells to ensure the entry of restriction enzymes. Optimal experimental conditions were found at a concentration of 0.271 units with a sampling time of 48 hours for the cell line C3H10T1/2.

2. Pvu II (blunt) is effective at producing a micronucleus dose response curve and is effective at cell killing. EcoR I (cohesive) has no significant effect in producing micronuclei or cell killing in the dose range studied (this result is similar with EcoR V (blunt) and BamH I (cohesive) restriction enzymes).

3. Analysing end type structure using filter elution showed that Pvu II was effective at producing significant molecular damage.

4. Pvu II shows a direct relationship to transformation frequency and this also seems to be determined by the type of end structures produced.

REFERENCES

Barrett, J.C. & Fletcher, W.F.,(1987),
Cellular and molecular mechanisms of multistep carcinogenesis in cell culture models,
In Mechanisms of Environmental Carcinogenesis, **2**, Multistep Models of Carcinogenesis, Barrett, J.C.(ed.) p. 73. CRC Press, Boca Raton.

Bertram, J.S., & Heidelberger, C., (1974),
Cancer Research, **34**, 526-537.

Borek, C., (1980),
X-ray-Induced In Vitro Neoplastic Transformation of Human Diploid Cells,
Nature, **283**, 776-778.

Bicknell, D. C., Sutherland, D. R., Stanbridge, E.J., & Greaves, M.F.,(1985),
Monoclonal Antibodies Specific for a Tumour Associated Membrane Phosphoprotein in Human Cell Hybrids,
Hybridoma, **4,** No. 2

Bishop, J.M., (1987) ,
Science, **235**, 305.

Bishop, D. T., Williamson, J. A., & Skolnick, M. H., (1983),
A Model for Restriction fragment Length Distributions,
American Journal of Human Genetics, **35**, 795-815.

Berwald, Y., & Sachs, L., (1965),
In Vitro Transformation of Normal Cells to Tumour Cells by Carcinogenic Hydrocarbons,
Journal of the National Cancer Institute, **35**, 641-661.

Blocher, D., & Pohlit, W., (1982),
DNA Double Strand Breaks in Ehrlich Ascites Tumour Cells at Low Doses of X-rays II. Can Cell Death be Attributed to Double Strand

Breaks?
International Journal of Radiation Biology, **42**, 329-338.

Bradley, M.O. & Kohn, K. W., (1979),
X-ray Induced DNA Double Strand Break Production and Repair in Mammalian Cells as Measured by Neutral Filter Elution,
Nucleic Acids Research, **7**, 793-804.

Bryant, P. E., (1984),
Enzymatic Restriction of Mammalian DNA Using Pvu II and BamH I: Evidence for the Double Strand Break Origin of Chromosomal Aberrations,
International Journal of Radiation Biology, **46**, 57-65.

Bryant, P. E., (1985),
Enzymatic Restriction of Mammalian DNA: Evidence for Double Strand Breaks as Potentially Lethal Lesions,
International Journal of Radiation Biology, **48,** 55-60.

Bryant, P. E., (1988)
Use of Restriction Endonucleases to Study Relationships Between DNA Double Strand Breaks, Chromosomal Aberrations and Other End-points in Mammalian Cells,
International Journal of Radiation Biology, 54, NO. 6, 869-890.

Bryant, P. E., (1986),
Immediate and Repair Induced DNA Double Strand Breaks in Mammalian Cells,
Mechanisms of DNA Damage and Repair; Implications for Carcinogenesis and Risk Assessment,
Plenum Press 1986.

Bryant, P. E. & Christie, A., F., (1989),
Induction of Chromosomal Aberrations in CHO Cells by Restriction Endonucleases: Effects of Blunt and Cohesive-Ended Double Strand Breaks in Cells Treated by 'Pellet' Methods,
Mutation Research, **213**, 233-241.

Bryant, P. E. & Riches, A. C , (1989) ,
Oncogenic Transformation of Murine C3H10T1/2 Cells Resulting from DNA Double-Strand Breaks Induced by a Restriction Endonuclease,
British Journal of Cancer, **60**, 852-854.

Bryant, P. E., (1992),
Induction of Chromosomal Damage by Restriction Endonuclease in CHO Cells Porated with Streptolysin O,
Mutation Research, **268**, 27-34.

