STUDY OF DNA DAMAGE INDUCED BY LOW AND HIGH LINEAR ENERGY TRANSFER (LET) RADIATION

ABSTRACT

By

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SUBMITTED
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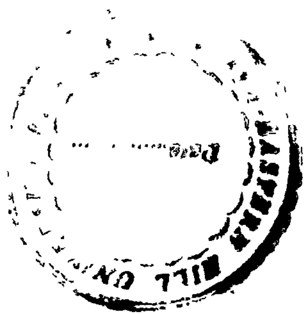

Biological effects of radiation emancipate from the interaction of radiation with matter comprising all levels of biological organization. The interaction causes subtle and obvious "changes" in various components of cells, tissues and organism. The effected living system usually "responds" to the "changes". The living system is normally endowed with ways and means of variable magnitude and types to counter such "changes". Consequently and depending on the quality and quantity of the changes, some such changes are reverted or repaired. The remaining component or "changes" are likely to effect the process of life and are commonly referred to as "damages". The outcome of these intricately balanced and, to a great extent not clearly understood, processes culminate into cancer, heritable changes and cell death.

The damages include a variety of changes in the genetic material, which, among others, includes base changes, mutations, strand breaks and transformation. Radiation also induces and influences the process of programmed cell death. Consequences of these changes culminate into genomic instability. These events and their molecular mechanisms are only partly understood in case of low-LET radiation. On the other hand, the situation for high-LET radiation remains far from being clear. A proper assessment and evaluation of biological effects of low- and high-LET radiation is therefore, highly relevant. It is not only important in fundamental understanding of cellular response to effects of radiation, but also has applied potentials.

The search for an effective way of applying radiation to human cancer radiotherapy has been probably one of the main objective of radiobiologists. Its obvious importance has led to extensive radiobiological studies. One universal aim and interest in radiobiology research is to understand the interaction and mechanisms of action of ionizing radiation and its effects on biological systems. It may be of low linear energy transfer (LET) radiation i.e., sparsely ionizing radiations, such as X-rays, gamma rays, electrons, ultra soft X-rays or high-LET radiation i.e., densely ionizing like alpha particles, protons, neutrons etc. and other heavier particles produced by certain types of high energy accelerators. The knowledge of this is important in medicinal application such as radiotherapy and radio-diagnostics, and also for radiation protection on earth and in the space environment.

Ever since the elucidation of structure of deoxyribonucleic acid (DNA) molecule in the early 1950's by Watson and Crick, studies related to understanding and manipulation of DNA has grown by leaps and bound in various branches of biological sciences. The focus has not been different in radiobiological investigations too. Because of the universal acceptance of DNA as the genetic material, it is considered to be a critical target for damage induced by radiation in cellular systems. The damages inflicted upon by radiation

are known to cause the consequent biological effect. Apoptosis, transformation and carcinogenesis via mutation and reproductive cell death are closely related to molecular damages in the DNA. The DNA damages have been studied experimentally and theoretically employing several approaches. Consequently, several models have been established in order to explain the biological observations. At molecular level, the studies involved different endpoints like cell survival, chromosomal aberration, DNA rejoining, mutation and DNA strand breaks both single strand breaks (SSB) and double strand breaks (DSB). Yet variation in results at different conditions defies working out a firm stand in explaining the mechanism. Most of the existing studies on DNA damage give quantitative insight such as, the yields of such damages and their dependence on radiation quality. The knowledge of the quality and nature of DNA damage is limited. Interaction of radiation with a matter is random. The interaction elicits changes in the target site, which may alter the normal course of cellular metabolism, their consequential repair and expression of the effects.

One general view is that it may be due to the differential repair capacity that each living cell possesses to repair damages particularly inflicted by radiation. This has an important implication since radiosensitivity of a cell is mostly defined depending on the ability of a cell to repair the wide spectrum of DNA damages. An increasing body of experimental evidences has accumulated indicating that distribution of the DNA radiation damage and the complexity and fluidity of the nuclear organization can affect repair. This has led to an indication that the structural organization of DNA or chromatin compactness determines the radiosensitivity of cells. Well within this line of observation, experimental reports also suggest that structural organization of DNA may not be the only factor influencing the radiosensitivity. Thus, because of such diverse observations, it is not surprising that a single concept has so far not emerged in defining radiosensitivity of a cell. The molecular basis of the variable inherent radiosensitivity and genomic instability, therefore, remains enigmatic. In this context, understanding the nature of the initial lesion to its DNA and its link to the eventual expression of biological damage is of utmost importance.

In the light of these information this work envisages to study the DNA damage induced by low- and high-LET radiation with the aim of contributing in the understanding of the molecular consequences of radiation induced DNA damages.

The study has been separated into two main sections. In the first section, a system was selected where the effect of radiation on DNA damage in non-cellular condition could be clearly observed at molecular level. For this reason, naked plasmid and bacterial genomic DNA were selected. The study attempts to understand the quality and nature of

DNA damage induced by low- and high-LET radiations.

In the latter section, the investigation covers study on effect of radiation on DNA and other components, and their response in cellular condition. Mammalian cells *in vitro* and *ex vivo* was selected for this purpose. The study attempts to describe and understand the radiation induced DNA damage in relation to the biological endpoints that is measured.

The study brings out the following main points:

* Low-LET ^{60}Co γ-radiation causes predominantly DNA SSB than DSB in pMTa4 plasmid. Since plasmid DNA was irradiated in aqueous solution, •OH seems to be the major determinant in the production of SSB.

* The formation of defined extra fragments from irradiated pMTa4 DNA by *Hae II* and *Nci I* (but not by others 7 RE that were used in the investigation) suggest a non-random manifestation of effect by radiation.

* Within the conditions and parameter studied it suggest that GC-rich nucleotides were being more affected or chemically modified upon exposure to ^{60}Co γ-radiation. It may be speculated that non-GC-motif were not affected by ^{60}Co γ-radiation due to which it did not afford any resistance to certain RE (*Acc I, Bgl I, Bgl II, Dra I, Hinf I, Ksp I* and *Pvu II*).

* Unlike in low-LET, high-LET ^{7}Li particle radiation induced more DSB than SSB in pMTa4. High-LET radiation lead to a denser deposition of ionization than γ-rays and this could explain the increase in the yield of DSB.

* Even though high-LET ^{7}Li particle radiation caused more DSB than SSB the nature of damage on pMTa4, as revealed by RE approach, appeared to be similar to that observed for low-LET ^{60}Co γ-radiation. RE fragmentation analysis indicates that, though the extent and impact of the damage induced by low- and high-LET may differ, the molecular basis of damage may likely follow a similar mechanism.

* The study suggests the likely possibility that radiomodified GC nucleotides would form important premutagenic lesions. This indication also points that clusters of GC in the DNA molecule might very likely form hotspots for radiation induced damages.

* While further detailed investigation would be required, it opens up the likely possibility that inherent radiosensitivity and genome instability may be at least partly determined by the GC-richness of nucleotide sequence in the DNA.

* Since in majority of eukaryotic genomes, especially human genome, there exists "CpG islands", our results suggest a likely possibility that "genes" are more radiosensitive than "non-gene" components of a genome.

* RE can be used in the partial characterization of radiation induced specific nucleotide damage in small defined DNA molecules. This approach may also be applicable in study of damages induced by other agents than radiation.

* RE approach does not reveal any apparent modification induced by radiation in *E. coli* genomic DNA. Due to the large number of undefined fragments produced as compared to the plasmid (pMTa4), the qualitative fragmentation analysis was not revealing. This makes RE approach and analysis by agarose gel electrophoresis non-sensitive for studying specific nucleotide damage in a large and highly complex DNA molecule.

* The alkaline comet assay was able to detect ^{137}Cs γ-radiation induced SSB in human kidney T1 cells. Under these conditions, SSB favored the relaxation or decondensation of chromatin.

* SSB, however, got partially repaired or rejoined during repair permissive conditions (20-min incubation at 37 °C). Under these conditions, repair or rejoining of SSB appeared to allow the chromatin reorganize its structural configuration favoring a re-condensation.

* Using a novel immuno-blot assay (Slot and Western blot), a general inhibition of poly-ADP-ribosylation (PAR) in *ex vivo* mouse spleen cells upon ^{60}Co γ-irradiation was observed.

* The PAR of total cellular protein was inhibited initially at 1 Gy after which the level increased gradually reaching the control level. For histone proteins, the inhibition was observed up to 2-4 Gy.

* Under identical experimental conditions maximum induction of apoptosis or programmed cell death was measured at about 2 Gy in *ex vivo* mouse spleen cells upon ^{60}Co γ-irradiation. It is suggested that reduction in the level of PAR of nuclear proteins occur during the initiation of radiation induced apoptosis.

* The lowering of PAR can be due to the loss of poly-ADP-ribose polymerase (PARP) activity. The results from *ex vivo* mouse spleen cells thus, support the theory of PARP cleavage by caspases during the execution phase of apoptosis. It can be inferred that PAR degrading enzymes such as, poly-ADP-ribose glycohydrolase may also be inactivated by ^{60}Co γ-radiation.

* The lowering of PAR may be proposed as a biomarker during early stages of radiation-induced apoptosis. Thus, the immuno-blot assay of PAR can potentially be used as a predictive assay for monitoring progression of radiotherapy.

* The study with ^{11}B charged particle on three cell lines (T1, R1H and HG) *in vitro* led to loss or inactivation of acetylcholine esterase enzyme as well as cell death. These cell membrane related biochemical parameters suggest that the accelerated charged ^{11}B particle effected cell membrane variantly in different cell types *in vitro*. Further investigations are necessary to see if these changes were only physiological or extended to molecular level.

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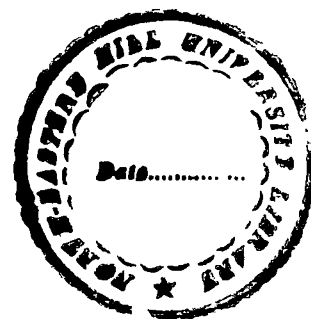

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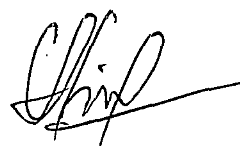

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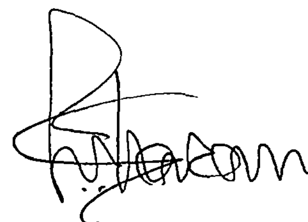

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Dedicated to

My beloved Apo and Ayo

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ABBREVIATIONS

A	Adenine
Ab	Antibody
ADP	Adenosine diphosphate
Ag	Antigen
Amp	Ampicillin
APS	Ammonium per sulphate
ATP	Adenosine triphosphate
^{11}B	Boron isotope
BME	Eagle's basal medium
bp	Base pair
BSA	Bovine serum albumin
DSB	Double strand break
C	Cytosine
°C	Degree centigrade
$CaCl_2$	Calcium chloride
cm	Centimetre
CTAB	Cetyltrimethylammonium bromide
DTNB	5,5'-Dithio-bis[2-nitrobenzoic acid]
DNA	Deoxyribonucleic acid
DNase I	Deoxyribonuclease I
DTT	Dithiothreitol
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme linked immunosorbent assay
Fig.	Figure
G	Guanine
gm	Gram
g	Centrifugal force
Gy	Gray
IMDM	Iscove's modified Dulbecco's medium
KeV	Kilo electron volt
^{7}Li	Lithium isotope

LB	Luria Bertani medium
LET	Linear energy transfer
lit.	Litre
min^{-1}	Per minute
ml^{-1}	Per millilitre
mM	Millimolar
NAD	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
NC	Nitrocellulose membrane
nm	Nanometre
O/N	Overnight
PAR	Poly-ADP-ribosylation
PARP	Poly-ADP-ribose polymerase
PBS	Phosphate buffered saline
PCD	Programmed cell death
RNase	Ribonuclease
rpm	Revolution per minute
RT	Room temperature
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SE	Standard error
sec	Second
SSB	Single strand break
T	Thymine
TBS	Tris buffered saline
TEMED	NNN'N-Tetramethylethylene diamine
TTBS	Tween-20 tris buffered saline
UV	Ultra violet
V	Volts
µg	Microgram
µl	Microlitre
µm	Micron

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SECTION A

GENERAL INTRODUCTION

Biological effects of radiation emancipate from the interaction of radiation with matter comprising all levels of biological organization. The interaction causes subtle and obvious "changes" in various components of cells, tissues and organism. The effected living system usually "responds" to the "changes". The living system is normally endowed with ways and means of variable magnitude and types to counter such "changes". Consequently and depending on the quality and quantity of the changes, some such changes are reverted or repaired. The remaining component or "changes" are likely to effect the process of life and are commonly referred to as "damages". The outcome of these intricately balanced and, to a great extent not clearly understood, processes culminate into cancer, heritable changes and cell death.

The damages include a variety of changes in the genetic material, which, among others, includes base changes, mutations, strand breaks and transformation. Radiation also induces and influences the process of programmed cell death. Consequences of these changes culminate into genomic instability. These events and their molecular mechanisms are only partly understood in case of low-LET radiation. On the other hand, the situation for high-LET radiation remains far from being clear. A proper assessment and evaluation of biological effects of low- and high-LET radiation is therefore, highly relevant. It is not only important in fundamental understanding of cellular response to effects of radiation, but also has applied potentials.

The search for an effective way of applying radiation to human cancer radiotherapy has been probably one of the main objective of radiobiologists. Its obvious importance has led to extensive radiobiological studies. One universal aim and interest in radiobiology research is to understand the interaction and mechanisms of action of ionizing radiation and its effects on biological systems. It may be of low linear energy transfer (LET) radiation i.e., sparsely ionizing radiation, such as X-rays, gamma rays, electrons, ultra soft X-rays or high-LET radiation i.e., densely ionizing like alpha particles, protons, neutrons etc. and other heavier particles produced by certain types of high energy accelerators. The knowledge of this is important in medicinal application such as radiotherapy and radio-diagnostics, and also for radiation protection on earth and in the space environment.

Ever since the elucidation of structure of deoxyribonucleic acid (DNA) molecule in the early 1950's by Watson and Crick, studies related to understanding and manipulation of DNA has grown by leaps and bound in various branches of biological sciences. The focus has not been different in radiobiological investigations too. Because of the universal acceptance of DNA as the genetic material, it is considered to be a critical target for damage induced by radiation in cellular systems. The damages inflicted upon by radiation are known to cause the consequent biological effect. Apoptosis, transformation and

carcinogenesis via mutation and reproductive cell death are closely related to molecular damages in the DNA. The DNA damages have been studied experimentally and theoretically employing several approaches. Consequently, several models have been established in order to explain the biological observations. At molecular level, the studies involved different endpoints like cell survival, chromosomal aberration, DNA rejoining, mutation and DNA strand breaks both single strand breaks (SSB) and double strand breaks (DSB). Yet variation in results at different conditions defies working out a firm stand in explaining the mechanism. Most of the existing studies on DNA damage give quantitative insight such as, the yields of such damages and their dependence on radiation quality. The knowledge of the quality and nature of DNA damage is limited. Interaction of radiation with a matter is random. The interaction elicits changes in the target site, which may alter the normal course of cellular metabolism, their consequential repair and expression of the effects.

One general view is that it may be due to the differential repair capacity that each living cell possesses to repair damages particularly inflicted by radiation. This has an important implication since radiosensitivity of a cell is mostly defined depending on the ability of a cell to repair the wide spectrum of DNA damages. An increasing body of experimental evidences has accumulated indicating that distribution of the DNA radiation damage and the complexity and fluidity of the nuclear organization can affect repair. This has led to an indication that the structural organization of DNA or chromatin compactness determines the radiosensitivity of cells. Well within this line of observation, experimental reports also suggest that structural organization of DNA may not be the only factor influencing the radiosensitivity. Thus, because of such diverse observations, it is not surprising that a single concept has so far not emerged in defining radiosensitivity of a cell. The molecular basis of the variable inherent radiosensitivity and genomic instability, therefore, remains enigmatic. In this context, understanding the nature of the initial lesion to its DNA and its link to the eventual expression of biological damage is of utmost importance.

In the light of these information, this work envisages to study the DNA damage induced by low- and high-LET radiation with the aim of contributing in the understanding of the molecular consequences of radiation induced DNA damages. It is to be noted that, the work with high-LET radiation in both the sections is limited due to scarce beam time that was available at Nuclear Science Center, New Delhi.

The study has been separated into two main sections:

(1). **Study of DNA damage in non-cellular system**:

In this first section, a system was selected where the effect of radiation on DNA damage in non-cellular condition could be clearly observed at molecular level. For this reason, naked plasmid and bacterial genomic DNA were selected. The study attempts to understand the quality and nature of DNA damage induced by low- and high-LET radiations. The results discuss certain likely factors within the DNA molecule that may be influencing its interaction with radiation.

(2). **Study of DNA damage in cellular system**:

In this latter section, the investigation covers study on effect of radiation on DNA and other components, and their response in cellular condition. Mammalian cells *in vitro* and *ex vivo* was selected for this purpose. In a likely effort to shed light on the mechanism of interaction of radiation with DNA in its *vivo* organization, the study attempts to describe and understand the radiation induced DNA damage in relation to the biological endpoint that is measured.

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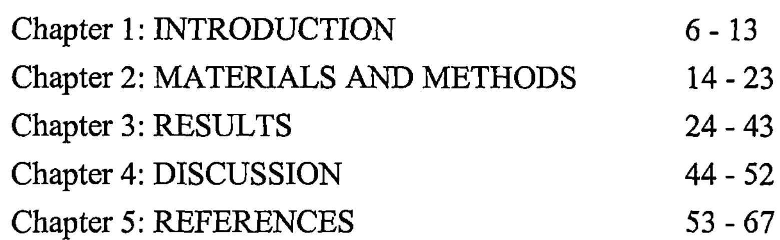
SECTION B

STUDY OF DNA DAMAGE IN NON-CELLULAR SYSTEM



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CHAPTER 1

INTRODUCTION

Ionizing radiation induces a wide range of lesions in DNA. Strong evidences indicate that such DNA damage are the source of the biological consequences of radiation (Painter, 1979; Ward, 1988; Makrigiorgas *et al.,* 1990). It results in the observed biological end points such as mutation, chromosomal aberration, oncogenic transformation, and cell death (Kiefer, 1990; Sharan, 1998; Blakeley and Kronenberg, 1998). In spite of enormous efforts devoted to DNA damage studies, the relationship between radiation induced DNA damage and the different biological effects are not coherently understood. The fact that it produces wide variety of lesions makes the understanding of the molecular processes controlling the response of cells to irradiation a difficult task. An intriguing question arising from such obscurity, which has serious bearing on clinical efficacy of radiotherapy, is the variable inherent radiosensitivity of different organs and species. The identification of the complex processes underlying these differences in radiosensitivity, therefore, remains a fundamental problem in radiobiology.

1.1 DNA DAMAGE INDUCTION:

Model for the initial events that lead to induction of DNA damage by radiation is based on the distribution of ionizing events within the molecule and its surrounding. The damage may be either by indirect or direct effect (Scholes *et al.,* 1969; Roots *et al.,* 1985; O'Niell and Fielden, 1993). The indirect effect is caused by reactive species like $^{\bullet}OH$, $^{\bullet}H$ and e_{aq}^{-} (Chapman *et al.,* 1973, Roots and Okada, 1975), produced from ionization or excitation of water molecules around the DNA molecule. Of the radicals, which interact with DNA, most of the $^{\bullet}H$ and all e_{aq}^{-} react with the nucleotide (NT) bases. While about 20 % of $^{\bullet}OH$ react with the sugar, the rest (80 %) are known to react with the bases. H_3O^+, which is one of the ionization product, however, does not react with DNA but can react with other water radicals (Holley and Chatterjee, 1991). The $^{\bullet}OH$ has been shown to be more effective than $^{\bullet}H$ or e_{aq}^{-} in inducing single strand breaks in DNA when irradiated in aqueous condition (Kuipers and Lafluer, 1998). DNA damages induced by low-linear energy transfer (LET) radiation are mainly via indirect effect (Ward and Kuo, 1970). In the direct effect category the damage is due to the deposition of energy directly onto the DNA molecule. The base ionization is known to be equally probable to the ionization of the sugar-phosphate backbone because of similar electron densities. The direct ionization is also suggested to lead to radical attack equivalent to the $^{\bullet}OH$ on DNA (Michalik, 1992). High-LET radiation is known to cause damage to DNA primarily via direct effect. Studies on neutron radiation have, however, suggested the involvement of $^{\bullet}OH$ in the damage induction (Spotheim-Maurizot *et al.,* 1990; Peak *et al.,* 1995). Regardless of the pathway, DNA lesions induced by such events include strand breaks, base damage, sugar-phosphate damage, multiple lesion, etc. The extent of energy and oxidative radical attack required for

such damages, however, depend on the nature and concentration of scavengers as well as the physical parameters of the incident radiation such as LET. Densely ionizing high-LET radiation that produces particle track of closely spaced radicals are known to cause damage that is more complex than the damage imparted by sparsely ionizing (low-LET) radiation (LaVerne, 1989). Correlation of DNA damage with other cellular end-points such as cell death is believed to be influenced by the damage complexity. According to the "multiply damaged site" and "clustered lesion" hypothesis by Ward (1985; 1994) and Goodhead (1994), respectively, complexity of damage is what accounts for radiation-induced cell death. A densely localized lesion in a small area is unlikely to be repaired. Due to this there is a greater likelihood that the lesion will be lethal. Considering ionization clusters as "spurs" from the radiation chemistry perspective, the term "locally multiply damage sites" has also been used to describe break or base alteration damage to each of the two strands of DNA (Ward, 1985). Damage in clusters has now been recognized as a characteristic feature of radiation damage that is deposited stochastically. Holley *et al.* (1998) have defined "cluster" as a group of closely damaged sites separated by undamaged regions of about 20 base pair on each side. Correlating these damages with cellular parameter, such as cell death, is believed to contribute a key point in the damage complexity.

1.2 DNA STRAND BREAK:

Strand breaks can be single strand break (SSB) i.e., those which affect only one strand of the double-helix or double strand break (DSB), those affecting both strands of the double-helix. Such strand breaks have been the most extensively studied parameter in radiation induced DNA damages (Fielden and O'Neill, 1991; Olive, 1998). SSB is believed to be produced in a very high number of about 1000 breaks per Gy per mammalian cell (McGrath and Williams, 1966), and is assumed in literature and research strategies to be of no practical importance. Generally, it is therefore, not considered radiobiologically as a critical damage. There are, however, reports showing the involvement of SSB in apoptosis (Peitsch *et al.,* 1993) and complexity of DNA damage (Cunniffe and O'Neill, 1999).