Carrano, A.V., (1973),
Chromosome Aberrations and Radiation Induced Cell Death, II. Predicted and Observed Cell Survival,
Mutation research, **17**, 355-357.

Chen, T.T. & Heidelberger, C., (1969),
Cultivation In Vitro of Cells Derived From Adult C3H Mouse Ventral Prostate,
Journal of the National Cancer Institute, **42**, 903-909.

Costa, N. D. & Bryant, P. E., (1990),
The Induction of DNA Double Strand Breaks in CHO Cells by Pvu II: Kinetics using Filter Elution (pH 9.6) ,
International Journal of Radiation Biology, **57**, NO. 5, 933-938.

Costa, N. D. & Bryant P. E., (1991),
Differences in Accumulation of Blunt and Cohesive-Ended Double-Strand Breaks Generated by Restriction Endonucleases in Electroporated Cells,
Mutation Research, DNA Repair, **254**, 239-246.

Elkind, M. M., Hill, C. K. & Han, A., (1985)
Repair and Misrepair in Radiation-Induced Neoplastic Transformation
Carcinogenesis, **10**.

Fenech, M. & Morley, A. A., (1985) ,
Measurement of Micronuclei in Lymphocytes,
Mutation Research, **147**, 29-36.

Frankenberg, D., Frankenberg-Schwager, M. & Harbich, R., (1984),
Interpretation of the Shape of Survival Curves in Terms of Induction and Repair-Misrepair of DNA Double Strand Breaks,
British Journal of Cancer, **49,** Suppl. **VI**, 233.

Gudrun, A., Bader, M., Bhakdi, S. & Gratzl, M., (1989),
Introduction of Macromolecules into Bovine Adrenal Medullary Chromaffin Cells and Rat Pheochromocytoma Cells (PC12) by

Permeabilisation with Streptolysin O; Inhibitory Effect of Tetanus Toxin on Catecholamine Secretion,
Journal of Neurochemistry,1989.

Han, A., Hill, C. K. & Elkind, M. M., (1979),
Transformation of Mouse C3H10T1/2 Cells by Single and Fractionated Doses of X-rays and Fission Spectrum Neutrons,
Cancer Research, **39**, 123-130.

Han, A., Hill, C. K., & Elkind, M. M., (1980),
Cancer Research, **40**, 3328-3332.

Han, A., Hill, C. K. & Elkind, M. M.,(1984),
Radiation Research, **99**, 249-269.

Hall, E. J. & Hei, T. K., (1985),
Oncogenic Transformation of Cells in Culture : Pragmatic Comparisons of Oncogenicity, Cellular and Molecular Mechanisms ,
International Journal of Radiation Oncology Biol. Phys.,**12** , 1909-1921.

Ho, K. Y., (1975),
Induction of Double Strand Breaks by X-rays in a Radiosensitive Strain of the Yeast *Saccaromyces cerevisae*,
Mutation Research, **30**, 327-334.

Joshi, G.P., Nelson, W.J., Revell, S.H. & Shaw, C. A., (1982),
X-ray Induced Chromosome Damage in Live Mammalian Cells, an Improved Measurement of its Colony Forming Ability,
International Journal of Radiation Biology, **41**, 161-181.

Kennedy, A. R., Fox, M. & Little, J.B.,(1980a),
Relationship Between X-ray Exposure and Malignant Transformation in C3H10T1/2 Cells,
Proceedings of the National Academy of Science, U.S.A., **77**, 7262-7266.

Kennedy, A. R. & Little J. B., (1984),
Evidence that a Second Step in X-ray Induced Oncogenic Transformation In Vitro Occurs During Cellular Proliferation
Radiation Research, **99**, 228-248.

Klein, G. & Klein, E., (1984),

Oncogenic Activation and Tumour Progression,
Carcinogenesis, **5,** 429.

Klein, G , (1987),
The Approaching Era of the Tumour Suppressor Genes,
Science, **238**, 1539-1545.

Kohn, K. W.,
Assessment of DNA Damage by Filter Elution Assays,
Mechanisms of DNA Damage and Repair; Implications for Carcinogenesis and Risk Assessment,
Plenum Press 1986.