On the other hand, a large number of studies suggest DNA DSB as the molecular lesion involved in initial events resulting in eventual radiobiological effects (Frankenberg *et al.,* 1982; Blocher and Pohlit, 1982; Elkind, 1985; Radford, 1986; Iliakis *et al.,* 1988). The damage, calculated to be about 20-40 DSB per Gy regardless of cell type (Blocher, 1982), leads to chromosomal aberration and cell death, if not repaired. The same magnitude of DSB may lead to mutation and cell transformation, if mis-repaired. A good number of theoretical and experimental studies on DNA DSB induction and lethality arising from such damages by different qualities of radiation is now well documented (Michalik, 1992; Goodhead, 1994; 1995; Barendson, 1994; Ward, 1994; Olive, 1998; Prise *et al.,* 1998). In

spite of these information, their correlation to radiosensitivity of different normal and mutant cells and their dependence on radiation quality has not been satisfactorily elucidated.

1.3 DNA BASE DAMAGE:

DNA base damage is one of several distinct lesions produced by radiation. It involves the chemical alteration of the bases that can result from severance of a side group from the base or damage to the ring structure itself. The damage is predominantly caused by •OH, which is supposed to react with the bases four times more efficiently than with sugar (Scholes, 1983). Even though it is not very clear if e_{aq}- is produced around DNA *in vivo*, it is thought to react with 100 % efficiency with bases without contributing to production of strand breaks (Lemaire *et al.,* 1984). The initial yield of damage types per Gray is approximated to be about 40 DSB, 1000 SSB and 2700 base damages (Charles *et al.,* 1995). These observations imply the probability of high base damage. A large number of base products have been identified and reported in literature (Hutchinson, 1985, Kuwabara, 1991). Such damages are produced by dihydroxylation of the 5, 6-double bond in pyrimidines, hydroxylation of the 5-methyl group in thymine, hydroxylation of purines at the 8- position and opening of the 5-membered ring in purines (Wiseman and Halliwell, 1996). A significant number of these damaged bases are stable and thus, when present in DNA, they could contribute to the biological consequences of radiation damage. The base damage alone, however, do not seem to play a major role in the biological effects of ionizing radiation (Ward *et al.,* 1985; Frankenberg-Schwarger, 1989) since such DNA modifications are understood to be efficiently repaired by the repair machinery of the cell (Radicella *et al.,* 1997). Radiation-induced base damage may, on the other hand, pose serious biological implications due to its role in multiple lesions such as "locally multiply damage sites" described by Ward (1994) and Goodhead (1994) or in local clusters (Holley *et al.,* 1998). Such clusters or group of damages, which can result from all ionizing radiation, is considered to be more severe than simple DSB. It has been suggested that such damages may be more difficult for the cell to repair with full fidelity (Goodhead, 1994).

In recent years, careful studies on identification of base damage have been carried out to define specific molecular damages in an attempt to understand the non-random biological response to radiation. One line of investigation of radiation induced base damage comes from the use of repair enzymes, for example formamidopyrimidine-DNA glycosylase (FPG) and endonuclease III (Endo III). These enzymes have been very useful in characterization of DNA damage induced by reactive oxygen species and photosensitizers in cellular and cell free systems (Muller *et al.,* 1990, Epe *et al.,* 1993) as well as by γ-radiation (Kuipers *and* Lafleur, 1998). The FPG predominantly recognizes

hydroxylated and open ring purines, while Endo III mostly recognizes oxidized and degraded pyrimidines. These modified bases are converted to SSB, which is then measured by several methods like sucrose gradient sedimentation (Hentosh, 1988), alkaline elution (Van Loon *et al.*, 1993), alkaline unwinding (Fohe *et al.*, 1994) or nick translation (Czene and Harms-Ringdahl, 1995). The yield of base damage can also be measured by gel electrophoresis (Milligan *et al.*, 1999), GC-MS technique (Jaruga *et al.*, 2000), or by comet assay (Collins *et al.*, 1996, Pflaum *et al.*, 1997, Banath *et al.*, 1999, Pouget *et al.*, 1999).

As opposed to an earlier view that radiation-induced strand breaks showed no preference in probability of breaks at all nucleotides sites of a B-DNA in solution (Henner *et al.*, 1982), current refinement in methodology allows the observation of sequence-dependent variations of breakage probability. A reduced probability of strand breaks has been reported at A/T sites (Barone *et al.*, 1994) and at AATT or AAATT sequences (Franchet-Beuzit *et al.*, 1993; Isabelle, 1995). On the other hand, there is a strong current contention that the probability of strand breakage does not depend on the chemical nature of the nucleotide/s (A, T, G or C), but on the sequence structure of the nucleotide (Sy *et al.*, 1997; Spotheim-Maurizot *et al.*, 1999). However, such reports are limited and would require further investigations in order to correlate the nucleotide sequence to radiation-induced DNA damage. Experimental evidences supporting a non-random distribution of DSB in DNA has recently been obtained for α-particles (Newman *et al.*, 1997) as well as for heavier ions (Lobrich *et al.*, 1996).

1.4 DNA DAMAGE, RADIOSENSITIVITY AND GENOMIC INSTABILITY:

Even though there are suggestions that the level of damage induced in DNA by radiation is the primary determinant of radiosensitivity (Bradley and Kohn, 1979), there is a belief that it is the processing or repair of such damages that is important (Iliakis *et al.*, 1988; Allalunis-Turner *et al.*, 1995). A difference in the induction of initial damage could not be attributed to the difference in radiosensitivity between M059J and M059K cells. Allalunis-Turner *et al.* (1995) concluded that the radiosensitivity of M059J cells was due to its defective repair mechanism. Studies from DNA DSB rejoining in human breast and bladder cancer cells support the hypothesis that repair process is a major determinant of cellular radiosensitivity (Nunez *et al.*, 1995). There are also studies indicating that the structural organization of DNA in chromatin has an intrinsic protective role against radiation (Sapora *et al.*, 1991; Ljungman *et al.*, 1991; Ljungman, 1991). In addition, chromatin compactness has also been thought to have a role in the radiosensitivity of cells (Nygren *et al.*, 1995). Earlier works have demonstrated that the actively transcribing genes are more radiosensitive due to partial decondensation of chromatin during the process of transcription (Chui *et al.*, 1982; Oleinick *et al.*, 1984; Warters *et al.*, 1987; Link *et al.*,

1992). It was observed by Woudstra *et al.* (1996) that the high cellular radiosensitivity could be accounted for by the sensitivity of chromatin leading to higher induction of DNA lesions (). In contrast, it has been reported that the kinetics of damage and repair were approximately the same in actively expressed and unexpressed genes (Bunch *et al.,* 1995). Recent work of Ljungman (1999) has shown that radiation-induced strand breaks does not occur preferentially in transcriptionally active genes. Half-life of radiation-induced strand breaks was observed to be similar in the dihydrofolate reductase (DHFR) gene, ribosomal gene, and in the genome as a whole. It was suggested that the repair of DNA strand breaks is fast and uniform in the genome of mammalian cells. While it has not been possible to relate any one of these observations consistently to radiosensitivity, it suggests that the structural conformation of DNA in the chromatin may not be the only factor in determining its susceptibility to radiation-induced damage.

Studies from mutation have suggested that the proximate nucleotide sequence and the general configuration of target DNA are two important factor in the production and processing of DNA damage (Waters *et al.,* 1991). They also indicate a non-random distribution of radiation effect. Similar observations have been made by other workers recently (Lobrich *et al.,* 1996; Sy *et al.,* 1997; Spotheim-Maurizot *et al.,* 1999). It is conceivable, therefore, that the determination and understanding of non-random DNA damage induced by radiation may perhaps contribute in explaining the complex inherent radiosensitivity of living cells.

A large body of evidence is available supporting the hypothesis that ionizing radiation induces a persistent state of genomic instability. It has been defined in a variety of biological markers such as karyotypic heterogeneity within a single clone (chromosomal instability) or in a variety of biochemical systems that include delayed reproductive death, gene amplification and delayed mutation (Kronenberg, 1994). Break points of chromosomal aberration observed immediately after irradiation is distributed randomly along chromosomes and is consistent with the introduction of strand breaks in the genome by action of radiation. However, the site of delayed chromosome instability is reported to involve telomere and interstitial telomeric elements indicating different mechanisms for the two categories of chromosome aberration (Morgan *et al.*, 1996; Yang *et al.*, 1998). Genetic instability also occurs as delayed mutation at the NT sequence level. Base damage induced by ionizing radiation might be bypassed during replication, leading to the inheritance of adducts in a subset of the daughter cells. Errors in replication might occur if these lesions were transmitted. Such mutations might result in the appearance of clone expressing a mutator phenotype, suggesting a trigger in destabilization of genome (Blakely and Kronenberg, 1998). Non-clonal spontaneous aberrations were observed in mouse haematopoietic stem cells (Kadhim *et al.*, 1992), and in human lymphocytes and fibroblasts

(Sabatier *et al.*, 1992) after irradiating them to densely ionizing radiation. The appearance of delayed aberration from these results was suggested to be due to densely ionizing radiation-induced phenotypic instability. Though such radiation-induced persistent and transmissible genetic instability in a variety of systems both *in vitro* and *in vivo* are known, the mechanism underlying such processes remains to be clearly understood.

1.5 RESTRICTION ENDONUCLEASE (RE) IN DNA DAMAGE STUDIES:

RE have been proven to be extremely useful tool in analyzing the genetic and physical organization of complex genomes. It has been employed in many ways in studying chemical- or radiation-induced DNA damages. Chung *et al.* (1991) have examined the effects of some inhibitors on chromosome aberration and DNA strand breaks using different restriction enzymes. They have been also used in studies of plasmid repair (Bien *et al.*, 1988), mutation induction (Muraiso *et al.*, 1993), alterations of DNA structures by alkylation (Gronenborg and Messing, 1978; Bird and Southern, 1978), drug-DNA interaction (Financsek *et al.*, 1984) and in measurement of DNA strand breaks (Lobrich *et al.*, 1994; 1996). They are enzymes that recognize specific short DNA sequences and cleave both strands of the duplex within or at some distance from the site. The fragments generated from such cleavage can be separated from one another on the basis of their size differences by several convenient methods. The most common is electrophoresis of the DNA fragments through a semisolid agarose gel. Generally, the mobility of a duplex fragment in electric field is inversely proportional to the logarithm of its size. Thus, using standard markers the size of the new fragments can be readily determined. RE is also highly specific in its action and cleaves only DNA that is not modified at its restriction sites. Therefore, by analyzing the fragments produced from the cleavage, one can not only deduce the order of the segments within the original DNA molecule, but also possibly study the alterations or modifications of the nucleotide sequence that may be induced by radiation.

1.6 AIMS AND OBJECTIVES:

Taking advantage of the specificity of RE, this part of the work was designed to study the relationship between nucleotide sequence and radiation-induced DNA damage. In this, fragment analysis was carried out on a pMTa4 plasmid and *E. coli* genomic DNA after exposing them to gamma (low-LET) and accelerated ^{7}Li particle (high-LET) radiation.

The following objectives were set forth:

1. To monitor the effect of γ-radiation on plasmid pMTa4 and *E. coli* genomic DNA and study its fragmentation pattern produced by different RE.

2. To monitor the effect of ^{7}Li particle on pMTa4 DNA and study its fragmentation pattern produced by RE.

3. In the light of data obtained from the above parameter, to analyze if there is any specific NT sequence related damage by low- and high-LET radiation.

*****

CHAPTER 2

MATERIALS AND METHODS

2.1 CHEMICALS:

Indigenous chemicals of analytical grade obtained from various chemical suppliers were used unless otherwise mentioned. Agarose (type I - low EEO), Ethylenediaminetetraacetic acid (EDTA), Tris[hydroxymethyl]- aminomethane (Tris base), Sodium dodecyl sulphate (SDS), Sodium acetate and Hexadecyltrimethylammonium bromide (cetyltrimethylammonium bromide, CTAB) were from Sigma Chemical Co. (USA) and Ethidium bromide from Merck (Germany).

2.2 OTHER MATERIALS:

Proteinase K was obtained from Boehringer Mannheim (Germany) while RNase and sodium salt Ampicillin was from Sigma Chemicals Co. (USA).

2.3 KIT:

High Pure Plasmid Isolation kit was procured from Boehringer Mannheim (Germany).

2.4 RESTRICTION ENZYMES AND DNA MARKERS:

Acc I, Bgl I, Bgl II, Dra I, Hae II, Hinf I, Ksp I, Nci I and *Pvu II* were obtained from Boehringer Mannheim (Germany); *Nco I*, λ DNA double digest marker were purchased from Genei (India).

2.5 IRRADIATION SOURCE:

2.5.1 Low LET:

Gamma Chamber 900 (Isotope Group, BARC, India) delivering γ-rays at a dose rate of ~ 0.2 Gy sec^{-1} from a cobalt-60 source was employed for low-LET experiments.

2.5.2 High LET:

^{7}Li particle generated from 15 UD tandem Pelletron accelerator (Nuclear Science Center, New Delhi) was used for high-LET studies. ^{7}Li particle (1×10^{6}, 5×10^{6}, 1×10^{7}, 5×10^{7}, 1×10^{8} and 5×10^{8} particles cm^{-2}) was delivered at LET, ~ 101 keV μm^{-1}.

2.6 BACTERIAL STRAIN AND PLASMID:

XL 1 *E. coli* strain harboring the plasmid construct, pMTa4, was generously

provided by Prof. Claus H. Schroeder, DKFZ, Heidelberg (Germany). Commercially available *E. coli* DH5α was purchased from Genei (India).

2.6.1 Plasmid Description:

The pBluescript (pBS) II SK phagemid is a 2958 bp derived from pUC19. It has an Ampicillin-resistance gene at 1975-2832 bp and a multiple cloning site (MCS) at 657-759 bp. A replication competent hepatitis B virus genome sequence, with a preX open reading frame (hpvprex), comprising 3215 bp, has been integrated at MCS [*Xho I* restriction site](Rakotomahanina *et al.*, 1994). This plasmid construct, totaling to 6173 bp, has been designated as pMTa4. RE listed in section 2.4 above have variable restriction sites on pMTa4. The number and size of the fragments that can be generated by these RE has been summarized in the table II.I. The complete sequence of pMTa4 is also provided in table II.II:

Table II.I. pMTa4 fragments characteristic generated by different RE.

RE	Number of fragments generated	Fragment size in bp
Acc I	3	3895, 1925, 353
Bgl I	3	3365, 1541, 1267
Bgl II	3	5734, 415, 24
Dra I	4	2862, 2600, 692, 19
Hae II	6	1938, 1680, 1593, 584, 370, 8
Hinf I	25	1074, 747, 662, 517, 453, 427, 393, 358, 278, 218, 177, 163, 148, 104, 83, 75, 71, 65, 51, 26, 24, 22, 19, 8, 7
Ksp I	2	4200, 1973
Nci I	7	2233, 1038, 1036, 818, 696, 351, 1
Nco I	1	6173
Pvu II	2	3660, 2513

Table II.II. The nucleotide (NT) sequence of pMTa4, $^{+}$ strand.

```
TCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCCTGCAGCCCGGGGGATCCACTAGTTCTAGAGCGGCCGCCACC
GCGGTGGAGCTCCAGCTTTTGTTCCCTTTAGTGAGGGTTAATTTCGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGT
GTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAG
TGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAA
TCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCG
TTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAA
GAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGC
CCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGT
TTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGG
AAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCA
CGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTAT
CGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTG
GCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTG
GTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAA
AAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTT
GGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATA
TGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCAT
AGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGC
GAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGC
AACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAA
CGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATC
AAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGT
TGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGT
GACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGG
ATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATC
TTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTT
CTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATAC
TCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAA
TAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGC
GTTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGAC
CGAGATAGGGTTGAGTGTTGTTCCAGTTTGGAACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGTCAAAGGGCGAA
AAACCGTCTATCAGGGCGATGGCCCACTACGTGAACCATCACCCTAATCAAGTTTTTTGGGGTCGAGGTGCCGTAAAGCA
CTAAATCGGAACCCTAAAGGGAGCCCCCGATTTAGAGCTTGACGGGGAAAGCCGGCGAACGTGGCGAGAAAGGAAGGG
AAGAAAGCGAAAGGAGCGGGCGCTAGGGCGCTGGCAAGTGTAGCGGTCACGCTGCGCGTAACCACCACACCCGCCGCGC
TTAATGCGCCGCTACAGGGCGCGTCCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCT
TCGCTATTACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACG
ACGTTGTAAAACGACGGCCAGTGAATTGTAATACGACTCACTATAGGGCGAATTGGGTACCGGGCCCCCCCTCGAGGACT
GGGGACCCTGCACCGAACATGGAGAGCACAACATCAGGATTCCTAGGACCCCTGCTCGTGTTACAGGCGGGGTTTTTCTT
GTTGACAAGAATCCTCACAATACCACAGAGTCTAGACTCGTGGTTGACTTCTCTCAATTTTCTAGGGGGAACACCCAAGT
GTCCTGGCCAAAATTCGCAGTCCCCAACCTCCAATCACTCACCAACCTCTTGTCCTCCAACTTGTCCTGGGTATCGCTGGA
TGTGTCTGCGGCGTTTTATCATATTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTGTTGGTTCTTCTGGACTACAAAGG
TATGTTGCCCGTTTGTCCTCTACTTCCAGGAACATCAACTACCAGCACGGGACCATGCAAGACCTGCACGATTCCTGCTCA
AAACACCTCTATGTTTCCCTCTTGTTGCTGTACAAAACCTTCGGACGGAAACTGCACTTGTATTCCCATCCCATCATCCTG
GGCTTTCGCAAGATTCCTATGGGAGTGGGCCTCAGTCCGTTTCTCCTGGCTCAGTTTACTAGTGCCATTTGTTCAGTGGTCT
GCAGGGCTTTCCCCCACTGTTTGGCTTTCAGTTATATGGACGATGTGGTATTGGGGGCCAAGTCTGTACAACATCTTGAGT
CCCTTTTTACCTCTATTACCAATTTTATGTTGTCTTTGGGCATACATTTGAACCCTAATAAAACCAAGCGTTGGGGCTACTC
CCTTAACTTCATGGGATATGTAATTGGAAGTTGGGGTACTTTACCACAAGAACATATTGTACAAAAACTCAAGCAATGTTT
TCGAAAACTGCCTGTCAATAGACCTATTGATTGGAAAGTATGTCAGAGAATTGTGGGTCTTTTAGGCTTTGCTGCCCCTTT
TACACAATGTGGCTATCCTGCCTTGATGCCTTTATATGCCTGTATACAATCTAAGCAGGCTTTCACTTTCTCGCCAACTTAC
AAGGCCTTTCTGTGTAAACAATATCTGAACCTTTACCCCGTTGCCCGGCAACGGTCAGGCCTCTGCCAAGTGTTTGCTGAT
GCAACCCCCACTGGATGGGGCTTGGCCATTGGCCATCAGCGCATGCGTGGAACCTTTGTGGCTCCTCTGCCGATCCATACT
GCGGAACTCCTAGCAGCTTGTTTTGCTCGCAGCCGGTCTGGAGCGACACTTATCGGAACCGACAACTCTGTTGTCCTCTCT
CGGAAATACACCTCCTTTCCATGGCTGCTAGGGTGTGCTGCCAACTGGATCCTGCGCGGGACGTCCTTTGTCTACGTCCCG
TCGGCGCTGAATCCCGCGGACGACCCGTCTCGGGGCCGTTTGGGCCTCTATCGTCCCCTTCTTCGTCTGTCGTTCCGGCCG
ACCACGGGGCGCACCTCTCTTTACGCGGTCTCCCCGTCTGTGCCTTCTCATCTGCCGGACCGTGTGCACTTCGCTTCACCT
CTGCACGTCGCATGGAGACCACCGTGAACGCCCACCAGGTCTTGCCCAAGGTCTTATATAAGAGGACTCTTGGACTCTCA
GCAATGTCAACGACCGACCTTGAGGCATACTTCAAAGACTGTTTGTTTAAAGACTGGGAGGAGTTGGGGGAGGAGATTAG
GTTAATGATCTTTGTACTAGGAGGCTGTAGGCATAAATTGGTCTGTTCACCAGCACCATGCAACTTTTTCACCTCTGCCTA
ATCATCTCATGTTCATGTCCTACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTAGGGCATGGACATTGACCCGTAT
AAAGAATTTGGAGCTTCTGTGGAGTTACTCTCTTTTTTGCCTTCTGACTTCTTTCCGTCTGTTCGAGATCTCCTCGACACCG
CCTCTGCTCTCTATCGGGAGGCCTTAGAGTCTCCGGAACATTGTTCACCTCACCATACAGCACTCAGGCAAGCTATTCTGT
GTTGGGTTGAGTTGATGAATCTGGCCACCTGGGTGGGAAGTAATTTGGAAGACCCAGCATCCAGGGAATTAGTAGTCAGC
TATGTCAATGTTAATATGGGCCTAAAAATCAGACAACTATTGTGGTTTCACATTTCCTGTCTTACTTTTGGAAGAGAAACT
GTTTTAGAGTATTTGGTGTCTTTTGGAGTGTGGATTCGCACTCCTCTCGCTTACAGACCACCAAATGCCCCTATCTTATCAA
CACTTCCGGAAACTACTGTTGTTAGACAACGAGGCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGCAGACGAAGATCT
CAATCGCCGCGTCGCAGAAGATCTAAATCTCGGGAATCTCAATGTTAGTATCCCTTGGACTCATAAGGTGGGAAACTTTA
CTGGACTTTATTCTTCTACTGTACCTGTCTTTAATCCTGAGTGGAAAACTCCCTCTTTTCCTAACATTCATTTACAGGAGGA
CATTATTGATAGATGTCAACAATATGTGGGCCCTCTTACAGTGAATGAAAAAAGGAGATTAAAATTAATTATGCCTGCTA
GGTTCTATCCTAACCTTACCAAGTATTTACCCTTGGATAAAGGCATTAAACCTTATTATCCTGAACATGCAGTTAATCATT
ACTTCCAAACTAGGCATTATTTACATACTCTGTGGAAGGCTGGCATTCTATATAAGAGAGAAACTACACGCAGTGCCTCA
TTTTGTGGGTCACCATATTCTTGGGAACAAGAGCTACAGCATGGGAGGTTGGTCTTCCAAACCTCGACAAGGCATGGGGA
CGAATCTTTCTGTTCCCAATCCTCTGGGATTCTTTCCCGATCACCAGTTGGACCCTGCGTTCGGAGCCAACTCAAACAATC
CAGATTGGGACTTCAACCCCAACAAGGATCACTGGCCAGAGGCAAAGCAGGTAGGAGCGGGAGCATTCGGGCCAGGGTT
CACCCCACCACACGGCGGCCTTTTGGGGTGGAGCCCTCAGGCTCAGGGCACATTGACAACAGTGCCAGCAGCGCCTCCTC
CTGCTTCCACCAATCGGCAGTCAGGAAGACAGCCTACTCCCATCTCTCCACCTCTAAGAGACAGTCATCCTCAGGCCATG
CAGTGGAACTCCACAACATTCCACCAAGCTCTGCTAGATCCAAGAGTGAGGGGCCTCTATTTCCCTGCTGGTGGCTCCAG
TTCCGGAACAGTAAACCCTGTTCCGACTACTGCCTCACCCATATCGTCAATCTTC
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2.7 BACTERIAL CULTURE COMPONENTS:

Yeast extract and tryptone were from Acumedia Manufacturers, Inc. Baltimore (USA) while Agar Agar was obtained from Qualigen (India).

2.8 MEDIUM PREPARATION AND BACTERIAL CULTURE:

Luria Bertani (LB) medium was prepared with 1 % Bacto-tryptone , 0.5 % Yeast extract and 1 % NaCl, pH 7.0. The medium was autoclaved at 15 lb for 20 min and was stored refrigerated. The XL 1 bacteria harboring the plasmid was selected from 1.5 % agar plate prepared in LB medium containing 100 µg ml^{-1} Ampicillin. A colony from the agar plate was picked and inoculated into a fresh LB medium containing 50 µg ml^{-1} Ampicillin. The bacteria was cultured overnight (O/N) with rigorous shaking in a Remi make (India) RS-24 rotary shaker maintained at 37 ^{0}C. Similarly, for genomic DNA isolation, a colony of DH5α grown on agar plate (without antibiotic) was inoculated and cultured O/N.

2.9 EXTRACTION OF DNA FROM BACTERIAL CULTURES:

2.9.1 Mini-Preparation of Plasmid DNA:

Plasmid DNA was isolated as per the protocol provided in the High Pure Plasmid Isolation kit. The buffer composition used in the isolation are shown below:

[a] Suspension buffer
- 50 mM Tris-HCl, pH 8.0
- 10 mM EDTA

[b] Lysis buffer
- 0.2 M NaOH
- 1 % SDS

[c] Binding buffer
- 4 M guanidine hydrochloride
- 0.5 M potassium acetate, pH 4.2

[d] Washing buffer
- 2 mM Tris-HCl, pH 7.5
- 20 mM NaCl
- Ethanol

Methodology:

Cells from O/N cultures were taken in a 1.5 ml eppendorf tube and were centrifuged for 10 sec in a 5414 S microfuge. The cell pellet collected was suspended and mixed properly in 250 µl of suspension buffer. Another 250 µl of lysis buffer was added and gently mixed. After 5 min of incubation at room temperature, 350 µl of chilled binding buffer was added and gently mixed by inverting the tube 3 to 6 times. This was followed by incubation on ice for 5 min. The cloudy solution was then centrifuged for 10 min at 13,000 x *g*. After combining the high pure filter tube and collection tube provided in the kit, the supernatant was pipetted into the upper reservoir and was centrifuged for 45 sec. The flow-through was discarded and 700 µl of wash buffer was added and centrifuged for 45 sec. Residual wash buffer was removed by an additional 45 sec centrifugation after which the filter tube was inserted into a clean sterile eppendorf tube. The plasmid DNA bound to the glass fleece was then eluted out with double distilled sterile water.

2.9.2 Mini-Preparation of Genomic DNA:

Genomic DNA from DH5α strain of *E. coli* was isolated by the method described by Ausubel *et al.* (1995). Buffer composition and solutions used for the preparation of genomic DNA are listed below:

[a] TE buffer

10 mM Tris, pH 8.0

1 mM EDTA

[b] CTAB/NaCl solution

10 % CTAB

0.7 M NaCl

(To the dissolved NaCl [distilled water], CTAB was added slowly while heating and stirring)

[c] Chloroform/ isoamyl alcohol

24 part of chloroform mixed with 1 part isoamyl alcohol

[d] Phenol/ chloroform/isoamyl alcohol

25 part of TE buffer saturated phenol mixed with 24-part chloroform and 1 part isoamyl alcohol

Methodology:

One and a half ml of O/N bacterial culture in an eppendorf tube was centrifuged for 10 sec in a SPINWIN microcentrifuge. The cell pellet was collected and suspended in 364

μl of TE buffer. After adding 30 μl of 10 % SDS and 6 μl of proteinase K (10 mg ml^{-1}), the tube was incubated at 37 ^{0}C for 60 min. To this 100 μl of 5 M NaCl was added and thoroughly mixed. The mixing was repeated after adding 80 μl CTAB/ NaCl solution. After incubating at 65 ^{0}C for 10 min, equal volume of chloroform/ isoamyl alcohol was added and mixed. The upper aqueous phase was collected into a fresh tube after centrifuging for 5 min (10,000 rpm) SPINWIN centrifuge. This was then phenol extracted by adding equal volume of phenol-chloroform-isoamyl alcohol mixture. After centrifuging for 5 min, the clear aqueous phase was collected and the DNA was recovered by gently mixing and precipitating in 0.6 volume of isopropanol. The precipitate was carefully fished out with a sterile toothpick into a new tube and was washed once in 70 % ethanol. The DNA collected by centrifuging for 1 min (10,000 rpm in SPINWIN microcentrifuge), was then dried and dissolved in sterile distilled water.

2.10 QUANTIFICATION OF DNA:

The yield and purity of the isolated plasmid and genomic DNA were determined by measuring the absorption ratio A_{260}:A_{280} nm in a double beam U-2001, HITACHI spectrophotometer. The DNA content was calculated by using the relationship of one unit measured at A_{260} in 1 cm light path length being equivalent to 50 μg ml^{-1} of DNA.

2.11 IRRADIATION CONDITION:

2.11.1 Low-LET:

Aqueous solution of pMTa4 in eppendorf tube (41.25 μg ml^{-1}) was γ-irradiated at room temperature to doses of ~ 30, 60, 120, and 240 Gy. The irradiated samples were stored on ice until analysis. Genomic DNA (1 μg μl^{-1}) was irradiated under similar condition.

2.11.2 High-LET:

Ten μl of aqueous solution of plasmid DNA construct (10 μg) was irradiated in specially designed polypropylene membrane pouches (1.5 x 1.5 cm). The pouch mounted on to the target plate and fixed to the beam window was exposed to varying fluence of ^{7}Li particle (1 x 10^6, 5 x 10^6, 1 x 10^7, 5 x 10^7, 1 x 10^8 and 5 x 10^8 particles cm^{-2}). Except during the irradiation, the pouches were maintained on ice. A non-irradiated pouch was maintained in similar condition as control. After the irradiation, 5 μl (5 μg DNA) each from the pouches were recovered and processed for analysis.

2.12 DNA FRAGMENTATION BY RESTRICTION ENDONUCLEASES:

Irradiated and non-irradiated plasmid DNA samples were fragmented with different restriction endonucleases (see section 2.4). The restriction conditions were as per manufacturer's recommendation (Genei, India; Boehinger Mannheim, Germany). Table II.III summarizes different RE with their respective buffers and restriction conditions. A parallel set of undigested plasmid DNA samples were used for monitoring the extent of strand breaks induced by irradiation.

Table II.III. Buffer compositions and conditions for DNA fragmentation by different RE.

RE	BUFFER (1 x recipe)	RE UNIT	CONDITION
*Acc I, Hae II**	Tris-acetate (pH. 7.9) 33 mM Magnesium acetate 10 mM Potassium acetate 66 mM Dithiothreitol (DTT) 0.5 mM	One unit of RE was used for every µg DNA (plasmid/genomic DNA).	60 min at 37 °C (*Digestion of plasmid DNA, after high-LET ^{7}Li particle radiation, with these RE were carried out for ~4 hr).
Ksp I, Nci I	Magnesium acetate (pH. 7.5) 10 mM Magnesium chloride 10 mM Dithioerythritol (DTE) 1 mM		
Bgl II, Dra I, Pvu II	Tris-HCl (pH. 7.5) 10 mM Magnesium chloride 10 mM Sodium chloride 50 mM DTE 1 mM		
*Bgl I *, Hinf I ** *Nco I*	Tris-HCl (pH. 7.5) 50 mM Magnesium chloride 10 mM Sodium chloride 100 mM DTE 1 mM		

2.13 AGAROSE GEL ELECTROPHORESIS:

Standard protocol for agarose gel electrophoresis was followed (Ausabel *et al.*, 1995). The buffer and solution used for the electrophoresis are given below:

[a] TAE buffer

40 mM Tris-acetate

1 mM EDTA

[b] Sample dye

40 % sucrose

0.2 % bromophenol blue

[c] Ethidium bromide solution
(Directly used from bottle)

2.13.1 Gel Preparation:

One % agarose in TAE buffer was mixed and melted by heating to ~ 90 ^{0}C. The gel solution was poured into a pre-assembled sealed gel casting platform (with inserted comb). After allowing it to solidify for about 20-30 min at RT, the sealing tape and the comb were removed from the gel casting platform. The gel platform was placed into the electrophoresis tank containing sufficient TAE buffer.

2.13.2 Sample Loading:

After diluting the restricted and non-restricted DNA samples with 1/6th volume of sample dye, appropriate volumes (~ 1 μg) of DNA were loaded into the wells.

2.13.3 Electrophoresis:

The gel submerged in TAE buffer was electrophoresed for 60 min at a constant voltage of 90 V in a Gel Electrophoresis Apparatus GNA-100 (Pharmacia, Sweden) unit.

2.13.4 Staining and Documentation:

Gels were stained in 0.3 μg ml^{-1} ethidium bromide for 10 min. After 30 min of destaining in double distilled water, the DNA-intercalated ethidium bromide flourescence was visualized on a UV transilluminator (Bio-Rad mini transilluminator). This was either photographed with AE-1 Canon camera using Alpha GP4 black and white negative film (gels from low-LET studies) or captured in a gel documentation system (gels from high-LET studies).

2.14 PLASMID DNA STRANDS BREAK ANALYSIS:

The relative amounts of DNA in each of the plasmid topological form resulting from irradiation were determined by scanning the photographs in a GS-690 Imaging Densitometer (Bio-Rad). Images were stored and analyzed in IBM-compatible computer using the Molecular Analyst™/PC Windows Software, version 1.5 (Bio-Rad). The relative amounts of DNA in each plasmid conformation were obtained from the sum of the pixel intensities of the DNA band.

2.15 DETERMINATION OF DNA FRAGMENT SIZE:

The approximate sizes of the DNA fragments generated by restriction

endonucleases were determined using the same software mentioned above. Based on the *Rf* values of the different fragments, their molecular weights (sizes) were obtained against known λ DNA double digest marker through third order cubic regression.

2.16 DATA AND STATISTICAL ANALYSIS:

Data obtained and analyzed were plotted using Cricket Graph software (version 1.3). The data from γ-rays-induced plasmid DNA strand breaks are expressed as the average of the means from two independent experiments with standard deviation (mean ± SD).

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CHAPTER 3

RESULTS

3.1 PLASMID DNA:

The plasmid used in this study, designated as pMTa4, was isolated from *E. coli* XL 1 blue strain. Miniprep protocol was followed for the isolation using the high pure plasmid isolation kit. The purity of the DNA was determined by 260/280 ratio of absorbency, which was then confirmed by agarose gel electrophoresis. Fig. 3.1 shows electropherogram of the plasmid DNA isolate. Plasmid DNA in its native form exists in a complex structural configuration known as supercoiled (SC) form. When a nick or a single strand break (SSB) is introduced, it uncoils or relaxes forming a relaxed structure or open circular (OC) form.