Kolman, A., Naslund, M., Siv, O., Gian-Paola, S. & Meyer, A. ,(1989),
Comparative Studies of In Vitro Transformation By Ethylene Oxide and Gamma-Radiation of C3H10T1/2 Cells ,
Mutagenesis, **4**, NO.4, 58-61.

Little, J. B.,(1977a),
Mouse Embryo Fibroblast Cell Lines in Studies of Transformation by Chemical and Physical agents,
In: Methods for Carcinogenisis Tests at the Cellular Level and their Evaluation for the Assessment of Occupational Cancer Hazards,
Proceedings of the Scientific Committee on Occupational and Environmental Health, Milan, Carlo Erba Foundation, p. 14-30.

Little, J. B. , (1985),
Cellular Mechanisms of Oncogenic Transformation In Vitro,
Reprinted from: Transformation Assay of Established Cell Lines: Mechanisms and Application,
IARC Scientific Publications No. 67, T. Kakunga & H. Yamasaki. Eds.

Mordan,L., J., Martner, J., E. & Bertram, J., S., (1983),
Quatitative Neoplastic Transformation of C3H10T1/2 Fibroblasts: Dependence Upon the Size of Initiated Cell Colony at Confluence,
Cancer Research, **43**, 4062-4067.

Moses, S. A. M., Christie, A. F. & Bryant, P. E., (1990),
Clastogenicity of Pvu II and EcoR I in Electroporated CHO Cells Assayed by Metaphase Chromosomal Aberrations and by Micronuclei using the Cytokinesis-Block Technique ,
Mutagenesis, **5**, NO. 6, 599-603.

Natarajan, A. T., Obe, G., Van Zeeland, A.A., Palitti, F., Meijers, M. & Verdegaal-Immerzeel, E.A.M., (1980),
Molecular Mechanisms Involved in the Production of Chromosomal Aberrations. II Utilisation of Neurospora endonuclease for the Study of Aberration Production by X-rays in G1 and G2 Stages of The Cell Cycle,
Mutation Research, **69,** 293.

Natarajan, A.T. & Obe, G., (1984),
Molecular Mechanisms Involved in the Production of Chromosomal Aberrrations,
Chromosoma, **90**, 120-127.

Obe, G. & Winkel, E., (1985)
The Chromosome Breaking Activity of the Restriction Endonuclease Alu I in CHO Cells is Independent of the S-Phase of the Cell Cycle,
Mutation Research, **152**, 25-29.

Okayasu, R. & Iliakis (1989),
Linear DNA Elution Dose-response Curves Obtained in CHO Cells with Non-unwinding Filter Elution after Appropriate Selection of the Lysis Conditions,
International Journal of Radiation Biology , **55**, 569-581.

Paquette, P. & Little, J.B. (1992),
Genomic Rearrangements in Mouse C3H10T1/2 Cells Transformed by X-rays, UVC-C, and 3-Methylcholanthrene, Detected by a DNA Fingerprint Assay,
cancer Research, **52**, 5788-5793.

Radford, I.R., (1985),
The Level of Induced DNA Double Strand Breakage Correlates with Cell Killing after X-irradiation,
International Journal of Radiation Biology, **48**, 45-54.

Redpath, J. L., Antoniono, R. J., Sun, C., Gerstenberg, H. M., Blakely, W. F. (1995),
Late Mitosis/ Early G1 Phase and Mid G1 Phase are not Hypersensitive Cell Cycle Phases for Neoplastic Transformation of Hela x Skin Fibroblast Human Hybrid Cells Induced by Fission Spectrum Neutrons,
Radiation Research, **141**, 37-43.

Reznikoff, C. A., Brankow, D. W. & Heidelberger, C., (1973),
Establishment and Characterisation of a Cloned Line of C3H Mouse Embryo Cells Sensitive to Postconfluence Inhibition of Division,
Cancer Research, **33**, 3231-3238.

Reznikoff, C. A., Bertram, J. S., Brankow, D. W. & Heidelberger, C., (1973),
Quantitative and Qualitative Studies of Chemical Transformation of Cloned C3H Mouse Embryo Cells Sensitive to Postconfluence Inhibition of Cell Division,
Cancer Research, **33**, 3239-3249.

Savage, J. R. K., (1988)a,
Some Comments on the Relationship between Chromosomal Aberrations and Micronuclei,
Clinical Cytogenetics Bulletin, **2**, 32-34.