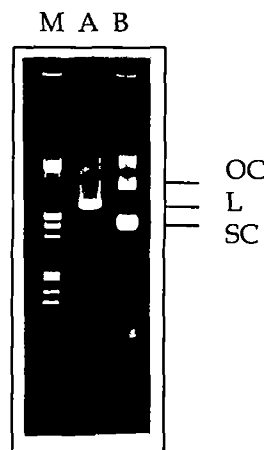

Figure 3.1: Gel electropherogram of pMTa4: Lane M represents λ DNA *Eco R I/ Hind III* double digest marker; lane A represents pMTa4 linearized by *Nco I* while lane B represents non-irradiated (control) pMTa4 isolate. The three forms - open circular, linearized and supercoiled are denoted as OC, L and SC, respectively.

Conventionally, when a single double strand break (DSB) is introduced to either of the two forms, a linearized (L) form is produced. This can also result when two SSB on opposite strands are very close to each other (Krisch *et al.,* 1985; Charlton *et al.,* 1989). The three forms are easily distinguished by their different mobility rates in an electric field (Fig. 3.1 lane B). To ascertain the authenticity and quality, pMTa4 was digested with an endonuclease, *Nco I,* that has a single restriction site in the plasmid (see Table II.I). Fig. 3.1 (lane A) shows the formation of a linearized form after restriction with *Nco I*.

3.2 EFFECT OF γ-IRRADIATION ON pMTa4:

Fig. 3.2 shows the gel electropherogram of pMTa4 after exposure to various dose of

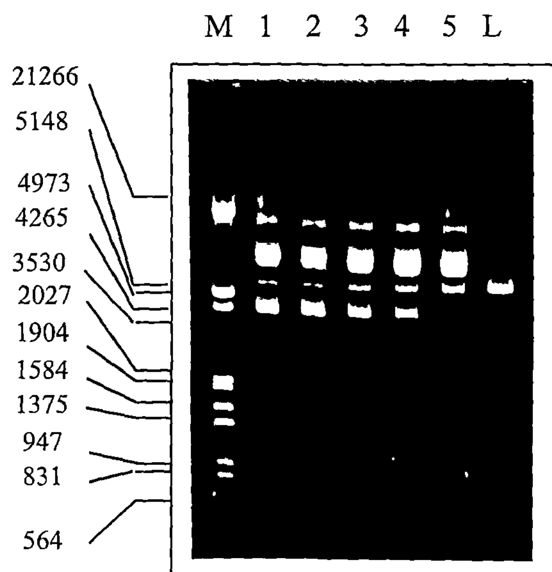

Figure 3.2: Agarose gel electropherogram of non-irradiated (control) pMTa4 (lane 1) and γ-irradiated plasmid DNA (lanes 2, 3, 4 and 5 corresponding to doses of 30, 60, 120 and 240 Gy, respectively). Lane M represents λ DNA *EcoR I* + *Hind III* double digest marker and L is the *Nco I* linearized form of pMTa4. The values on the left are sizes (bp) of marker DNA fragments.

γ-radiation. It reveals the changes in the three forms of plasmid in its non-irradiated (control) state and that of following irradiation. Densitometric analysis of the gel is summarized in table III.I. Fig. 3.3 depicts the plot. It indicates dose dependent reduction in the quantity of SC form, while dose dependent increase in the quantity of OC and L form were observed.

Table III.I: Densitometric quantification of the three forms of pMTa4 in its non-irradiated (control) and following γ-radiation.

Dose (Gy)	**Density OD x mm^2 (% of control)**		
	OC	L	SC
0 (control)	100	100	100
30	103.15 ± 9.25	103.47 ± 2.43	93.66 ± 6.39
60	108.41 ± 3.36	102.92 ± 5.59	76.53 ± 1.6
120	117.35 ± 1.18	107.48 ± 9.81	64.86 ± 8.59
240	131.68 ± 7.99	114.41 ± 6.96	50.33 ± 6.07

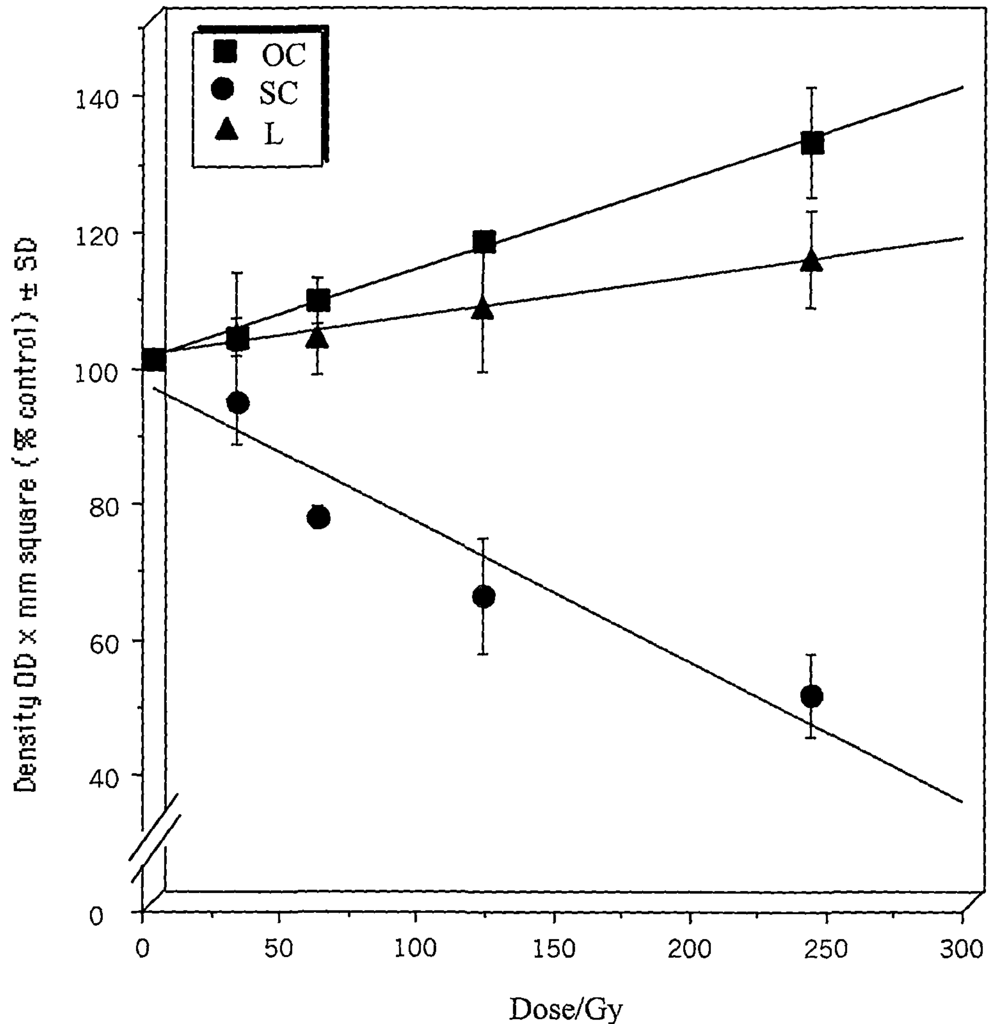

Figure 3.3: Densitometric analysis of pMTa4 after γ-radiation. Data points are from two independent experimental sets and represent the mean ± SD.

3.3 EFFECT OF γ-IRRADIATION ON THE RESTRICTION SEQUENCE OF DIFFERENT RESTRICTION ENDONUCLEASE IN pMTa4:

Table III.II shows the different restriction endonucleases along with their restriction sequence and NT sequence at fragment-end in the pMTa4 plasmid.

Fig. 3.4 shows the restriction fragmentation patterns of non-irradiated (control) pMTa4 generated by *Bgl I*, *Hinf I* and *Hae II* and that after exposure of plasmid to doses of

TABLE III.II. Characteristics of the different restriction endonucleases (RE): RE were selected depending on their restriction sites: blunt- and staggered-ends as well as their NT composition in the site.

Restriction endonuclease (RE)	**Restriction site**	**Fragment-end generated by RE cleavage**	**Effect on the restriction following radiation**
Acc I	-GC/ (A,C)(T,G)AC- -CA(T,G)(A,C)/ TG-	-GC -CA(T,G)(A,C)	-
Bgl I	-GCCGTTT/ GGGC- -CGGC/ AAACCCG-	-GCCGTTT -CGGC	-
Bgl II	-A/ GATCT- -TCTAG/ A-	-A -TCTAG	-
Dra I	-TTT/ AAA- -AAA/ TTT-	-TTT -AAA	-
Hae II	-GGCGC/ (T,C)- -C/ CGCG(A,G)-	-GGCGC -C	+
Hinf I	-G/ ANTC- -CTNA/ G-	-G -CTNA	-
Ksp I	-CCGC/ GG- -GG/ CGCC-	-CCGC -GG	-
Nci I	-CC/ (C,G)GG- -GG(G,C)/ CC-	-CC -GG(G,C)	+
Pvu II	-CAG/ CTG- -GTC/ GAC-	-CAG -GTC	-

30, 60, 120 and 240 Gy. In the *Bgl I* (Fig. 3.4 A) and *Hinf I* (Fig. 3.4 B) restricted plasmid, there was no observable change in restriction patterns for non-irradiated and irradiated samples. In contrast, the irradiated plasmid restricted with *Hae II* (Fig. 3.4 C) showed additional slow migrating bands or large plasmid DNA pieces on the gel which were entirely absent in the non-irradiated control. The formation of the extra fragments (Fig. 3.4 C lanes 2 through 5) remained unchanged even at the maximum dose of 240 Gy.

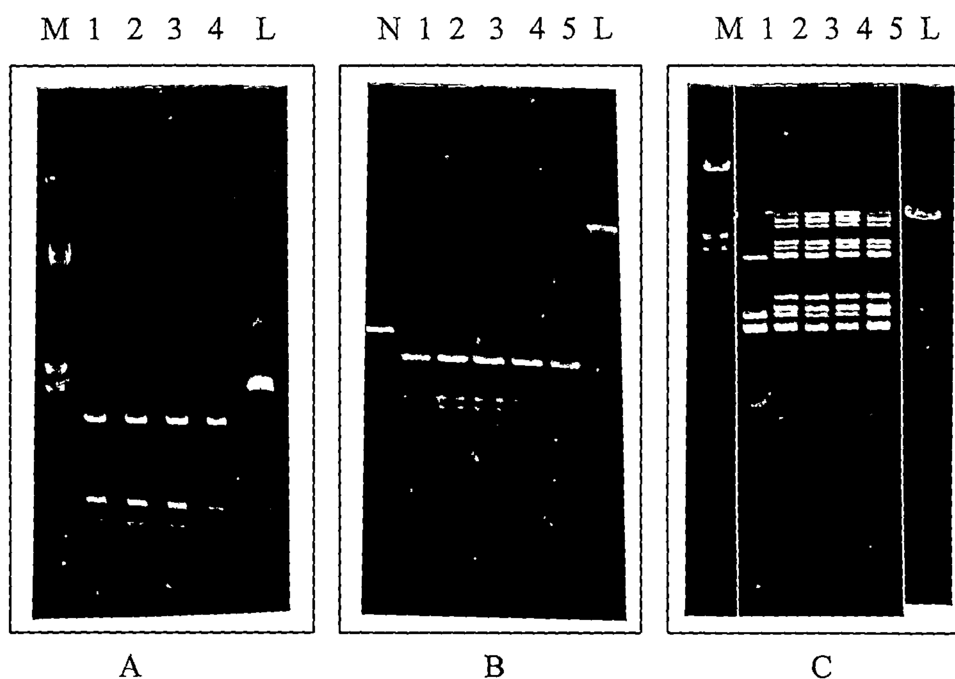

Figure 3.4: Agarose gel electropherogram of pMTa4 restricted with different RE in control and after irradiation. Panel A is restriction with *Bgl I*, B with *Hinf I* and C with *Hae II*. M and N represents λ DNA double digest and pBR322 *Hae III* digest markers; lane 1, 2, 3, 4 and 5 represent non-irradiated control, and 30, 60, 120 and 240 Gy of γ-radiation, respectively. Lane L represents *Nco I* linearized non-irradiated pMTa4.

Fig. 3.5 show results of restriction fragment patterns of plasmid generated by *Dra I*, *Pvu II*, *Acc I* and *Bgl II*,. In all the cases there was no major observable changes in the restriction fragmentation patterns for both non-irradiated (control) and irradiated samples except for an insignificant faint band which appears due *Acc I* and *Pvu II* restrictions. This band which appears to be a linearized form has, however, not been considered as an abnormal restriction. Fig. 3.6 shows restriction fragment pattern of plasmid generated by *Ksp I* and *Nci I* endonuclease. In *Ksp I* restricted plasmid there was no changes in its restriction patterns for non-irradiated and irradiated samples. The *Nci I* restriction, on the other hand,

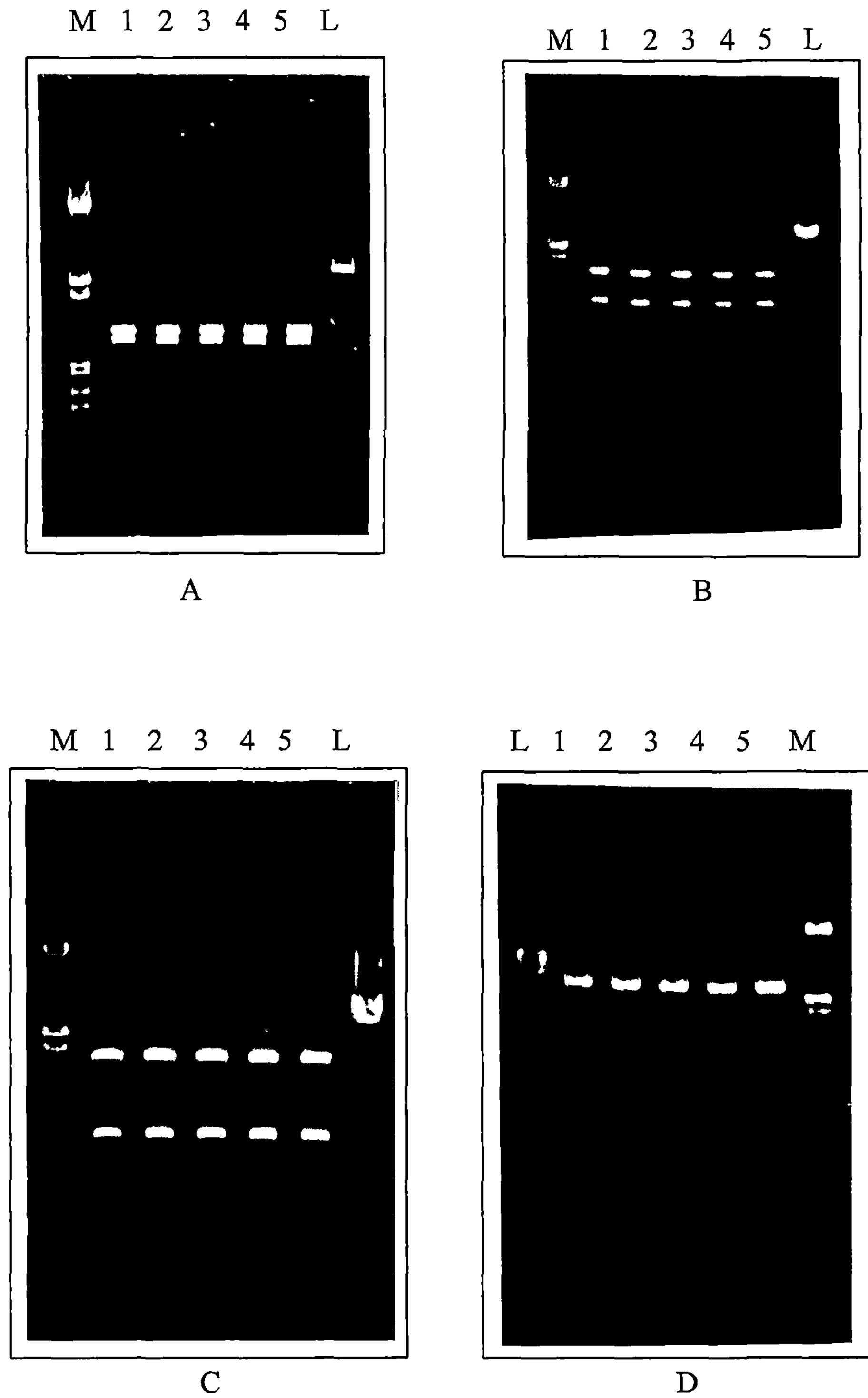

Figure 3.5: Agarose gel electropherogram of pMTa4 restricted with different RE in control and after irradiation. Panel A is restriction with *Dra I*, B with *Pvu II*, C with *Acc I* and D with *Bgl II*. M represents λ DNA double digest marker, lanes 1, 2, 3, 4 and 5 are non-irradiated control, 30, 60, 120 and 240 Gy of γ-radiation, respectively. Lane L represents *Nco I* linearized non-irradiated pMTa4.

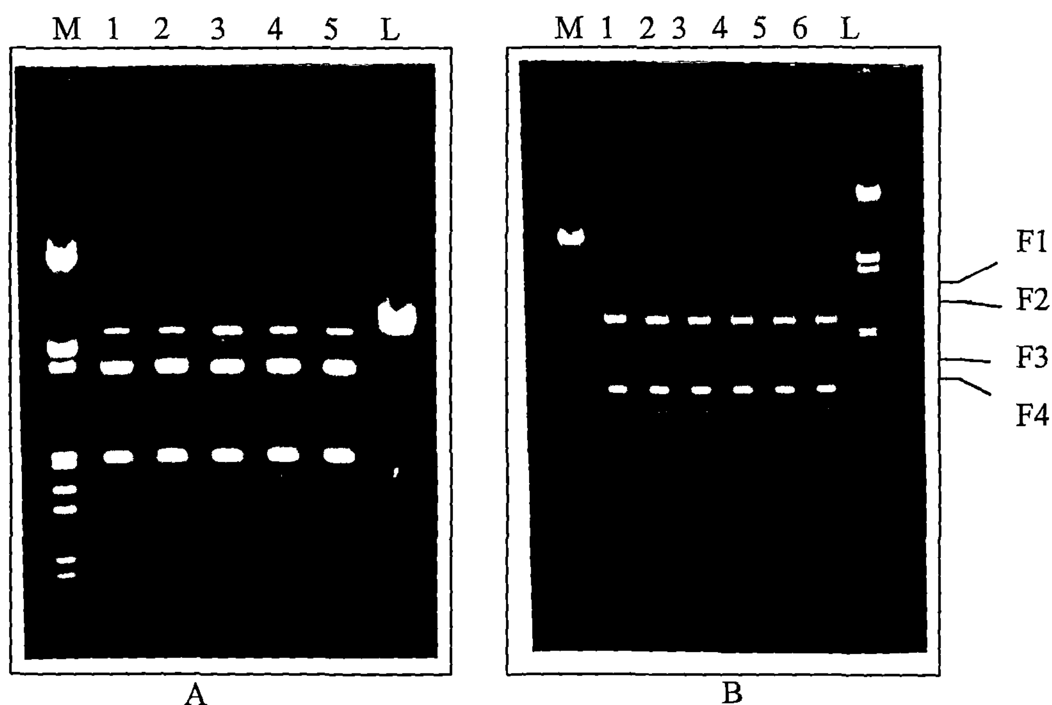

Figure 3.6: Agarose gel electropherogram of pMTa4 restricted with *Ksp I* (A) and *Nci I* (B) in control and after irradiation. M represents λ DNA double digest marker, lanes 1, 2, 3, 4 and 5 are non-irradiated (control), 30, 60, 120 and 240 Gy of γ-radiation, respectively. Lane L represents *Nco I* linearized non-irradiated pMTa4. The four extra bands generated by *Nci I* are indicated as F1, F2, F3 and F4.

showed the formation of four distinct extra bands or relatively larger plasmid DNA fragments on the gel. The quantity of these bands increased with radiation dose exhibiting dose dependence (Table III.III), as revealed by densitometric quantification.

Table III.III: Densitometric quantification of the four extra fragments produced by *Nci I* after γ-irradiation. The four fragments in order of decreasing size is denoted as F1, F2, F3 and F4 Data points are from a single experimental set

Dose (Gy)	**Density OD x mm^{-1} (% control)**			
	F1	**F2**	**F3**	**F4**
0 (control)	100	100	100	100
15	193.85	186.49	181.14	173.33
30	228.07	213.79	206.20	200.74
60	230.99	242.52	231.26	225.18
120	260.23	254.59	241.43	234.81
240	296.78	281.60	294.54	288.64

3.4 ANALYSIS OF *HAE II* AND *NCI I* GENERATED FRAGMENT PATTERN FOLLOWING γ-IRRADIATION:

In order to understand and evaluate the abnormal fragmentation produced by *Hae II* (Fig. 3.4 C) and *Nci I* (Fig. 3.6 B) following γ-irradiation, it was envisaged to look for those factors that were possibly causing such effects. To get an insight in this direction, attempt was made to identify the extra fragments that were generated. Table III.IV shows the size of *Hae II* generated extra fragment that were calculated using Molecular Analyst (MA)

Table III.IV. pMTa4 size analysis of the extra fragments generated by *Hae II* restriction after γ-irradiation. Left column represents the values obtained from the Molecular Analyst software, while right column represents the values of predicted/ expected fragments generated by possible combinations that would result due to incomplete digestion.

Approximate fragment size (bp) calculated by MA software	**Predicted actual size in bp with their possible fragment combinations**	
γ-radiation		
6356	6173	(I + II + III + IV + V + VI)
6055	5802	(III + II + I + VI + V)
5605	5589	(II + I + VI + V + IV)
4300	4234/ 4326	(IV + III + II + I /VI)
4126	3864/ 3856	(III + II + I /VI)
3721	3539	(I + VI + V)
2462	2588	(II + III + IV)
2261	2316/ 2303	(IV + V /VI)

software (see section 2.15) in comparison to the theoretical sizes that should be generated. *Hae II* restriction map of pMTa4 was constructed as illustrated in Fig. 3.7. Out of the several possible theoretical combinations of fragments generated by *Hae II*, the values falling nearest to the observed sizes calculated by software were taken as their actual fragment size (Table III.IV). Fig. 3.8 shows the different plasmid bands generated by *Hae II* restriction with the sizes likely to be generated by their possible fragment combinations.

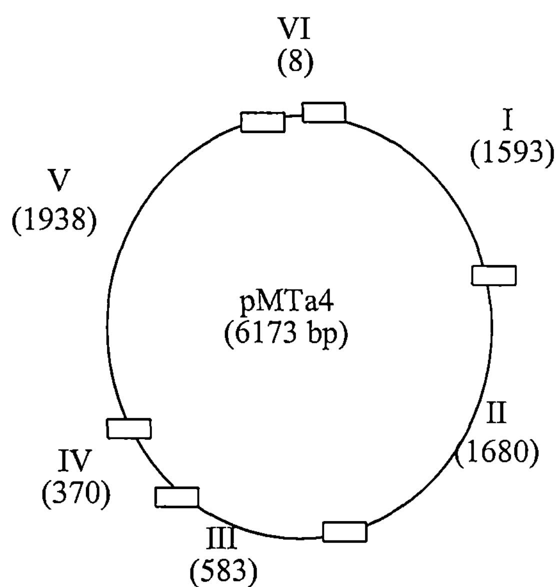

Figure 3.7: A circular representation of pMTa4 showing location of *Hae II* restriction site in open blocks. Fragments generated along with their corresponding sizes in bp are indicated as I, II, III, IV, V and VI.

Similarly, for *Nci I*, identification of the extra fragments was also done. An approach similar to that of the *Hae II* fragment analysis was employed to create *Nci I* restriction map of pMTa4 (Fig. 3.9) and determine extra fragment sizes (Table III.V).

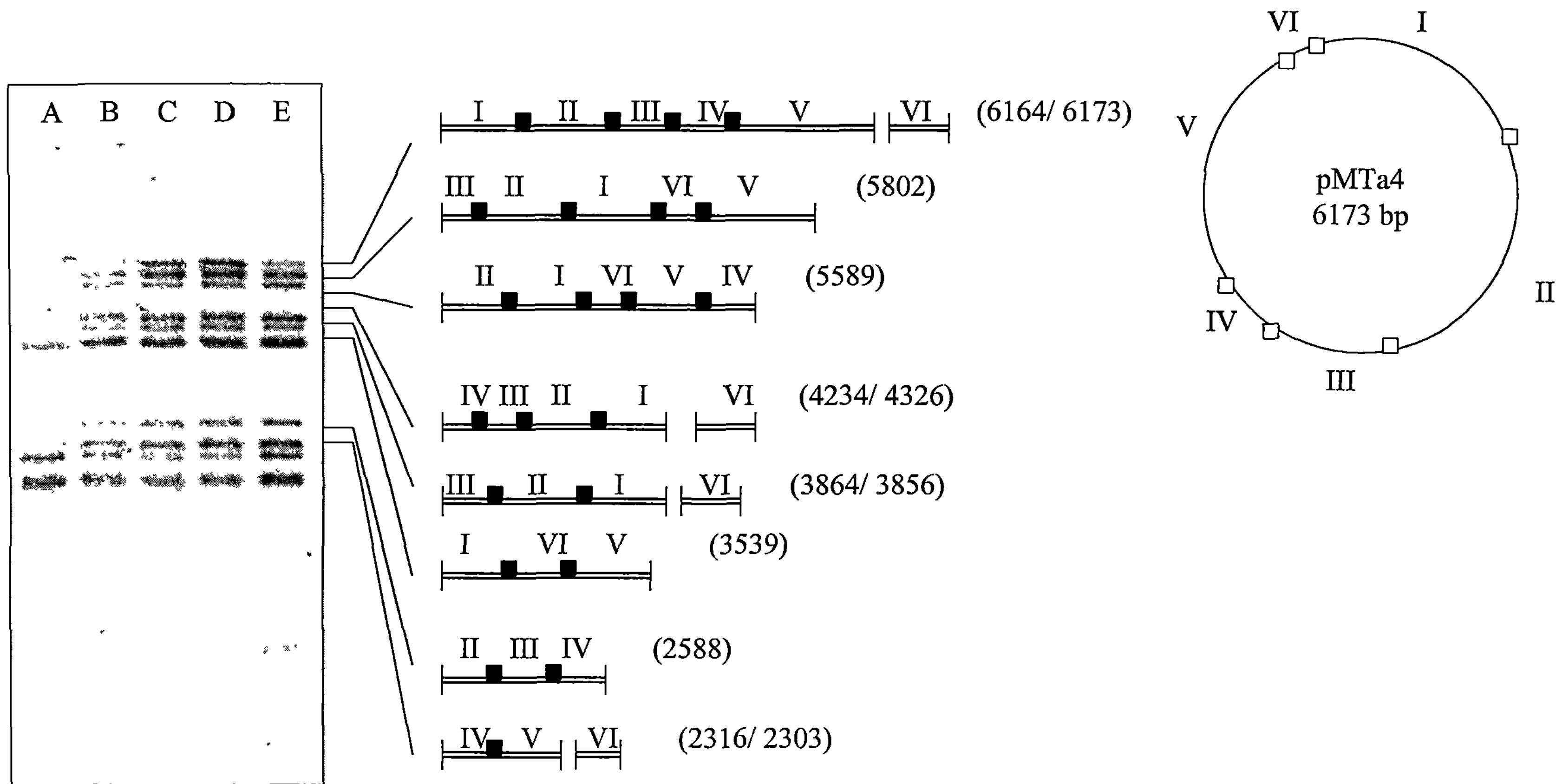

Figure 3.8: Densitometric image scan of Fig. 3.4 C showing fragments generated by *Hae II* after low-LET ^{60}Co γ-irradiation. Corresponding fragment size for each indicated bands are represented by numericals obtained by various possible fragment combination (see also table III.IV). Lanes A, B, C, D and E represent radiation doses of 0, 30, 60, 120 and 240 Gy, respectively. Shaded blocks indicated in the fragments represents restriction sites that were apparently affording resistance to *Hae II* cutting.

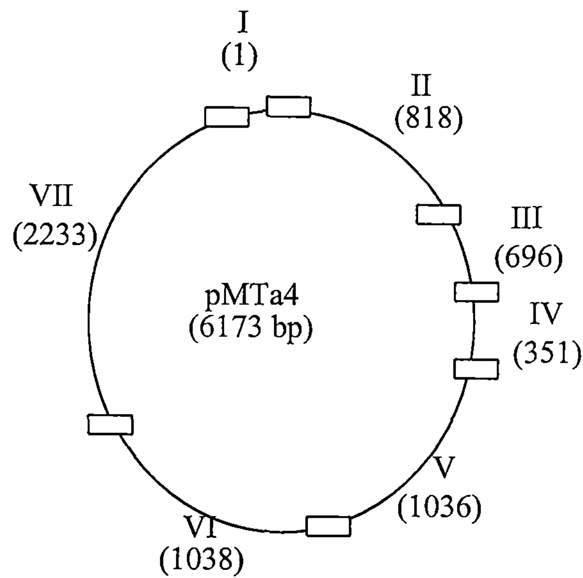

Figure 3.9: A circular representation of pMTa4 showing location of *Nci I* restriction sites in open blocks. Fragments along with their corresponding sizes in bp are indicated as I, II, III, IV, V, VI and VII.

Fig. 3.10 shows the different DNA bands generated by *Nci I.* The four extra bands generated after irradiation are indicated with their possible fragment combinations along with their respective sizes.

Table III.V. pMTa4 size analysis of the extra fragments generated by *Nci I* restriction after γ-irradiation. Left column represents the values obtained from the Molecular Analyst software, while right column represents the values of predicted/ expected fragments generated by possible combinations that would result due to incomplete digestion.

Approximate fragment size (bp) calculated by MA software	**Predicted actual size in bp with their possible fragment combinations**
γ-radiation	
3208	3272 (VI + VII + I)
2966	2901 (II + III + IV + V)
1537	1514 (II + III)
1387	1387 (IV + V)

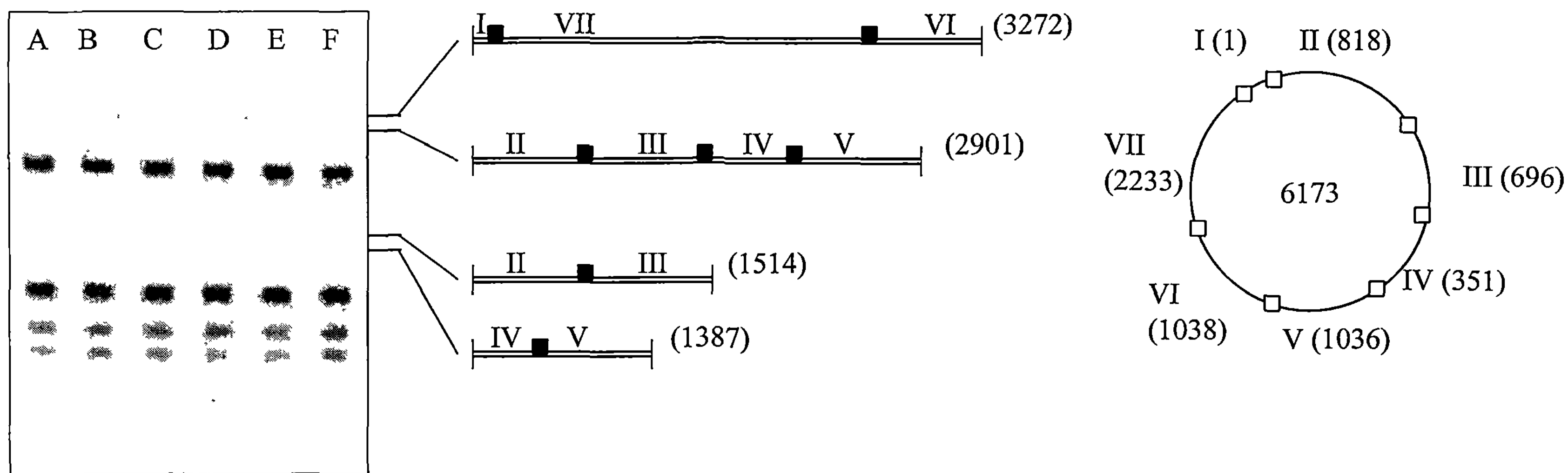

Figure 3.10: Densitometric image of Fig. 3.6 B showing fragments generated by *Nci I* after low-LET ^{60}Co γ-irradiation. Corresponding fragment size for each indicated extra bands are represented by numericals obtained by possible fragment combination (see also table III.V). Lanes A, B, C, D, E and F represent radiation doses of 0, 15, 30, 60, 120 and 240 Gy, respectively. Shaded blocks indicated in the fragments represent restriction sites that were apparently not cut by *Nci I*.

3.5 EFFECT OF ^{7}LI PARTICLES ON PLASMIDS DNA:

Fig. 3.11 shows densitometric scan image of gel electropherogram of pMTa4 after exposure to varying fluence of accelerated charged ^{7}Li particle. Fig. 3.12 depicts the plots

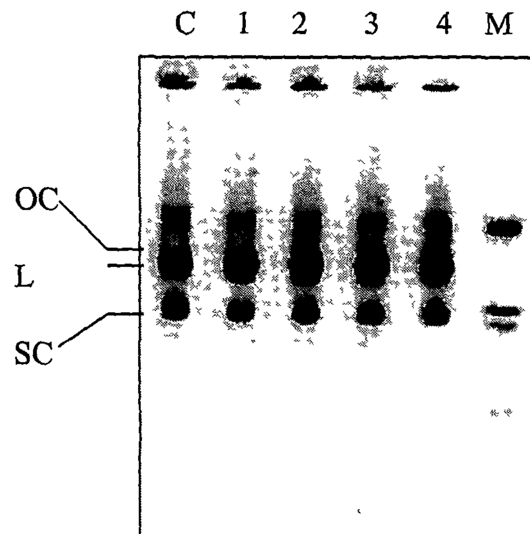

Figure 3.11: Electropherogram of the three forms of plasmid pMTa4 following ^{7}Li particle irradiation. OC, L and SC represents open circular, linearized and supercoiled form of plasmid DNA, respectively. Fluence of 1 x 10^6, 5 x 10^6, 1 x 10^7 and 1 x 10^8 particles cm^{-1} delivered at LET of ~ 101 KeV/μm is represented by 1, 2, 3 and 4. C represents non-irradiated (control) and M is λDNA double digest marker.

of the three forms of plasmid as quantified by densitometry against fluence. While SC form of plasmid decreased, its L form increased in a dose dependent manner. There was no apparent increase in the OC form.

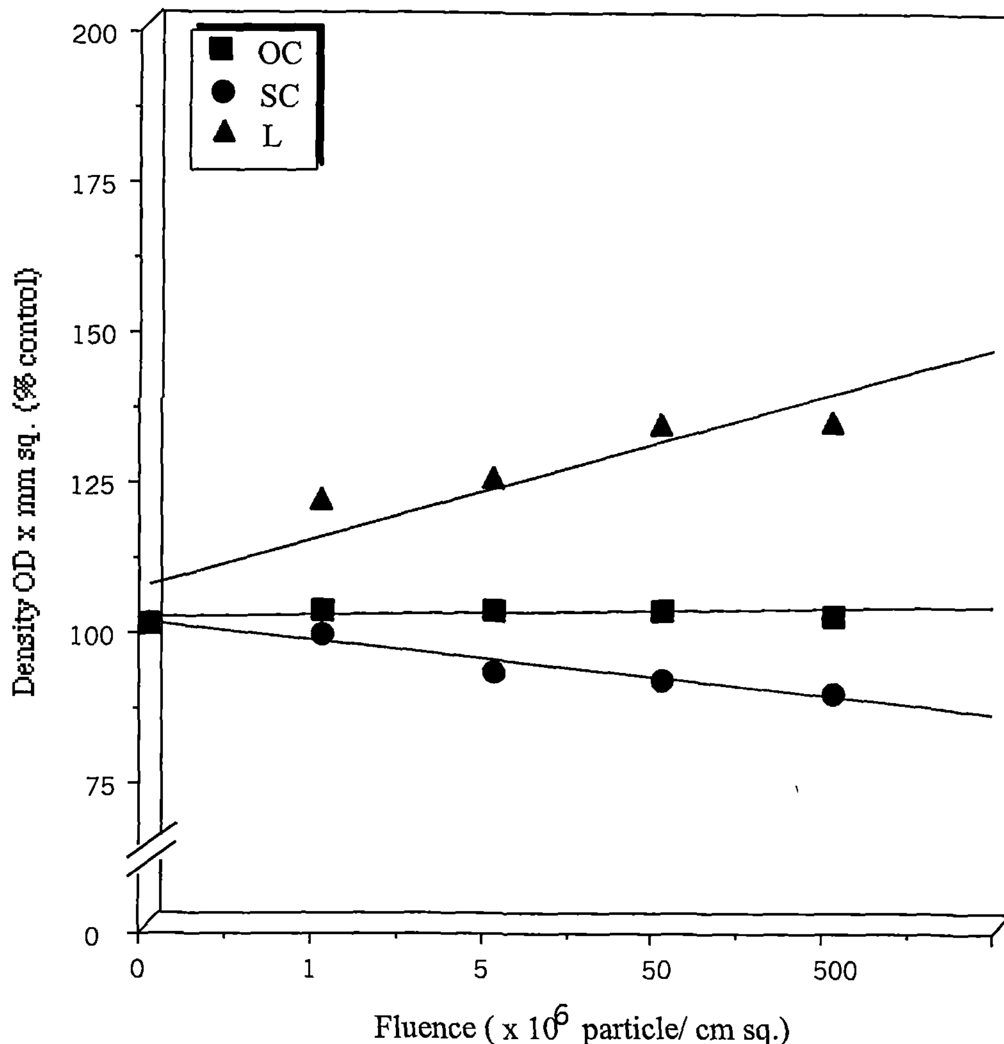

Figure 3.12: Densitometric analysis of pMTa4 depicting the change in the three different forms of pMTa4 after ^{7}Li particle irradiation. Data points are from single experimental set.

3.6 EFFECT OF ^{7}LI PARTICLES ON THE RESTRICTION SEQUENCES OF DIFFERENT RESTRICTION ENDONUCLEASE IN pMTa4:

Fig. 3.13 shows restriction fragmentation pattern of pMTa4 generated by *Bgl I, Hinf I*, and *Hae II* following ^{7}Li particle irradiation. Similar to results from low-LET, there was no differences in the fragmentation patterns generated by *Bgl I* and *Hinf I*. In contrast, the irradiated plasmid restricted with *Hae II* showed additional bands or large DNA pieces on the gel which were entirely absent in the non-irradiated control. A dose dependent abnormal fragmentation was observed in the pMTa4 restricted by *Hae II*. While the lowest fluence of 1 x 10^{6} particle cm^{-1} showed no effect (Fig. 3.13 C, lane 1), abnormal restriction

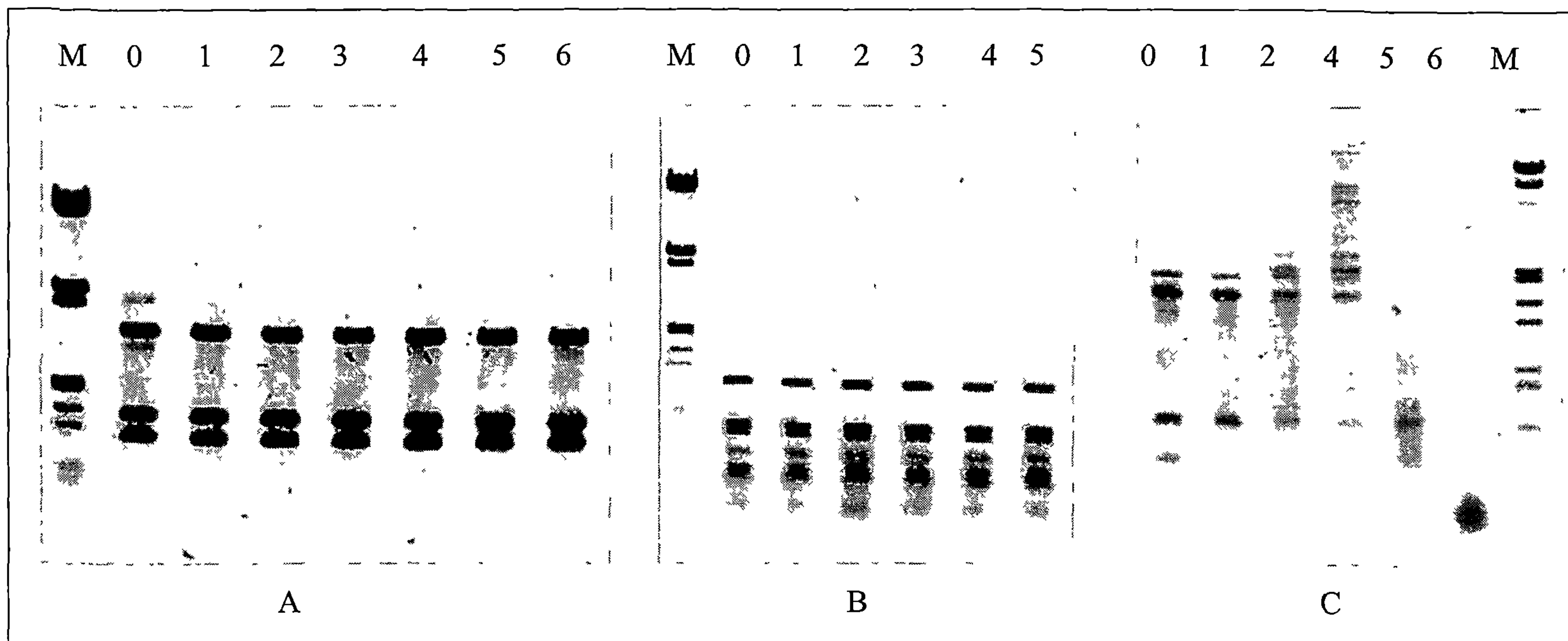

Figure 3.13: Electropherogram of ^{7}Li irradiated pMTa4 DNA restricted with *Bgl I* (A), *Hinf I* (B) and *Hae II* (C). Doses: Lanes 0 = non-irradiated (control), 1 = $1x10^{6}$, 2 = $5x10^{6}$, 3 = $1x10^{7}$, 4 = $5x10^{7}$, 5 = $1x10^{8}$ and 6 = $5x10^{8}$ particles cm^{-2}. Lane M represents λDNA double digest marker.

was observed in plasmid exposed to 5 x 10^6, 5 x 10^7 and 1 x 10^8 particle cm^{-1} (Fig. 3.13 C, lanes 2, 4, 5). The highest fluence of 5 x 10^8 particle cm^{-1} on the other hand, caused plasmid DNA undergo nonspecific fragmentation forming a tear-drop smear (Fig. 3.13 C, lane 6). In order to identify the fragments, size analysis for *Hae II*, similar to that of low-LET, was done as shown in table III.VI. Fig. 3.14 shows the different DNA bands generated by *Hae II* after ^{7}Li particle irradiation. The extra bands generated are indicated with their possible fragment combinations along with their respective sizes.

Table III.VI. pMTa4 size analysis of the extra fragments generated by *Hae II* restriction after ^{7}Li particle irradiation. Left column represents the values obtained from the Molecular Analyst software, while right column represents the values of predicted/ expected fragments generated by possible combinations that would result due to incomplete digestion.

Approximate fragment size (bp) calculated by MA software ^{7}Li particle-radiation	**Predicted actual size in bp with their possible fragment combinations**	
6381	6173	(I + II + III + IV + V + VI)
5763	5802	(III + II + I + VI + V)
4351	4234/ 4326	(IV + III + II + I /VI)
4114	3864/ 3856	(III + II + I /VI)
3717	3539	(I + VI + V)
2652	2588	(II + III + IV)
2239	2316/ 2303	(IV + V /VI)

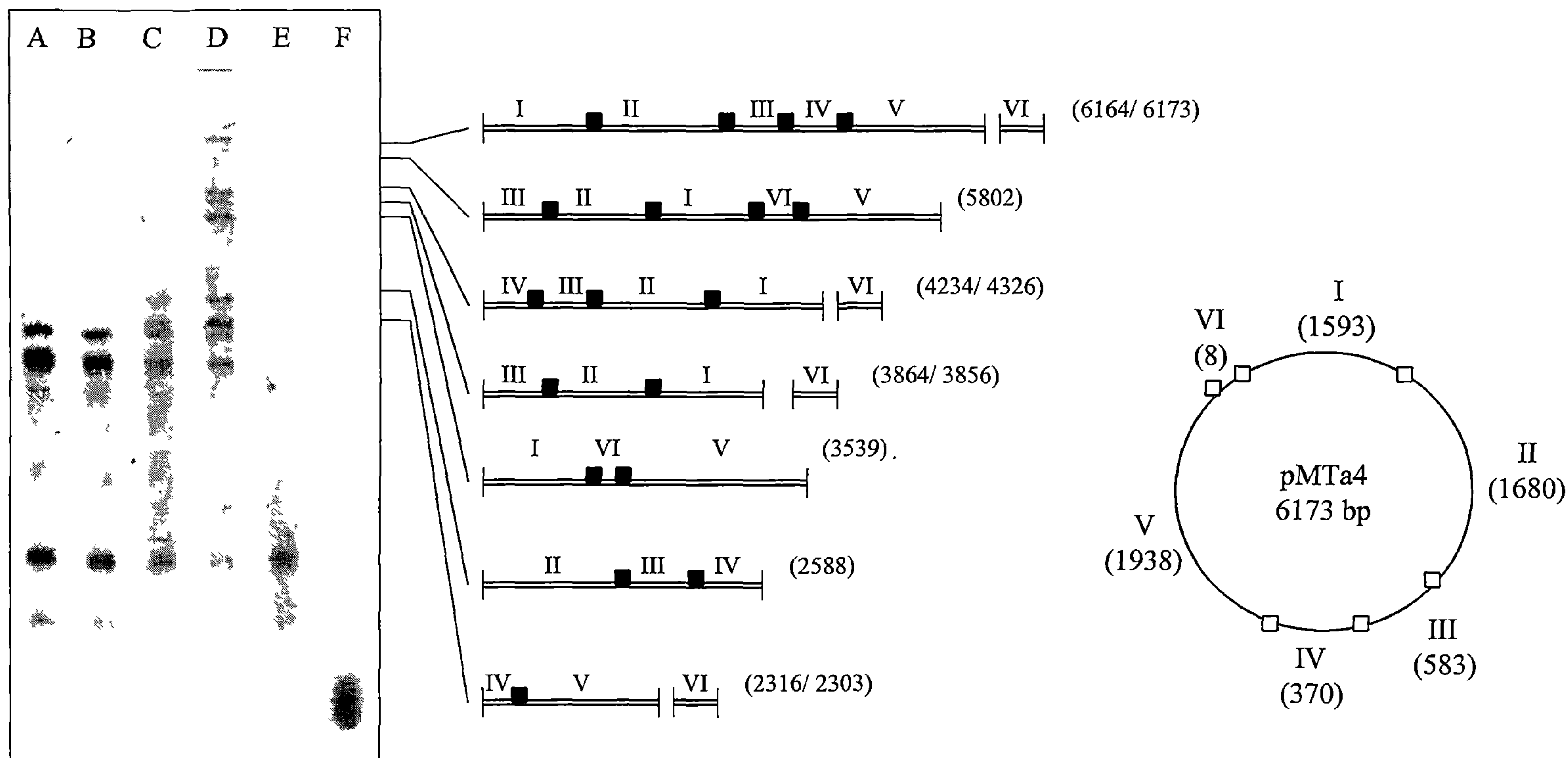

Figure 3.14: Densitometric image of pMTa4 restriction by *Hae II* after charged ^{7}Li particle irradiation. Corresponding size for each indicated band are represented by numericals (see also table III.VI). Lanes A, B, C, D, E and F represent fluence of 1 x 10^6, 5 x 10^6, 1 x 10^7, 5 x 10^7, 1 x 10^8 and 5 x 10^8 particles cm^{-2}, respectively. Shaded blocks in the fragments indicate restriction sites that were apparently affording resistance to *Hae II* cutting.

3.7 EFFECT OF γ-RADIATION ON *E. coli* GENOMIC DNA:

Fig. 3.15 shows gel electropherogram of genomic DNA of non-irradiated (control) and following γ-irradiation. Exposure to dose of 15, 30, 60, 120 and 240 Gy, respectively, showed a change in the distribution pattern of DNA fragments on the agarose gel. As the dose was increased the smear, which is an indication of non-random degradation, starts to slide down gradually under the condition studied.

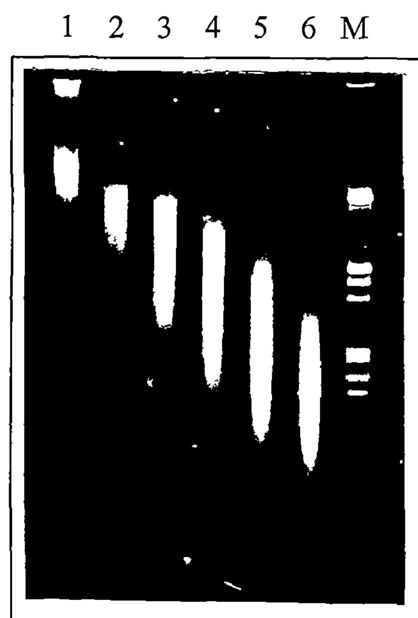

Figure 3.15: Agarose gel electropherogram of bacterial genomic DNA. Lane 1 represent non-irradiated (control) genomic DNA, while lane 2, 3, 4, 5 and 6 represent doses of 15, 30, 60, 120 and 240 Gy of γ-radiation, respectively. M represents λ DNA double digest marker.

3.8 EFFECT OF γ-RADIATION ON GENOMIC DNA FRAGMENTATION BY DIFFERENT RESTRICTION ENDONUCLEASE:

Fig. 3.16 shows gel electropherograms of restriction fragmentation pattern of *E. coli* genomic DNA generated by *Nci I, Hinf I, Hae II* and *Bgl I.* Unlike in plasmids system, there was no observable changes in the fragmentation patterns that would indicate abnormal restriction in irradiated samples.

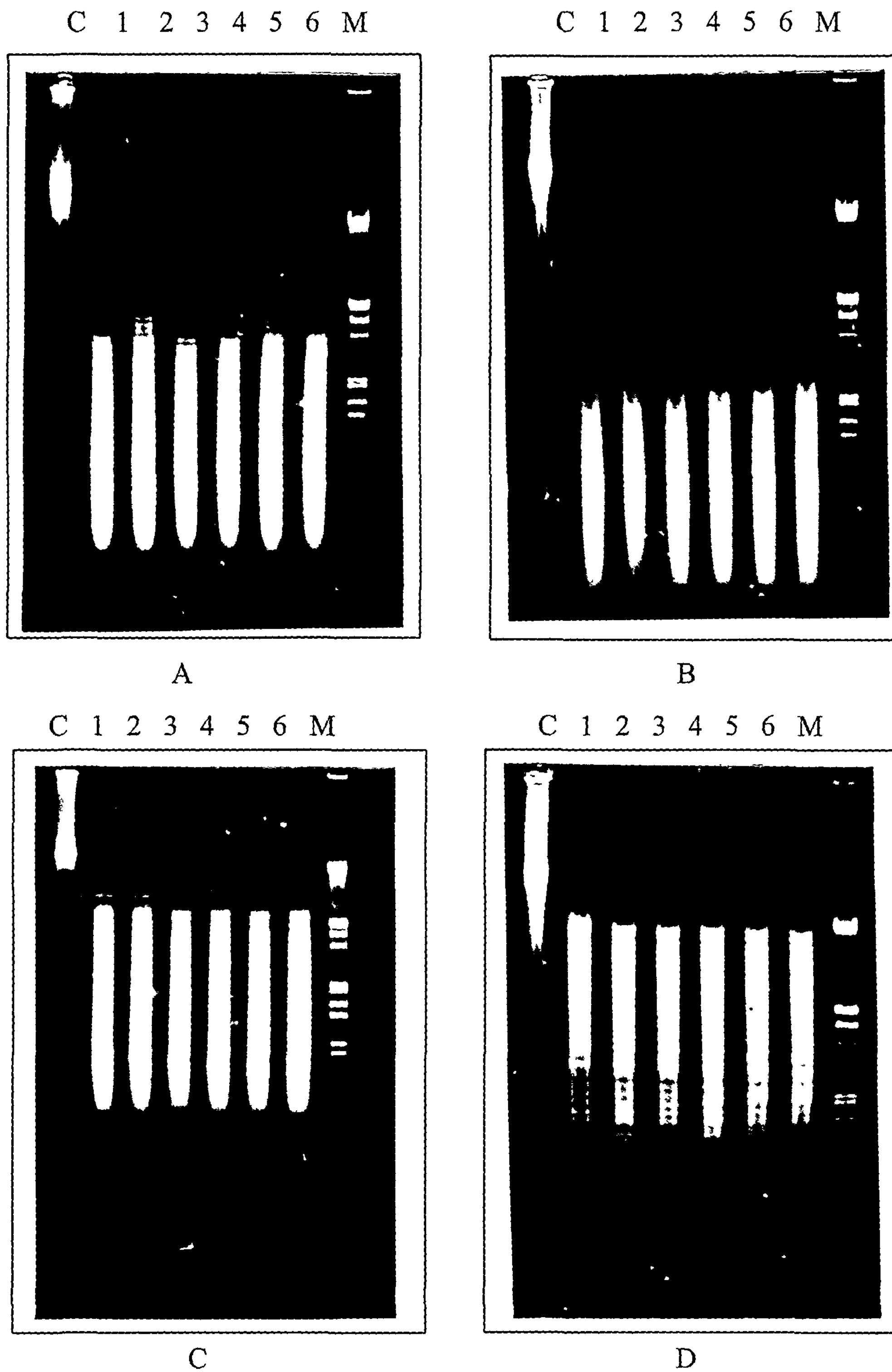

Figure 3.16: Agarose gel electropherogram of *E. coli* genomic DNA fragmentation pattern by different restriction endonuclease. Panel A, B, C and D are restriction by *Nci I, Hinf I, Hae II* and *Bgl I*, respectively. Lanes 1, 2, 3, 4, 5, and 6 represent non-irradiated (control), and 15, 30, 60, 120 and 240 Gy of γ-radiation, respectively. Lane C is control undigest while lane M is λ DNA double digest marker.

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CHAPTER 4

DISCUSSION

The damage of the molecular DNA by ionizing radiation is generally considered to be the prime lesion in a living system. Owing to its implication in major biological events, extensive studies have subsequently being carried out with different qualities of low- and high-LET radiation. Indeed, considerable insights in DNA damages induced by radiation have been gained in the process. Yet, the intricacy and subtlety of damage mechanism have prevented the unambiguous understanding of the quality and nature of DNA damage and its correlation to the eventual biological response. This piece of work was undertaken in an attempt to gain more insight into the interaction and nature of DNA damage induced by low-LET γ- and high-LET ^{7}Li particle radiation.

Plasmids DNA besides being used as a vector, can be a very useful model for studying radiation-induced DNA damages. Because of its simple form, it provides the possibility of determining the number and types of DNA lesions without much complication. Since the entire nucleotide sequence of the plasmid (pMTa4) used in this study is known (see table II.II), it provides the advantage of accurate prediction of NT sequence in different parts of plasmid making fragment analysis realistic. The plasmid isolate consisting of three geometrically different forms (Fig. 3.1 lane B) was used throughout the analysis. The slow migrating DNA observed above the OC form might be multimeric plasmids produced due to the intra-inter strand interactions. Such DNA structures have been observed by other investigators in plasmid (Washino and Schnabel, 1982; Herskind, 1987) and in calf thymus DNA studies (Georgakilas *et al.*, 1998; 2000). Perhaps the methodology manipulations and the experimental condition that induced SSB and DSB during isolation creating 3 forms of plasmid (Fig. 3.1) had probably favored the interaction resulting into a complex multimeric configuration. This is seen as a high molecular size DNA on the gel. Such multimeric form of plasmid DNA does not interfere with the normal restriction by endonucleases. This is evident from results shown in Fig. 3.1, which shows that the restriction of the plasmid preparation by *Nco I* (lane A) and control digestions by different restriction endonucleases (RE) were complete (Figs. 3.4, 3.5, 3.6, 3.13).

The four nucleotides (G, A, T and C) in DNA are constant targets of damage by chemical processes such as methylation, depurination, deamination and oxidation. Induction of DNA damage by radiation is mostly caused by oxidation via $^{\bullet}$OH mediated indirect effect (Ward and Kuo, 1970; Roots and Okada, 1975; Fielden and O'Neill, 1991). The reaction sites for $^{\bullet}$OH to the purine and pyrimidine DNA bases have been studied and discussed by several workers (Fugita and Steenken, 1981; O'Neill, 1983; Von Sonntag, 1987; Steenken, 1989). The 5-C and 6-C of the pyrimidine and the 4-C and 8-C of purines are the most probable sites known for the attack resulting into a plethora of modified bases (Fielden and O'Neill, 1991). Besides, the production of a wide range of base products

(Hutchinson, 1985; von Sonntag, 1987; Wallace, 1998), such damages include predominantly strand breaks. Fig. 3.2 shows the change in the plasmid DNA conformation after exposure to γ-rays indicating strand breaks. The increase in the OC form at higher doses of radiation (Table III.I) is an indicator of induction of SSB. The •OH has been reported to be much more effective in the production of SSB than •H and e_{aq}^- that are formed when DNA is γ-irradiated in aqueous solution (Kuipers and Lafleur, 1998). Observations have also been made that •H contributes in the formation of SSB (Lafleur *et al.*, 1978) although at a smaller rate. Since in this study DNA was irradiated in aqueous solution, it will be reasonable to assume that the significant induction of SSB (Fig. 3.3), is primarily due to the water derived •OH and to a lesser extent •H (Kuipers and Lafleur, 1998). This is in good agreement with existing dose dependent response to radiation (Schans and Bleichrodt, 1973; Roots *et al.*, 1990; Milligan, 1993; Weinfeld, 1997).

Result from Figs. 3.4, 3.5 and 3.6 show variation in the restriction capabilities of endonucleases after plasmid DNA has been exposed to γ-rays. While efficiency of the plasmid restriction by *Hinf I*, *Bgl I* (Fig. 3.4 A, B), *Dra I, Pvu II*, *Acc I* and *Bgl II* (Fig. 3.5) and *Ksp I* (Fig. 3.6 A) remained unchanged, it was reduced for *Hae II* (Fig. 3.4 C) and *Nci I* (Fig. 3.6 B) after the plasmid DNA was γ-irradiated. This can be attributed to the possible γ-rays-induced effects on the restriction sequence of *Hae II* and *Nci I* (Table III.II) which could be a possible cause of the restriction sequences offering resistance to its cleavage by the two RE. This has probably led to sub optimal restriction. Such restriction can result when either the restriction site is shielded by DNA binding chemical compound (Financsek *et al.*, 1978) or due to possible radiation-induced base modification (Sinheimer, 1954; Dizdaroglu *et al.*, 1978; Paul *et al.*, 1990). It is highly likely that the latter possibility holds true for the experimental set up used in this investigation since it lacks any other component which may interact with the plasmid DNA in aqueous solution. The partial restriction by *Hae II* appears to be dose independent as fragments generated after the lowest dose of 30 Gy did not apparently change even after the highest dose of 240 Gy (Fig. 3.4 C, lanes 2 through 5) that was used in this investigation. Result from *Nci I* restriction (Fig. 3.6 B) also followed a similar pattern. The appearance of four distinct extra bands (Fig. 3.6 B, lanes 2 through 6) again suggest dose independent effect on the production of fragments due to partial restriction. The increase in the intensity of the additional fragments (Table III.III) may, on the other hand, indicate a dose dependent but non-random manifestation of effect. Dose dependent production of radiation-induced base damage in intact cells is also known (Le *et al.*, 1998). Although, these effects remains not very clear, the non-random generation of fragments due to abnormal restriction suggest that all restriction site that were available for *Hae II* and *Nci I* were not equally susceptible to the effect by radiation. Therefore, for such abnormal restriction process to occur, it is likely that plasmid DNA underwent certain specific alteration or modification in the *Hae II* and *Nci I* restriction

sequence such that some of these sites became less accessible for its efficient restriction. This alteration or modification on specific sites, therefore, resulted in partial restrictions producing larger DNA fragments, which are seen on the gel as slow migrating bands (Figs. 3.8 and 3.10). This assumption logically allows us to look for the specific nucleotides composition constituting the restriction sites for *Hae II* and *Nci I*, in order to explore the possibilities of identifying changes that may be responsible for such partial fragmentation. A closer look into *Hae II* and *Nci I* restriction site revealed its GC-richness (Table III.II). Unlike the others, except *Ksp I*, it was notable that *Hae II* and *Nci I* produced 100 % GC staggered-ended DNA pieces. The other RE that generated either staggered- or blunt-ended pieces did not show the GC-motif feature. The nucleotide composition in the flanking region around the restriction sequence of *Hae II* and *Nci I*, as shown below in table IV.I and table IV.II, too indicated higher GC contents.

Table IV.I. Twenty nucleotide flanking region sequence around restriction sites for *Hae II* on pMTa4 (refer to table II.II for complete NT sequence of pMTa4).

Restriction site between (see Fig. 3.7)	**Nucleotide sequence**	**GC content (%)**
VI & I	GCTAGGGCGC/ TGGCAAGTGT	65
I & II	CCGTCGGCGC/ TGAATCCCGC	75
II & III	CCAGCAGCGC/ CTCCTCCTGC	75
III & IV	TATTGGGCGC/ TCTTCCGCAA	55
IV & V	AGCGTGGCGC/ TTTCTCATAG	55
V & VI	GAGCGGGCGC/ TAGGGCGCTG	80

Within these parameters it is reasonable speculation to say that GC-rich nucleotides were being more effected or chemically modified upon exposure to γ-radiation. In spite of *Ksp I* recognition sequence (CCGC/GC) being similar to *Nci I* (CC/[CG]GG) and *Hae II* (GGCGC/[TC]), there was no abnormal fragmentation observed in this (Fig. 3.6 A). This can be partially explained by the difference in the number of restriction sites these enzymes have on plasmid pMTa4. Since *Nci I* and *Hae II* cut the plasmid DNA more frequently than *Ksp I* enzyme (Table II.I), the probability of γ-rays interacting with a restriction site is higher at *Nci I* and *Hae II* sites than at *Ksp I* site. This could be the reason why *Ksp I* restriction site was unaffected by γ-irradiation. Furthermore, the lack of abnormal restriction of pMTa4 by the other RE supports the possible NT modification hypothesis of

certain specific bases by γ-radiation. It also indicates that non-GC-motif were not affected by γ-radiation such that it afforded any resistance to the enzymes.

Table IV.II. Twenty nucleotides flanking region around restriction sites of *Nci I* on pMTa4 (refer to table II.II for complete NT sequence of pMTa4).

Restriction site between (see Fig. 3.9)	**Nucleotide sequence**	**GC content (%)**
VI & I	TCCTGCAGCC/ CGGGGGATCC	75
I & II	CCTGCAGCCC/ GGGGGCTCCA	75
II & III	GAGTCCAACC/ CGGTAAGACA	60
III & IV	AATTGTTGCC/ GGGAAGCTAG	50
IV & V	TGCTCTTGCC/ CGGCGTCAAT	60
V & VI	ATTGGGTACC/ GGGCCCCCCC	75
VI & VII	CCCCGTTGCC/ CGGCAACGGT	75

In the experimental conditions, the frequency of strand breaks induced by ^{7}Li particle was qualitatively different from low-LET γ-radiation. The L form of plasmid DNA is produced largely due to a single DSB and, therefore, the percentage of linear DNA in the sample can be taken as a measurement of the frequency of DSB induction. Fig. 3.12 indicates that, unlike in low-LET, high-LET ^{7}Li particle induced more DSB than SSB. This is concurrent to existing report that high-LET radiation is more efficient than low-LET radiation in inducing DSB (Christensen *et al.*, 1972; Nygren and Ahnstrom, 1996). The production of DSB has been suggested mainly due to the clusters of ionization (Barendsen, 1964; Ward, 1981; Goodhead, 1994). After γ-ray irradiation, the ionization in a cluster is close enough to permit one hit in each strand. On the other hand, high-LET radiation leads to a denser deposition of ionization than γ-rays and this could explain both an increase in yield of DSB and a more devastating damage on a certain DNA segment. This may be the reason for the increased production of DSB observed as the result of high-LET radiation. However, going by restriction fragment analysis, the nature of plasmid damage after high-LET ^{7}Li particle irradiation appears to be similar to that observed from low-LET γ-radiation. The result from Fig. 3.13 shows no abnormal restriction by *Bgl I* and *Hinf I* after ^{7}Li particle irradiation. This indicates that even with high-LET radiation induced damages, there was no apparent alteration in GCCGTTT/GGGC and G/ANTC sequences that would

effect the restriction efficiency of *Bgl I* and *Hinf I,* respectively (Table III.II). On the other hand, high-LET radiation too influenced GGCGC/(TC), the restriction site for *Hae II.* However, unlike low-LET effects, a dose dependent effect was observed in this case. While the lowest fluence of 1 x 10^6 particles cm^{-2} showed no effect (Fig 3.13, C lane 1), exposure to 5 x 10^6 particles $cm^{-2}$and higher effected differently the extent of restriction by *Hae II.* At a fluence of 5 x 10^8 particles cm^{-2}, the whole plasmid got fragmented into very small DNA pieces (Fig. 3.13, C lane 6). The sizes of the extra fragments were calculated and were found to be similar to ones resulting from low-LET radiation (Table III.VI). These observations allow the possible inference that though the extent and quality of damage induced by low- and high-LET may differ, the molecular basis of damage may likely follow a similar mechanism.

Even though the observations made above are in the realm of endonuclease restriction, they indicate the likelihood of specific nucleotide sequences being more susceptible to radiation-induced damages. Sy *et al.* (1997) have reported that the probability of strand breakage at a nucleotide site is not determined by the chemical nature of the nucleotide (G, A, T or C) but by the sequence to which the nucleotide belongs. Such suggestions come from the variation in the accessibility of H4' and H5' of the nucleotides by the •OH (Fratini *et al.*, 1982; Souza and Goodfellow, 1994). Accessibility of the radicals to such sites have been known to lead to strand breaks (von Sonntag, 1987). It is presumable, therefore, that CC- or GG-motif which favors the major groove of DNA duplex facing outward, will be more accessible to the oxidative reactions by radicals. The decreased probability of strand breakage observed at A/T (Barone *et al.*, 1994) and at AATT or AAATT sequence (Franchet-Beuzit *et al.*, 1993; Isabel *et al.*, 1993) has been suggested to be due to reduced accessibility of •OH to H4' located in the minor groove of the DNA.

Although the type of the modified base was not determined in this study, a wide range of radiation-induced bases damages are known (Hutchinson, 1985; Wallace, 1998). Of the several types, 8-oxoguanine has been identified as the major product (Kasai *et al.*, 1984). Fuciarelli *et al.* (1990) found that when DNA was γ-irradiated in oxic condition, high amount of 8-oxoguanine were formed. Under conditions where •OH are the only damaging species, formamidopyrimidine glycosylase (Fpg) sensitive sites were formed in high amounts (Kuipers and Lafleur, 1998) indicating a high formation of guanine product. Deamination or demethylation of C nucleotide is also known to be induced by radiation. Since the NT sequences effected are GC-rich, the resistance to restriction after irradiation may also be due to deamination of C nucleotide. This provides a possibility to assume that such products are likely to be induced when aqueous plasmid DNA was γ-irradiated. Regardless of the type and nature of modification, the observation indicates GC-rich motif

to be more frequently modified or damaged upon interaction with radiation.

Such indications may have important bearing on the understanding of stability and integrity of the genomic DNA. Structural alterations in the DNA due to base modification may lead to conformational changes in the DNA, which may, in turn, retard the efficiency of DNA polymerase and other DNA repair enzymes. The modified forms of C and G have been implicated in mutagenesis, carcinogenesis and ageing (Fraga *et al.,* 1990; Shibutani *et al.,* 1991; Shigenaga *et al.,* 1994; Wiseman and Halliwell, 1996). Fig. 4.1 shows possible pathways for mutation arising from a damaged base (Wiseman and Halliwell, 1996).