Savage, J. R. K., (1988)b,
A Comment on the Quantitative Relationship Between Micronuclei and Chromosomal Aberrations,
Radiation Research, **207**, 33-36.

Stanbridge, E.J., Der, C.J., Doersen, C.J., Nishimi, R.Y., Peehl, D.M., Weissman, B.E., and Wilkinson, J.E., (1982),
Human Cell Hybrids: Analysis of Transformation and Tumorigenicity,
Science, **215**, 252-259.

Sun, C., Redpath, M., Colman, M., Stanbridge, E. J., (1988),
Further Studies on the Radiation-Induced Expression of a Tumor-Specific Antigen in Human Cell Hybrids,
Radiation Research, **114**, 84-93.

Sutherland, D. R., Bicknell, D. C., Downward, J., Parker, P., Waterfield, M.D., Baker, M.A., Greaves, M.F.,& Stanbridge, E.J., (1986),
Stuctural and Functional Features of a Cell Surface Phosphoglycoprotein Associated with Tumorigenic Phenotype in Human Fibroblast x HeLa Cell Hybrids,
The Journal of Biological Chemistry, Vol. **261**, No. 5, 2418-2424.

Tanaka, K., Sekiguchi, M., & Okada, Y., (1975),
Restoration of Ultraviolet-induced Unscheduled DNA Synthesis of *Xeroderma pigmentosum* Cells by the Concomitant Treatment with Bacteriophage T4 Endonuclease V and HVJ (Sendai virus),

Proceedings of the National Acadamy of Sciences, U.S.A., **72**, 4071-4075.

Terzaghi, M. & Little, J.B. , (1976) ,
X-radiation-induced Transformation in a C3H Mouse embryo-derived cell Line,
Cancer Research, **36**,1367-1374.

Thomson , J.F., Williamson, F.S., Grahn, D., & Ainsworth, (1981),
Radiation research, **86**, 572-588 .

Tuschy, S. & Obe, G., (1988),
Potentiation of Alu I Induced Chromosome Aberrations by High Salt Concentrations in Chinese Hamster Ovary Cells,
Mutation Research, **207**, 83-87.

Weibezahn, K.F., & Coquerelle, T., (1981),
Radiation Induced DNA Double Strand Breaks are Rejoined by Ligation and Recombination Processes,
Nucleic Acids Research, **13**, 3139-3150.

Winegar, R. A. & Preston, R. J., (1988),
The Induction of Chromosome Aberration by Restriction Endonucleases that Produce Blunt-End or Cohesive-End Double-Strand Breaks ,
Mutation Research, **197**, 141-149.

Winegar, R. A., Philips, J.W., Youngblom, J.H., & Morgan, W.F., (1989),
Cell Electroporation is a Highly Efficient Method for Introducing Restiction Endonucleases into Cells,
Mutation Research, **225**, 49-53.

APPENDIX

Compilation of Data Obtained to Produce Graphs

Graph1

X-irradiation: damage measured by production of micronuclei sampled at 24 hours. A set of 3 experiments.

	Experiment				
Dose (Gy)	1	2	3	Mean	S.E. (+/-)
0	4	3	4	3.7	0.3
2	57	47	52	52.0	2.9
4	94	110	92	100.3	4.9
6	127	159	148	144.7	9.4

Graph 2

X-Irradiation: damage measured by production of micronuclei sampled at 48 hours. A set of 3 experiments.

	Experiment					
Dose (Gy)	1	2	3	4	Mean	S.E. (+/-)
0	6	5	5		5.3	0.3
2	58	42	69		56.3	7.8
4	68	105	113		95.3	13.9
6	204	179	157	201	185.3	10.9

Graph 3

Gamma Irradiation: damage measured by cell killing sampled at 48 hours. A set of 3 experiments.

	Experiment				
Dose (Gy)	1	2	3	Mean	S.E (+/-)
0	2	5	3	3.3	0.8
2	32	44	38	38.00	6.00
4	59	63	69	63.67	2.91
6	73	78	100	83.67	8.29
8	96	108	106	103.33	3.71

Graph 4

X-Irradiation: damage measured by cell killing. A set of 2 experiments of at least three replicates replicates.