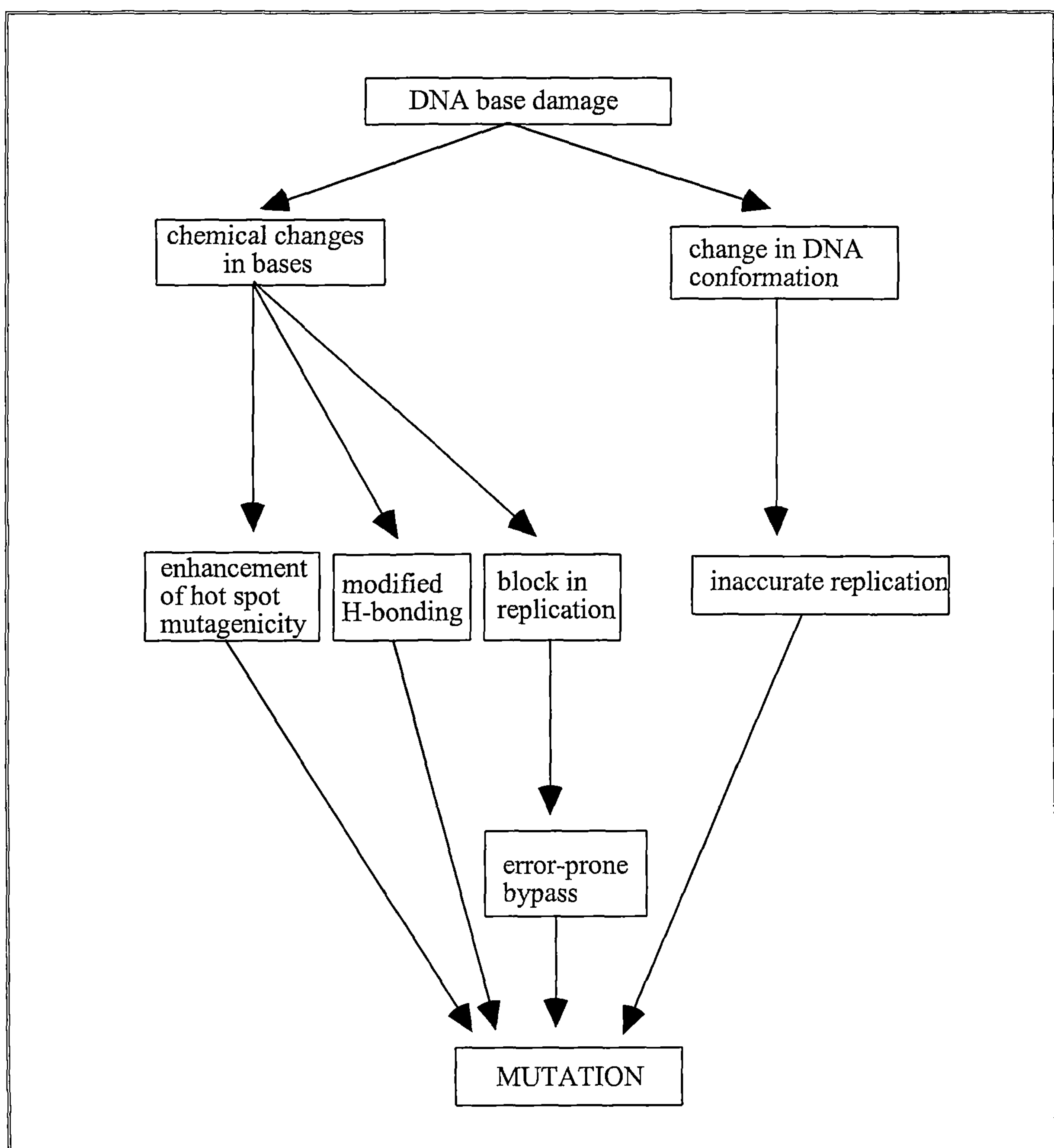

Figure 4.1: Pathways leading from initial base damage to subsequent mutation (reproduced from Wiseman and Halliwell, 1996).

Large numbers of studies have reported high mutagenecity at GC than AT. Mutation studies using MSH2 deficient mice have indicated a greater percent of the mutation in GC than AT (Phung *et al.,* 1998; Rada *et al.,* 1998). Chemically modified G or C causes mis-pairing, where an error-prone polymerase frequently introduces a wrong base opposite these nucleotides. 8-oxoguanine preferentially pairs with A and causes G to T transversion (Kasai and Nishimura, 1984). The 5-methyl C in a CpG motif can undergo deamination to produce C to T transition (Coulondre *et al.,* 1978). C nucleotide could also spontaneously de-aminate to produce uracil (Lindahl, 1993) or be oxidized to 5-hydroxycytidine (Wang and Essigmann, 1997) leading to a C to T transition. High frequency of GC mutations have been observed in several mutation studies (Levy *et al.,* 1992; Seetharam *et al.,* 1990; Murata- kamiya *et al.,* 1997; Burcham and Harkin, 1999).

With such evidences of GC-vulnerability to mutation, it is likely that radiomodified GC nucleotides would form important premutagenic lesions. This indication also points that clusters of GC in the DNA molecule may very likely form hotspots for radiation-induced damages. While further detailed investigation would be required, it opens up the likely possibility that inherent radiosensitivity and genome instability may be at least partly determined by the GC-richness of nucleotide sequence in the DNA.

GC-rich sequence of DNA has been reported to occur frequently in many human genes that have been identified (Meneveri et *al.*, 1993; Dunham *et al.*, 1999; Hattori *et al.*, 2000). A distinct feature, which is now suggested to be useful in the identification of genes, is the "CpG islands" (Reeves, 2000). Such regions, consisting of about 55 % G-C and spanning in about 400 bp, is reported to be associated with about 60 % of known human genes. The GC composition and its nature of occurrence are, therefore, likely to be associated in the expression of different genes. If the hypothesis of GC-vulnerability to radiation-induced damage holds true, it is a likely possibility to speculate that CpG islands shall be more effected by radiation. This would suggest that most genes (rich in CpG islands), regardless of its state of transition (transcribing or non-transcribing), might be effected by radiation with higher probability. This perhaps partially explains the current observation of inducing almost uniform damage and repair processes in expressed or unexpressed genes (Bunch *et al.*, 1995; Ljungman, 1999). It is also not unlikely to suggest that humans, with constant exposure to radiation in different forms, may in the course of time undergo alterations in most genes. Perhaps the evolutionary change that is observed in many living forms follows such mechanisms.

The results from Fig. 3.15 show a dose dependent damage to the bacterial genomic DNA irradiated in aqueous solution. The downward shift of the smear indicates a non-specific fragmentation of the DNA as the dose was increased. Similar to a situation

discussed above, the effects are mainly due to the attack by •OH resulting from water radiolysis. DNA oxidation by the •OH results in abstraction of a hydrogen atom from sugar or bases, in addition to the bases, or in radical transfer from bases, generating a sugar or base radical (Siddique and Bothe, 1987). Thus, in this case such reactions may lead to SSB or DSB causing the DNA molecule to undergo fragmentation, which is seen as a smear. However, unlike in plasmid system, RE analysis does not reveal any apparent modification that would be induced by radiation (Fig. 3.16). Unexpected though, however, such results have to be cautiously considered as the analysis is in the realm of enzyme restriction only. Due to the large number of undefined fragments produced as compared to the plasmid, the qualitative fragmentation analysis was not feasible. Therefore, the observations made cannot be merely dismissed. This makes obvious that under the applied condition RE approach is not sensitive when analyzing large and more complex DNA molecules by agarose gel electrophoresis. On the broader view this permits the presumption that the similarity of the interaction and molecular damage event observed from plasmid model to the higher and complex genomic DNA system may not be totally implausible.

In summary, the study suggests the possible involvement of specific nucleotide (G or C) in radiation-induced damage. Under the parameters studied, it also indicates a possibility of similar molecular basis of damage for low- and high-LET even though the impact of the damage may differ.

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CHAPTER 5

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SECTION C

STUDY OF DNA DAMAGE IN CELLULAR SYSTEM

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CHAPTER 6

INTRODUCTION

One major target of radiation assault on living cells, which may have serious immediate and long-term consequences, is the genome. In the present radiation paradigm, the ultimate implications of such assaults are apoptosis or carcinogenesis via mutations (Sugahara, 1994). The interactions of radiation with the genome may cause changes that do not have noticeable impact on the process of life. It may also cause serious impedance to life processes. In this later event the induced changes are conventionally designated as "damages". Thus, "damages" refers to a specific condition of radiation-induced changes in the living system.

The cellular endpoints following irradiation are believed to be primarily due to induction, processing and manifestation of DNA damage. Much efforts has consequently been focused on understanding the link between DNA damage and cells response (Szumiel, 1998; Woudstra *et al.,* 1998; Nunez *et al.,* 1998). In the preceding section, studies from lower organism in non-cellular conditions have indicated the possible preferences of specific nucleotide sequence in DNA damage. Such phenomenon may partially be involved in determining the extent of DNA damage and, thereby, influence inherent radiosensitivity and genomic instability of an organism. A notable difference between non-cellular and cellular systems is complete absence of any repair in the former. In contrast, a cellular system has an in-built repair capacity and other organizational intricacies that may influence the final manifestation of radiation-induced changes. Therefore, direct extrapolation of such information from a non-cellular system to cellular systems, shall be appropriate only after sufficient insights into the complexities of cellular response in relation to DNA damage has been obtained. Knowledge of the biochemical and molecular basis of sensitivity to ionizing radiation can provide useful information in the understanding of carcinogenesis, cancer susceptibility and patients' response to radiotherapy. Therefore, in this chapter attempt has been made to evaluate some biochemical parameters in relation to DNA damage in different mammalian cell systems.

Exposure of mammalian cells to ionizing radiation results in DNA strand breaks either through a direct deposition of energy or indirectly via the action of free radicals produced predominantly from the radiolysis of water in the cell (Chapman *et al.,* 1973; Roots and Okada, 1975). Variation in susceptibility to the induction of such damages have been suggested to be important in determining tumor-cell radiosensitivity (Radford, 1986; McMillan *et al.,* 1990). Studies from different radiosensitive cell lines shows distinct dose-response curves for DNA damage (Taylor *et al.,* 1991; Malyapa *et al.,* 1996; Woudstra *et al.,* 1996). Both the presence of DNA-bound proteins and the higher order chromatin structural organization markedly influence such effects (Warters and Lyons, 1990; Ljungman, 1991). These reports suggest that cellular radiosensitivity may be partially dependent on the fragility of chromatin structure. Therefore, to fully comprehend the

induction and repair of DNA damage in mammalian cell, a good understanding of chromatin structure is necessary. In this context, it may be worthwhile to look into the status of chromatin organization in relation to strand breaks following radiation assault.

6.1 DETECTION OF DNA DAMAGE:

Over the years, a number of techniques have been developed to quantify both SSB and DSB (Singh *et al.,* 1995, reference therein; Prise *et al.,* 1998). In such assays, the sensitivity and specificity of the methods are of obvious importance. A convenient way of measuring SSB is the single cell gel electrophoresis (SCGE) or comet assay. The method, conceived in 1984 (Ostling and Johanson, 1984), is a rapid, simple, visual and sensitive technique which is frequently used for detection of strand breaks of DNA in mammalian cells (Singh *et al.,* 1988; Olive *et al.,* 1990a). One particular advantage of the assay is that it is possible to measure the level of SSB in individual cells and the detection is very reliable even at low levels of damage (Malyapa *et al.,* 1998). Apart from image analysis, which greatly facilitates the possibilities of comet measurements, the cost of performing the assay is low. In addition, the results can be obtained in a single day. The sensitivity for detecting damage in single cells analyzed in the comet assay is shown to be comparable to the sensitivity of other methods which measures average damage to a population of cells (Olive *et al.,* 1990b). The application of the technique has, therefore, increased in wide areas like bio-monitoring (Somorovska *et al.*, 1999; Kassie *et al.*, 2000), clinical practice (Olive *et al.*, 1999), genetic toxicology and carcinogenicity (Tsuda *et al.*, 2000; Tice *et al.*, 2000); radiation biology (Olive, 1999) and genetic ecotoxicology (Cotelle and Ferard, 1999). The application of the assay in the identification of dietary protective factors in clinical studies and in monitoring the risk of DNA damage resulting from occupational, environmental or life style exposure has been reviewed (Kassie *et al.,* 2000). The influence of antioxidants on cigarette smoke-induced DNA SSB in lungs, liver and stomach of mouse has been studied using the comet assay (Tsuda *et al.*, 2000). The assay has been very widely employed in studying radiation-induced DNA damages. Alkaline and neutral conditions are used to measure DNA SSB (Singh *et al.,* 1988) and DSB (Olive *et al.*, 1991; Marples *et al.*, 1998: Sarkaria *et al.*, 1998), respectively.

6.2 CHROMATIN:

The fundamental unit of chromatin structure in eukaryotic cells is the nucleosome in which DNA is wound around an octamer of the core histones H2A, H2B, H3 and H4. Interaction with linker histones H1 and various non-histone proteins provide DNA compaction, which in the defined regions of the genome, culminates in a highly condensed state termed as heterochromatin (Kornberg and Klug, 1981; Singer and Berg, 1991;

Jackson, 1997). The packaging is known to effect processes such as DNA replication, transcription and the maintenance of genomic integrity depending on its state of transitions from highly condensed to more unfolded, open or relaxed status of chromatin structures (Singer and Berg, 1991). The structural flexibility of chromatin is essentially achieved by the electrostatic and weak force interactions between the chromosomal proteins, mainly basic histones proteins, and the DNA strand which is acidic (Weisbrod, 1982). The strength of the interaction between these two components of the chromatin decides the condensed or relaxed status of the chromatin.

Transitional status of chromatin structure can be conveniently monitored by several approaches. A convenient and widely used method is chromatin activity assay using exogenous DNase I that randomly degrades DNA of chromatin (Weisbrod and Weintraub, 1979; Chakraborty, 1997; Saikia *et al.*, 1999). The fragmentation efficiency of chromatin by DNase I depends on the accessibility of DNase I to DNA for cutting. This, in turn, is greatly dependent on the status of the chromatin structural configuration. Depending on the level of condensation or relaxation of the chromatin, accessibility of DNase I fragmentable DNA sites will be either few or more, respectively. Therefore, DNase I mediated fragmentation is a convenient way of monitoring the status of chromatin conformation. Using DNase I fragmentation approach, a natural chemical alkaloid, arecoline was found to influence the chromatin organization (Saikia *et al.*, 1999). Chromatin isolated from spleen and bone marrow cells of mouse was observed to undergo progressive relaxation following chronic exposure to arecoline. Schneeweiss *et al.* (2000) have shown in T1 (human kidney) and L929 (murine fibroblast) cells that high mobility group (HMG) proteins depleted chromatin became resistant to cleavage by DNase I. It is known that HMG proteins bind with the nucleosome in either the core or the linker region. Therefore, the reduction in chromatin cleavage by DNase I has been suggested to reflect a condensed organization of the chromatin structure.

6.3 APOPTOSIS:

Apoptosis or programmed cell death (PCD) is a morphologically and biochemically distinct form of cell death that occurs in many different cell types under a wide variety of conditions (Kerr *et al.*, 1972; Wyllie *et al.*, 1980; Begum *et al.*, 1999). It is a genetically controlled cellular process that may occur in response to diverse range of specific developmental and environmental stimuli, including DNA damage, growth factor deficiency, neurotransmitters, oncogenes, tumor suppressor gene p53, ligation of cell surface receptors, heat shock, ionizing radiation, bacterial or viral infections, *etc.* (Vaux, 1993). The process of apoptosis is controlled through the expression of an increasing number of genes conserved throughout evolution from mammals to nematodes, flies, and

viruses. Some gene products are activators of apoptosis whereas others are inhibitors. Several such products and other physiological factors that are involved in the regulation of apoptosis are now largely understood (Begum *et al.*, 1999). The well defined morphological sequence of events in apoptosis is characterized by surface membrane blebbing, cytoplasmic contraction, activation of endogenous endonucleases, nuclear condensation and packaging of cellular compounds within the membrane prior to their budding from cell as apoptotic bodies (Wyllie *et al.*, 1980; Arends and Wyllie, 1991). The Ca_2^+ and Mg_2^+ dependent nuclease cleaves double stranded DNA at the most accessible inter-nucleosomal regions producing DNA fragments that are discrete multiples of 180 bp subunits. They are detected as a "DNA ladder" on agarose gel electrophoresis, and is usually considered an important biochemical hallmark of apoptosis in both intact and *in vitro* models (Wyllie and Morris, 1982; Lazebnik *et al.*, 1993).