Dose (Gy)	Experiment	Cells Plated	Survivors	% Survival
0	1	212	95 89 85 91 104 96	37.6
	2	150	56 36 48 50	31.6
2	1	266	65 72 82	27.4
	2	150	29 37 35 40	23.5
4	1	480	74 79 83	16.4
	2	300	20 23 27 20	7.5
6	1	3100	116 136 145	4.3
	2	1500	34 82 73 70	4.3

The survival figures are related to the controls which are taken as 100% and the means and standard errors of the experiments are taken from these percentages.

Dose (Gy)	% Survivors	S.E. (+/-)
0	100	
2	73.6	0.7
4	33.7	10.0
6	12.5	1.1

Graph 5

Streptolysin-O: damage measured by cell killing. A set of 2 experiments of 3 replicates.

SLO (Units)	Experiment	Cells Plated	Survivors	% Survival
0.000	1	284	37 49 54	16.4
	2	305	73 68	23.1
0.090	1	282	56 62 59	20.9
	2	353	48 55 42	13.7
0.181	1	319	39 45 44	13.4
	2	412	20 25 13	4.7
0.271	1	318	20 12 20	5.5
	2	1446	60 61 37	3.6
0.361	1	336	11 11 9	3.1
	2	2240	59 27 30	1.7
0.452	1	334	7 2 4	1.3
	2	4974	70 71 77	1.5

The survival figures are related to the controls which are taken as 100% and the means and standard errors of the experiments are taken from these percentages.

Streptolysin-O (Units)	% Survivors	S.E. (+/-)
0.000	100	18.5
0.090	86.5	12.4
0.181	45.2	21.6
0.271	22.5	4.6
0.361	12.1	3.5
0.452	7.0	0.5

Graph 6

Streptolysin-O Titration: damage measured by production of micronuclei. A set of 2 experiments.

	Experiment			
Streptolysin-O (Units)	1	2	Mean	S.E. (+/-)
0.0	1	0	0.0	0.0
0.036	7	6	6.5	0.5
0.072	19	12	15.5	3.5
0.108	10	20	15.0	5.0
0.145	9	22	15.5	6.5
0.166	28	31	29.5	1.5
0.199	39	37	38.0	1.0
0.217	61	65	63.0	2.0
0.235	80	70	75.0	5.0
0.253	92	86	89.0	3.0
0.271	94	85	89.5	4.5
0.289	76	67	71.5	4.0
0.325	36	26	31.0	5.0

Graph7

Time Sampling: damage measured by production of micronuclei. A set of 2 experiments.

	Experiment			
Time (Hours)	1	2	Mean	S.E. (+/-)
0	3	4	3.5	0.5
6	4	10	7.0	3.0
12	11	16	13.5	2.5
18	9	20	14.5	5.5
24	29	20	24.5	4.4
30	15	21	18.0	1.7
36	47	39	43.0	4.0
42	56	41	48.5	7.5
46	53	40	46.5	6.5
52	44	48	46.0	2.0
61	43	49	46.0	3.0

Graph 8

Restriction Enzymes: damage measured by production of micronuclei at varying doses of Pvu II and EcoR I.

	Experiment							
Dose	1	2	3	4	5	6	Mean	S.E. (+/-)
PvuII								
0	6	4	5				5.0	0.6
1	12	20					16.0	4.0
3	65	29					47.0	18.0
6	64	42					53.0	11.0
10	78	65	73				72.0	3.0
20	87	78	90	53	99	66	76.8	7.3
30	122	95	64	63			86.0	14.1
EcoR I								
0	2	4					3	1.0
10	7	6					6.5	0.5
20	6	6					6.0	0.0
30	8	7					7.5	0.5
40	13	8					10.5	2.5

Graph 9

Restriction Enzymes: damage measured by cell killing. A set of 3 experiments of 3 replicates. Survivors with number of cells plated in brackets.

	Experiment		
Dose (units)	1	2	3
Pvu II			
0	52 69 63 (2200)	90 81 70 (1125)	152 149 144 (2249)
3	37 26 28 (1623)		
10	15 14 16 (1973)		57 65 62 (2389)
20	15 8 10 (1813)	9 16 12 (900)	60 42 38 (2457)
30	11 19 12 (1943)		41 43 37 (2457)
40		2 4 7 (925)	23 28 23 19 (2319)
EcoR I			
0	79 87 75 (2225)	164 148 167 (1521)	
3		138 152 139 (1408)	
10	66 58 57 (1853)	127 138 140 (1478)	
20	71 63 60 (1977)	131 165 151 (1486)	
30	74 74 57 (1983)	155 133 146 (1436)	
40	55 67 62 (2072)		

The survival figures are related to the controls which are taken as 100% and the means and standard errors of the experiments are taken from these percentages.