Extensive DNA damages by certain genotoxic agents and radiation often lead to cell death by PCD or apoptosis (Soldatenkov *et al.*, 1995). Studies from sensitivities of some cell types to the induction of apoptosis by DNA targeted radiation damage, relationship between p53 status and radiation response, and the influence of enzymatic repair capability on susceptibility have strongly suggested that DNA damage is an important initiator in radiation-induced apoptosis (Radford and Aldridge, 1999). The work of Vral *et al.* (1998) on apoptosis induced by fast neutrons in human peripheral blood lymphocytes have also suggested the role of initial DNA damages in the induction of apoptosis. Evaluation of apoptotic induction by radiation, therefore, may indicate the severity of DNA damage. Peitcsh *et al.* (1993) have proposed that DNA fragmentation induced during apoptosis is not due to double strand cutting enzyme, but rather the result of SSB. A radiosensitive cell line, NMT-1, has been shown to undergo apoptosis as a function of time within 24 hr after irradiation (Mitsuhashi *et al.*, 1997). A significant increase in the amount of apoptosis between 2 to 5 Gy was observed, while increase in the radiation dose from 5 to 10 Gy did not result in increased apoptosis. Irradiated lymphocytes samples analyzed after 24 hr, culture showed similar dose-response curves for both neutron and γ-radiation (Vral *et al.*, 1998). An initial steep increase in the number of apoptotic cells below 1 Gy, with a flattening of the curve at higher dose towards 5 Gy was observed. It is suggested that initial DNA damage, as opposed to DNA repair, dominate the induction of apoptosis in resting lymphocytes radiation (Vral *et al.*, 1998).

Since chromatin is DNA complexed with overwhelming amounts of chromosomal proteins, it will be relevant to look into the influence of modifications of chromosomal proteins on radiation-induced DNA damage. Poly-ADP-ribosylation (PAR) is one of the various avenues of post-translational modification of chromosomal proteins, especially histones, in response to DNA damages (Boulikas, 1989; de Murcia and de Murcia, 1994).

This process has been shown to respond to DNA damage to a variety of agents including ionizing radiation (Berger, 1985; Oleinick and Evans, 1985; Schneeweiss and Sharan, 1999). Similar response to neutron and γ-radiation induced cellular damages have been reported (Sharan *et al.,* 1996; 1998b). Therefore, the simultaneous measurement of radiation-induced apoptosis and monitoring the status of PAR of total cellular and chromosomal proteins would be vital as it is likely to shed light on the mechanism of interaction of radiation with DNA in its *in vivo* organization.

6.4 POLY-ADP-RIBOSYLATION:

Poly-ADP-ribosylation (PAR) is a post-translational modification of mainly nuclear proteins. During this process, molecules of ADP-ribose are added successively on to acceptor proteins to form unbranched or branched polymers of variable lengths (Althaus, 1992; Saikia, 1996; Chakraborty, 1997). The reaction is enzymatic and is catalyzed by poly-ADP-ribose polymerase (PARP). This modification is transient but very extensive *in vivo*, as unbranched and branched polymer chains can reach a length of up to 200 units on certain protein target. While PAR of histones, topoisomerases and many chromosomal proteins have has been shown to occur in living cells (heteromodification), the major target of PAR *in vivo* is PARP itself (automodification) (Ferro and Olivera, 1984). The effect of this modification is crucial, as the chromatin tends to relax or decondense depending on the net negative charge accumulated on the nuclear proteins, primarily the histones. The enzymes, poly-ADP-ribose glycohydrolase and ADP-ribosyl protein lyase are reported to break down the ADP-ribose polymers to reverse the reaction (Gonzalez and Althaus, 1989; Sharan *et al.*, 1996). This post-translational modification of chromosomal proteins has been suggested as one of the most important post-translational processes known to affect interaction of chromosomal proteins with DNA in chromatin (Poirier *et al.,* 1982; Neidergang *et al.,* 1985; De Murcia *et al.,* 1988). Therefore, the PAR reaction is considered to have a significant bearing on the stability and state of organization of chromatin (Poirier *et al.,* 1982). Such modification is also known to be responsible for the formation of hypersensitive sites in chromatin that becomes available for nuclease cleavage during apoptotic cell death (Khodarev *et al.,* 1998).

PAR is catalyzed by the nuclear enzyme PARP in response to DNA strand breaks with NAD^+ serving as the substrate (Althaus and Richter, 1987; Lindahl *et al.,* 1995). PARP has a NH_2-terminal DNA-binding domain comprising two zinc fingers, which mediate the binding to DNA double strand or single-strand breaks. Binding to DNA strand breaks drastically stimulates the catalytic center of PARP, which resides in the C-terminal NAD^+-binding domain. PARP is known to undergo rapid caspase mediated proteolysis during the execution phase of apoptosis (Kaufmann *et al.,* 1993; Shah *et al.,* 1996;

D'Amours *et al.,* 1998). The cleavage of the enzyme is proposed to prevent the depletion of NAD^+ (PARP substrate) and ATP, which are thought to be required for the later events in apoptosis (Simbulan-Rosenthal *et al.,* 1998; Ha and Snyder, 1999), and in facilitating cellular disassembly (Oliver *et al.,* 1998). However, its role in apoptosis is not clearly understood with contradictory data suggesting that in some cell lines neither cleavage nor activation of PARP has any role in apoptotic cell death (Leist *et al.,* 1997).

Poly-ADP-ribose synthesis and turnover are thought to modulate cellular responses to DNA damage. Its involvement has been shown in DNA repair (Durkaez *et al.,* 1980; Satoh and Lindahl, 1992; Molinete *et al.,* 1993), DNA amplification (Burkle *et al.,* 1987; Kupper *et al.,* 1996), cell cycle perturbations (Jacobson *et al.,* 1985), malignant transformation (Borek *et al.,* 1984; Takahashi *et al.,* 1984; Kasid *et al.,* 1986; Saikia *et al.,* 1999), necrotic cell death (Berger and Berger, 1986; Zhang *et al.,* 1994), regulation of p53 functions (Simbulan-Rosenthal *et al.,* 1999a; Kumari *et al.,* 1998), carcinogenesis (Miwa and Sugimura, 1990; Boulikas, 1991; Saikia, 1996; Saikia *et al.,* 1999) and apoptosis (Kaufmann *et al.,* 1993; Lazebnik *et al.,* 1994; Nicholson *et al.,* 1995; Tewari *et al.,* 1995; Simbulan-Rosenthal, 1999b). However, despite accepted importance of this molecular event, there has not been any clear understanding of PAR influence on a variety of cellular processes. Increasing body of evidences suggest that one reason for this could be the lack of reliable quantification of the metabolic level of cellular PAR. The radioactive assay of PAR, employed widely, appears to be inappropriate to give correct assessment of metabolic level of PAR (Schneeweiss *et al.*, 1995; Sharan *et al.*, 1998b; Humtsoe *et al.*, 1999). The assay uses radiolabeled NAD^+ (^{32}P-NAD^+) as the substrate. During the process of assay of PAR, part of the radiolabeled activity of ^{32}P-NAD^+ pool is taken up to the newly synthesized ADP-ribose polymer on a target protein. The total cellular uptake of labeled NAD^+ can be monitored by scintillation counting (Schneeweiss *et al.,* 1995) or by autoradiography (Sharan *et al.,* 1998b). However, such a measurement does not consider the cellular pool of cold NAD^+ present in the system that would be participating in the PAR reaction. The method also requires physical intervention such as preparation of single cell suspension, centrifugation and hypotonic shock treatment in order to facilitate the internalization of labeled NAD^+ into the cell for the reaction. Such manipulations, however, have been shown to influence the PAR reaction (Schneeweiss *et al.,* 1995). Therefore, the measurement of PAR after γ-radiation would represent PAR level induced by such manipulations as well as by radiation. In order to overcome some of these limitations, a non-radioactive immuno-blot assay developed by Sharan *et al.* (1998a) seems appropriate. This assay may likely provide the correct estimation of metabolic PAR. The ELISA-based method consists of immobilizing protein samples on nitrocellulose membrane and processing for immuno-detection using polyclonal antibody against ADP-ribose polymers. The assay has been able to sensitively detect ADP-ribosylated proteins in mouse liver

samples (homogenates and supernatants), spleen cell homogenates and ascitic fluids (Sharan *et al.*, 1998a). Besides sensitivity, it eliminates some of the problems addressed to by Schneeweiss *et al.* (1995) and Schneeweiss and Sharan (1999). This method also offers the potential advantage of screening PAR reaction to any tissue sample or biopsy.

6.5 MEMBRANE DAMAGE STUDIES:

While it is generally accepted that the critical target for radiation within the cell is DNA, there is also a body of experimental data that does not fit with the idea of a simple direct action on DNA alone. Alper (1979) has discussed these evidences in depth. It has been postulated that besides primary lesions in DNA, free radical mediated damages, that are responsible for sensitization by oxygen, lead to lipid peroxidation of membranes (Koteles, 1986) and oxidation of proteins (Dean *et al.*, 1993). It is envisaged that these lesions are associated with damage to cell membranes or with the points of attachment between DNA and the nuclear membrane. Changes observable at the cellular level does not appear until later, although few ultrastructural changes in some cells can be observed quite early. The changes are accompanied by lipid peroxidation (Wolters *et al.*, 1987), modifications in membrane permeability (Patrick, 1977; Edwards *et al.*, 1984) and loss of some membrane bound enzymes (Bacq and Alexander, 1966). Liver membrane has been reported to undergo lipid peroxidation upon heavy charged particle irradiation in a fluence dependent manner (Choudhary *et al.*, 1998). Oxidative damage to membrane of human lymphocytes has been demonstrated to trigger apoptosis through a rapid induction process (Cregan *et al.*, 1999). Szumiel (1981) had reported that there might be an indirect influence of membrane damage on repair of DNA. Therefore, while it is unlikely that membrane damage is directly responsible for cell death following irradiation, its influence on other functions may be important. Radiation is known to disrupt and distort membranes. This has been attributed to the oxidation of lipids due to a chain reaction induced by the radiolysis products of water (Stark, 1991; Ziegler, 1994). Therefore, to assess the impact of radiation on a living cell, membrane damage analysis is also a relevant approach.

The cellular membranes are vital elements and their integrity is extremely essential for maintaining the microenvironment of the cell and, thereby, the viability of the cells. Cell viability after radiation has been employed in several studies as an important parameter for assessment of radiation induced damages. The dye exclusion technique by Phillips (1973) to monitor viability of cells has been very widely used (Wary and Sharan, 1988; Sharan *et al.*, 1995; Chakraborty, 1997; Kawai *et al.*, 1998). The test is based on the principle that live (viable) cells take up and actively exclude certain dyes, whereas dead (non-viable) cells fails to exclude the dyes. This results in continued presence of dye within dead cells only. The cells that are stained, therefore, can be counted under microscope.

Alteration of enzyme activity has been observed to be influenced by ionizing radiation (Bacq and Alexander, 1966; Sharan and Srivastava, 1984; Wary and Sharan, 1988). The indirect radiation effect is considered to offer major contribution to the inactivation of most enzymes. Acetylcholine esterase is an oligomeric membrane bound enzyme (Messoulie and Toutant, 1988), which mediates cholinergic neurotransmission by rapid hydrolysis of transmitter acetylcholine to acetate and choline (Jurss and Maclicke, 1981; Bazelyansky *et al.,* 1986). By estimating the change in the enzyme activity of irradiated cells, the metabolic changes occurring as a consequence of radiation can be elucidated. Since radiation is known to disrupt membrane, monitoring the activity of AchE in a cell lysate serves as a useful biochemical parameter for the assessment of radiation-induced membrane damage in a mammalian cell system.

6.6 AIMS AND OBJECTIVES:

With this information, this part of the work embodies studies done *in vitro* and *ex vivo* mammalian cells.

The following aims were set forth for this investigation:

1. Study of effect of γ-radiation-induced DNA damage on chromatin organization.
2. Study of effect of γ-radiation-induced on the PAR of chromosomal proteins.
3. Induction of apoptosis by γ-radiation, and
4. Study of membrane damage induced by high-LET ^{11}B particle radiation.

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CHAPTER 7

MATERIALS AND METHODS

7.1 CHEMICALS:

Indigenous chemicals of analytical grade obtained from various chemical suppliers were used unless otherwise mentioned. Agarose (type I: low EEO), Ethylenediaminetetra acetic acid (EDTA), Ammonium persulphate, Tris[hydroxymethyl]aminomethane, Acrylamide, Bis-acrylamide, Sodium dodecyl sulphate (SDS), NNN",N' Tetramethylethylenediamine (TEMED), ß-Mercaptoethanol, PBS tablets, 5,5'-Dithio-bis[2-nitrobenzoic acid] (DTNB), Acetylcholine, Bovine serum albumin (BSA), Polyoxyethylene-Sorbitan monolaurate (Tween-20), Coomassie brilliant blue R, Alkaline phosphatase conjugated goat anti-rabbit IgG and SigmaFast BCIP/NBT buffered substrate tablet were obtained from Sigma Chemical Co. (USA). Dithiothreitol (DTT), (N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]) (HEPES) and sodium sarcosinate were obtained from Serva Chemicals (Germany) while Sephadex G-25 was from Pharmacia Fine Chemicals (Sweden) and ethidium bromide and propidium iodide were from Merck (Germany).

7.2 OTHER MATERIALS:

Adjuvant: Superfos, DK-2950, Vedback, Denmark; Nitrocellulose membrane: Immobilon-NC (pore size: 0.45 μm) of Sigma Chemicals Co., USA and staining ink of Rotring-werke Riepe KG, Germany were used.

7.3 KIT:

Cell Death Detection ELISA Kit was purchased from Boehringer Mannheim (Germany).

7.4 IRRADIATION SOURCE:

7.4.1 Low-LET:

^{60}Co gamma chamber 900, at a dose rate of 8.4 Gy min^{-1} (Isotope group, BARC, India) and ^{137}Cs GammaCell 40, at a dose rate of 0.9 Gy min^{-1} (Atomic Energy of Canada Limited) were employed for experiments.

7.4.2 High-LET:

^{11}B beam from 15 UD tandem accelerator Pelletron (Nuclear Science Center, New Delhi) was used for high-LET experiments. ^{11}B particle (9×10^5 particles cm^{-2}) was delivered at LET of about 198 KeV μm^{-1}.

7.5 ANIMALS:

Swiss albino mice (Balb/c) were obtained from Assam Veterinary Biological, Guwahati. Its inbred line was maintained in an animal room at 22 ± 2 °C on standard dry pellet and water *ad libitum*. Russian Chhinchilian strain rabbit for raising polyclonal antibody was purchased from Indian Council of Agriculture Research, Barapani, Meghalaya.

7.6 CELL LINES:

Human kidney (T1), human glioblastoma (86HG-39) and rat rhabdomyosarcoma (R1H) cell lines, used in this investigation, were generously provided by Dr. F. H. A. Schneeweiss, IME, FZ, Juelich, Germany.

7.7 CULTURE MEDIA:

Minimum essential medium (MEM; HIMEDIA, India), Eagle's basal medium (BME) and Iscove's modified Dulbecco's medium (IMDM; both Biochrom, Germany) and fetal calf serum (FCS; Gibco BRL, England) were used.

7.8 CELL CULTURE:

7.8.1 Low-LET Experiment:

Human kidney T1 cells were cultured as a monolayer in Leighton tubes (NUNC, Denmark) at 37 °C in a CO_2-incubator (5 % CO_2 and 95 % humidity). The cells were grown to confluent for five days in BME supplemented with 10 % FCS, 0.1% antibiotics and 0.9 % glutamine.

7.8.2 High-LET Experiment:

Keeping in view the horizontal beam line for high-LET particle irradiation, cells were grown in a special polypropylene Leighton tubes (COSTAR, USA). The COSTAR tube that measures 16 x 93 mm with a screw cap has a 9 x 55 mm insert for cell anchorage and growth. This insert has two segments covering an area of 2.5 cm^2 each on which cells grow as monolayer (see Fig. 7.1). The cell lines - human glioblastoma (86HG-39), rat rhabdomyosarcoma (R1H) and human kidney T1 cells were cultured in the COSTAR tubes. Approximately 0.3 x 10^6 cells were seeded in each tubes in 2.5 ml of appropriate media and were grown to confluence for 5-6 days at 37 °C in a CO_2-incubator (5 % CO_2 and 95 % humidity). For HG and R1H the medium, IMDM, was supplemented with 10 % FCS and