Dose (Units)	Pvu II	S.E. (+/-)	EcoR I	S.E. (+/-)
0	100.0		100.0	
3	67.4	0.5	97.1	
10	31.1	3.9	89.8	0.8
20	24.0	2.0	93.1	2.4
30	23.4	1.5	95.7	0.5
40	10.7	4.7	82.2	

Graph 10
Restriction Enzymes: measured by the formation of micronuclei sampled at 48 hours.

	Experiment				
Dose (20 units)	1	2	3	Mean	S.E. (+/-)
Pvu II	99	60	53	70.6	14.3
EcoR I	3	8	4	5.0	1.5
BamH I	12	11	12	11.7	0.3
EcoR V	22	41	31	31.3	5.5

Graph 11
Restriction Enzymes: damage measured by cell killing.

Dose (20 units)	Cells Plated	Survivors	Survivors (per 1000 plated)	S.E. (+/-)
Pvu II	1996	26 21 20 22	11.2	0.7
EcoR I	2056	68 71 63 68	32.8	0.8
BamH I	2076	76 64 56 57	30.4	2.2
EcoR V	2083	65 58 53 55	27.1	1.6

Graph 12

X- Irradiation: damage measured by filter elution. A set of 2 experiments. Data shows control values subtracted.

	Experiment			
Dose (Gy)	1	2	Mean	S.E (+/-)
0	0.024	0.027		
5	0.22	0.27	0.24	0.03
10	0.49	0.41	0.45	0.04
15	0.49	0.43	0.46	0.03
20	0.75	0.63	0.69	0.06

Graph 13

Gamma Irradiation: damage measured by filter elution. A set of 2 experiments. Data shows control values subtracted.

	Experiment			
Dose (Gy)	1	2	Mean	S.E (+/-)
0	0.21	0.18		
5	0.024	0.13	0.08	0.05
10	0.33	0.44	0.38	0.05
15	0.45			
20	0.57	0.63	0.60	0.03

Graph 14

Restriction Enzyme: damage measured by filter elution. A set of 2 experiments. Data shows control values subtracted.

Dose (Gy)	Experiment 1	2	Mean	S.E (+/-)
0	0.075	0.098		
Pvu II (units)				
50	0.25	0.05	0.15	0.1
100	0.31	0.36	0.34	0.02
150	0.27	0.33	0.30	0.03
200	0.34	0.32	0.33	0.01
EcoR I (units)				
100	0.19	0.12	0.15	0.04
200	0.13	0.13	0.13	

Graph 15

X-irradiation: measured by the transformation of normal cells. A set of 2 experiments of 10 replicates. Survival curve data from graph 4 data.

Dose (Gy)	Experiment	Survivors (per flask)	Transformants (per flask)	T.F./ 1000 survivors
0	1	1128	3/10	0.26
	2	950	0/10	0.00
2	1	823	9/10	1.09
	2	705	1/10	0.14
4	1	492	6/10	1.22
	2	225	5/10	2.22
6	1	128	4/10	3.13
	2	130	3/10	2.31

The mean and standard error of the transformation frequency of the two experiments is shown below.

Dose (Gy)	Transformation Frequency (TF) (per 1000 survivors)	S.E. (+/-)
0	0.13	
2	0.62	0.47
4	1.72	0.50
6	2.72	0.41

Graph 16

Restriction Enzyme Pvu II: damage measured by the transformation of normal cells. One experiment of 10 replicates. The number of cells plated to obtain survivors are in brackets and transformation frequency (T F) is per 1000 surviving cells.

Dose (units)	Survivors (per no.plated)	Cells Seeded (per transformation flask)	Transformants (per flask)	TF
0	51 30 83 (1080)	3600	3/10	1.64
SLO	90 81 70 (1125)	4500	7/10	2.19
20	9 16 12 (900)	3600	4/10	8.33
40	2 4 7 (925)	3700	5/10	31.25