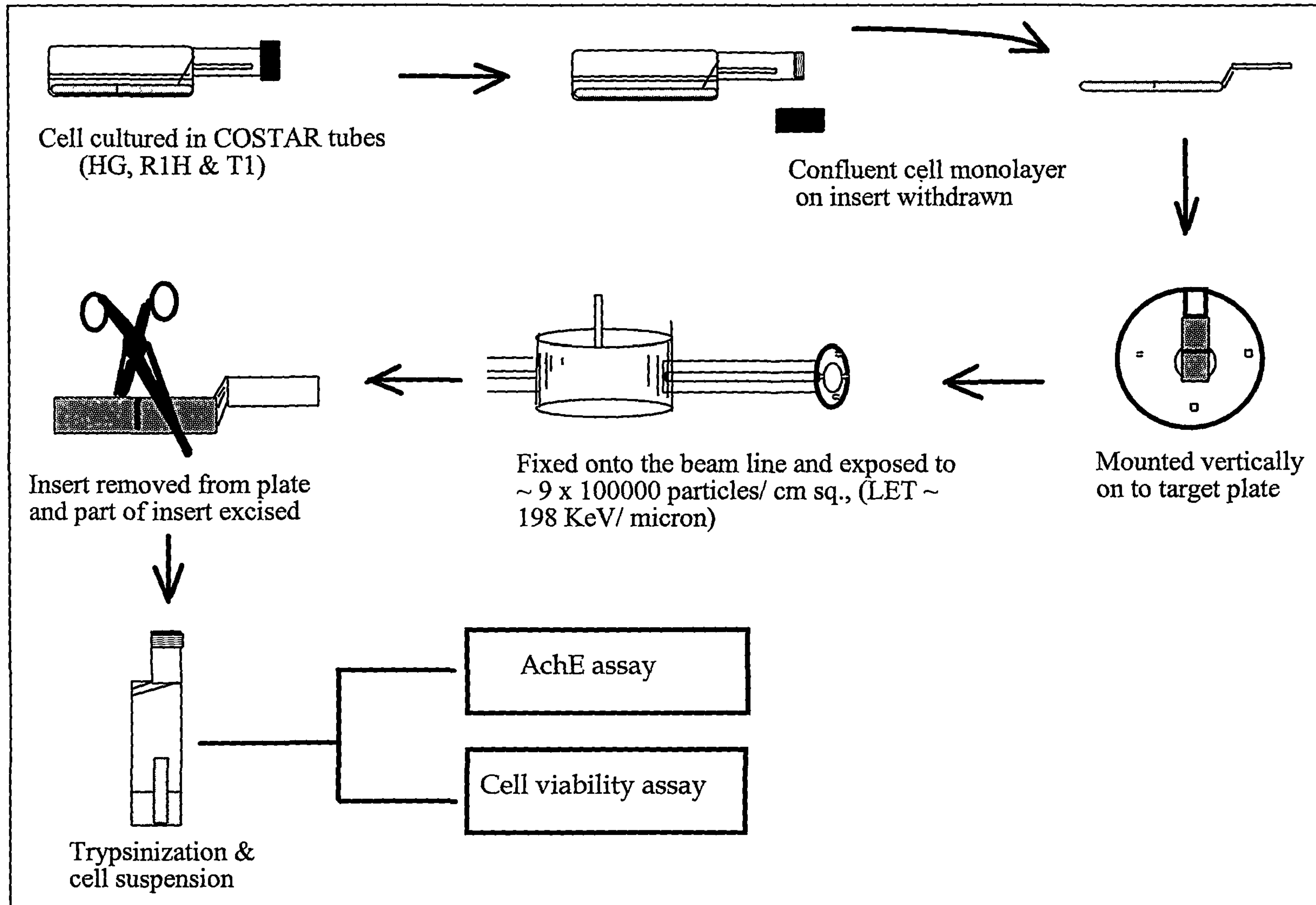

Figure 7.1: Cartoon illustration of the experimental set up for high-LET ^{11}B particle irradiation.

1% penicillin/streptomycin while for T1 cells the medium, BME, was supplemented with 10 % FCS, 0.1% antibiotics and 0.9 % glutamine.

7.9 PREPARATION OF SPLEEN CELLS:

Normal male mouse (~ 12 weeks) was sacrificed by cervical dislocation. The spleen excised and washed in cold minimal essential medium (MEM) was then transferred to 1 ml fresh MEM. The spleen was cut and the cells were squashed into the medium. Single cell suspension was then made by repeated mild agitation with a micropipette.

7.10 CELL COUNTING:

After taking a small aliquot and diluting it by 1,000 folds, cells were counted in a phase-contrast microscope using a Buerker cell counting chamber. The number of cells was calculated using the equation:

No. of cells ml^{-1} = average cells per square x dilution factor x 25,000

7.11 IRRADIATION AND POST-IRRADIATION CONDITION:

7.11.1 Low-LET:

In vitro system: T1 cell monolayers in Leighton tubes were γ-irradiated in full medium using the ^{137}Cs source. After exposure to doses of 0.25, 0.5, 1, 2 and 4 Gy at room temperature, culture tubes were incubated for 20 min at 37 °C. Cells were then processed for chromatin activity assay and comet assay.

Ex-vivo system: Mouse spleen cell suspension in MEM (10 x 10^6 cells ml^{-1}) were irradiated at room temperature in an eppendorf tube using the ^{60}Co source. The doses of 0.5, 1, 2, 4 and 8 were delivered at a dose rate of 8.4 Gy min^{-1}. Samples were incubated for 20 min at 37 °C after irradiation. Cells were then processed for apoptosis and poly-ADP-ribosylation analysis.

7.11.2 High-LET:

In vitro system: The experimental set up for the accelerated ^{11}B particle irradiation is illustrated in Fig.7.1. Confluent cell monolayer growing on insert was withdrawn with the help of a pair of forceps. After dripping off the medium, the insert was vertically mounted on a specially designed circular plastic target plate. The plate was fixed on the beam window and about 4 x 10^5 cells on the insert were directly exposed to approximately 9 x 10^5 ^{11}B particles cm^{-2}. Immediately after irradiation, the insert was removed and a fixed area of

the insert was cut out. The cells from this piece of insert were recovered after trypsinization. AchE activity and cell viability was assayed using the cell suspension.

7.12 CHROMATIN ACTIVITY ASSAY:

The method by Bellard *et al.* (1989) was followed for studying chromatin structure in intact nuclei. The buffer compositions for nucleus preparation and nuclease digestion are given below:

[a] Phosphate Buffer Saline (PBS), pH 7.4.

[b] TK Buffer
10 mM Tris-HCl, pH 7.5
1 mM $CaCl_2$

[c] Digestion Buffer for DNase I
85 mM KCl
1 mM $CaCl_2$
5 mM HEPES
5 % Sucrose

[d] Stop Buffer
50 mM Tris-HCl, pH 7.5
150 mM NaCl
15 mM EDTA
0.3 % SDS

[e] Sample Buffer
40 % of sucrose
0.25 % bromophenol blue

7.12.1 Nuclei Preparation:

Nuclei were prepared by the method described by Bellard *et al.* (1989) with slight changes. Cell monolayers was first rinsed with PBS after decanting the medium. Cells were then detached from the culture tubes into 1 ml of PBS with the help of plastic scrappers. After centrifuging at 158 x *g* for 3 min at 4 °C in a Hareaus refrigerated Varifuge, the cell pellet was suspended in 0.5 ml TK buffer (final concentration of approximately 4 x 10^6 cells ml^{-1}). The suspension was incubated for 10 min on ice and cells were mechanically lysed by homogenization (COLORA, Germany) for 2 min on ice. The pellet was collected

after 5 min centrifugation at 158 x *g*, at 4 °C. This was followed by two rounds of resuspension and centrifugation until the pellet was recovered as nuclei. This nucleus preparation was suspended in 0.5 ml of digestion buffer.

7.12.2 DNase I Digestion and Analysis:

DNase I digestion of the nuclei was carried out for 2 min at 37 °C at an enzyme concentration of 20 units for each ml of isolate (Bellard *et al.*, 1989). The digestion reaction was terminated by adding equal volume of stop buffer. After diluting with $1/6^{th}$ volume sample buffer, the sample mixtures were analyzed by 1 % agarose gel electrophoresis using Bio-Rad Sub-cell model 192 cell and POWERPAC 3000 power supply. The gel, stained in 0.7 $\mu g\ ml^{-1}$ ethidium bromide, was photographed under UV illumination with Polaroid MP-4 Land Camera using polapan 55 PN films.

7.13 DNA DAMAGE ANALYSIS BY COMET ASSAY:

DNA damage was analyzed by the alkaline comet assay (Singh *et al.*, 1988). The buffer composition and solutions for the comet assay are given below:

[a] Lysing solution
- 10 mM Tris, pH 10
- 2.5 M NaCl
- 100 mM Na_2-EDTA
- 1 % sodium sacrosinate and 1 % Triton-X-100 (added fresh before use)

[b] Electrophoresis buffer (pH >12)
- 0.3 M NaOH
- 1 mM Na_2-EDTA

[c] Neutralizing solution
- 0.4 M Tris-HCl, pH 7.5

7.13.1 Methodology:

The method described by Singh *et al.* (1988) was followed with minor changes. One percent normal agarose prepared in PBS was poured on a pre-warmed glass slides by spreading a thin film with the help of a cover slip. Care was taken for homogeneous spreading of the gel on the slide. The gel was then dried on a hot plate for about 15 min. Cell suspended in BME (1×10^6 cells ml^{-1}) was diluted ten folds with 0.75 % of low melting agarose prepared in PBS. These cell-agarose mixtures were spread on the prepared slides and were immediately covered with cover slips. After solidifying the gel on ice for

10 min the cover slips were removed and the slides were transferred to a tray. The cells embedded in agarose were lysed by immersing the slides in the lysis solution and incubating it for 60 min in a refrigerator. The slides were removed and subjected to DNA-unwinding by incubating them in the electrophoresis buffer for 25 min. Electrophoresis was then carried out for 25 min at 0.8 V cm^{-1}. The slides were carefully withdrawn from the electrophoresis tank and were neutralized by gentle repeated washings in the neutralizing solution. DNA was stained by layering with 1-2 drops of propidium iodide (20 μg ml^{-1}) on the slides. Immediately after covering with a cover slip the slides were taken for visualization and analysis.

7.13.2 Visualization and Analysis of Comets:

The propidium iodide stained slides were visualized and analyzed under a fluorescence microscope (Zeiss, Germany) hooked to IBM-compatible PC using Kinetic 3.1 software (OPTILAS, Germany). The tail moment, defined as the product of the fraction of DNA (fluorescence) in the "tail" and the length of the "tail", was used as the parameter to monitor the DNA damage. DNA damage was quantified as an increase in tail moment, an indicator of damage that is proportional to the number of strand breaks per cell. Tail moment distributions for 350-400 comets were used to determine the mean value of tail moment for each dose point.

7.14 APOPTOSIS ANALYSIS:

Apoptotic induction was measured using the Cell Death Detection ELISA kit. It essentially quantifies histone-associated DNA fragments (mono- and oligonucleosomes) present in the cytoplasm of cells undergoing apoptosis. All components used were provided in the kit unless otherwise noted and the reagents were prepared as recommended using double distilled water.

7.14.1 Sample Processing:

Samples were processed as per the protocol provided with the kit for *ex-vivo* cells system with minor modifications. Non-irradiated and irradiated cell samples in tubes (1×10^6 cells in MEM) were first pelleted by centrifuging for 5 min at 3,000 rpm in a SPINWIN microcentrifuge. The medium was discarded and the cell pellet was resuspended in 500 μl of ready to use solution supplied with the kit as incubation buffer. After mixing thoroughly, the suspensions were incubated for 30 min at 4 °C for lysis. The lysate was centrifuged at 10,000 rpm (SPINWIN microcentrifuge) for 12 min after which 400 μl of the supernatant (cytoplasmic fraction) was collected carefully for immunoassay.

7.14.2 Immunoassay:

The assay is based on the quantitative sandwich-enzyme-immunoassay-principle using mouse monoclonal antibodies directed against DNA and histones, respectively. It allows the specific determination of mono-and oligonucleosomes in the cytoplasmic fraction of cell lysates.

The working procedure of the ELISA provided in the kit was again followed. The cytoplasmic fraction or supernatant was directly used for the assay. One hundred μl of the fraction corresponding to approximately 0.2 x 10^6 cells was used for each assay. All samples were measured and titrated in duplicate.

In the first incubation step, anti-histone antibody was fixed by adsorption on the walls of the microtiter plate. After blocking non-specific binding sites, the second incubation step allows binding of nucleosomes present in the sample via their histone components to the immobilized antibody. In the final incubation step, anti-DNA-peroxidase was reacted with the DNA-part of the nucleosome. The unbound peroxidase conjugate was removed by a washing step. After reaction with ABTS (2,2'-azino-di-[3-ethylbenzthiazoline sulfonate]) as substrate, the amount of peroxidase retained in the immunocomplex was determined photometrically at 405 nm against substrate solution as blank in a Multiskan MS (version 4.0) ELISA reader (Labsystem). The change in absorbence, which reflects the detection of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes), was used as the parameter for monitoring the induction of apoptosis.

7.15 POLY-ADP-RIBOSYLATION (PAR) ANALYSIS:

7.15.1 Sample Processing for Western and Slot Blotting:

Cells numbering approximately 2 x 10^6 were first pelleted by centrifuging for 5 min at 3,000 rpm in a SPINWIN microcentrifuge. The cells pellet suspended in 50 μl non-ionic detergent, 0.5 % Triton X-100 (40,000 cells μl^{-1}) were then lysed by incubating on ice for 5 min. The lysate, obtained as crude protein extract, was aliquoted for Slot and Western blotting.

7.15.2 Western Blotting:

7.15.2.1 ***SDS-Polyacrylamide Gel Electrophoresis*:**

The method described by Laemmli (1970) for gel electrophoresis was followed.

(I) Requirements for Gel Preparation:

[a] Acrylamide solution (Acrylamide 30 %, Bisacrylamide 0.8 %; usually prepared in advance, filtered through Whatmann paper and stored refrigerated.

[b] 1 M Tris-HCl, pH 8.8

[c] 1 M Tris-HCl, pH 6.8

[d] 10 % SDS

[e] 10 % ammonium persulphate (freshly prepared)

[f] TEMED

(II) Electrophoresis Buffer:

25 mM Tris-HCl, pH 8.8
192 mM glycine
0.1 % SDS

(III) Sample Buffer:

0.5 M Tris Cl pH 6.8	1 ml
Glycerol	0.8 ml
10 % SDS	1.6 ml
2-mercaptoethanol	0.4 ml
0.05 % bromophenol blue	0.4 ml
Double distilled water	3.8 ml

(IV) Gel Preparation:

(i) Separating gel: For preparing 10 ml of separating gel solution of 12 % acrylamide concentration, the following were mixed together:

Acrylamide solution	4 ml
1 M Tris-Cl, pH 8.8	3.73 ml
10 % SDS	100 μl
Double distilled water	2.10 ml

After degassing the mixture for 15 min, 30 μl each of TEMED and freshly prepared 10 % ammonium persulphate were added. The solution was poured into pre-assembled casting glass plate sandwiches (Bio-Rad) and was allowed to polymerize for about 20 min

after overlaying with water.

(ii) Stacking gel: Similarly, for 5 ml of 3 % stacking gel solution, the following mixture was prepared:

Acrylamide solution	500 µl
1 M Tris-HCl, pH 6.8	625 µl
10 % SDS	50 µl
Double distilled water	3.8 ml
TEMED	12 µl
10 % APS.	12 µl

After removing the water layer atop the polymerized separating gel, comb was fixed and stacking gel solution was poured. It was allowed to polymerize for about 45 min.

(v) Electrophoresis:

Crude protein extract samples diluted to 1:5 with sample buffer was heated in boiling water bath for 3 min. Twelve and a half µl of the samples (equivalent to ~ 0.5×10^6 cells) were loaded in each well of the gel. The electrophoresis was carried out at a constant voltage of 25 V cm^{-1} for 1 hr in a Mini-PROTEAN® II Electrophoresis Cell (Bio-Rad).

7.15.2.2 *Electroblotting*:

The sample separated on SDS-PAG was electrotransferred onto a nitrocellulose (NC) membrane in a Mini Trans-Blot® Electrophoretic transfer cell (Bio-Rad). The electroblotting was carried out in chilled Towbin buffer (25 mM Tris, 192 mM glycine and 20 % methanol, pH 8.3) at a constant voltage of 100 V for 1 hr.

7.15.3 Slot Blotting:

Samples were heat inactivated by boiling in a water bath for 5 min in order to minimize endogenous phosphatase enzyme activity (Sharan *et al.*, 1998a). Samples were appropriately diluted with double distilled sterile water to get final cell concentration of 1,000 cells μl^{-1}. One hundred µl of this (equivalent to ~ 0.1×10^6 cells) were slot blotted onto NC membrane using Bio-Dot SF® Microfiltration Apparatus (Bio-Rad) connected to a vacuum pump.

7.15.4 Immunodetection:

Standard immunodetection protocol was employed with slight modifications (Sharan *et al.*, 1998a). The working solutions for the assay are shown below:

[a] Tris buffered saline (TBS)
20 mM Tris-Cl, pH 7.5
500 mM NaCl

[b] Wash solution (TTBS)
20 mM Tris, pH 7.5
500 mM NaCl
0.05 % Tween-20

[c] Blocking solution
5 % non-fat dry milk in TBS

[d] First antibody solution
Polyclonal rabbit IgG diluted to 1:1000 with blocking solution

[e] Second antibody solution
Alkaline phosphatase-conjugated anti-rabbit IgG diluted to 1:15,000 with blocking solution.

Methodology:

Non-specific binding sites of electro- and slot-blotted membranes were first saturated for 45 min with blocking solution. The solution was decanted and the membranes were washed twice in TTBS for 5 min each. The membranes were incubated in first- and second-antibody solution for 45 min each with a similar wash with TTBS in between. Immediately after washing the unbound second-antibody with TTBS and TBS for 10 min each, the membranes were incubated in BCIP/NBT substrate solution. The incubation was allowed till optimum intensity of color was developed (about 7-15 min). The membranes were washed twice with double distilled water for 5 min each. After air drying the pixel intensities were quantified and analyzed by an imaging densitometer (Bio-Rad GS-690 Imaging Densitometer/ Molecular Analyst PC software 1.5). All incubations and reaction steps were maintained at 37 °C with gentle agitation.

7.15.5 Total Protein Ink Stain:

A replica of the immunodetected membranes were stained for 3-4 hr in 0.2 % Rotring ink prepared in PBS containing 0.3 % Tween 20.

7.16 ISOLATION OF POLY ADP-RIBOSE POLYMERS:

Hakem and Kun's (1985) method for ADP-ribose polymers isolation was followed

with slight changes (Saikia, 1996). Spleen from a normal adult mouse was excised and cell suspension was made after squashing out the cells into pre-chilled PBS. The cell suspension was centrifuged at 1,300 x *g* for 15 min (all centrifugation steps were carried out at 4 °C). The cell pellet was collected, resuspended in 10 % TCA and incubated for 30 min on ice. The pellet collected after centrifuging for 15 min at 20,000 x *g*, was suspended in 0.1 M Tris glycine, pH 10.5 and incubated at 37 °C for 60 min with regular gentle shaking. After centrifuging for 60 min at 20,000 x *g*, the supernatant was collected and was desalted through Sephadex G-25 column at a flow rate of 1 ml 5 min^{-1}. The eluent fractions of the peak were pooled and dialyzed extensively in double distilled water for about 24 hr. The sample was then frozen, lyophilized and stored as an antigen.

7.17 GENERATION OF POLYCLONAL ANTIBODIES:

7.17.1 Immunization of Rabbit:

The lyophilized antigen, dissolved in PBS (2 mg ml^{-1}), was homogeneously suspended and emulsified thoroughly in equal volume of aluminium hydroxide gel (Superfos) adjuvant. An aliquot of the emulsion was further diluted ten times with PBS making the final antigen concentration to 0.1 μg μl^{-1}. Five hundred μl (50 μg) of the emulsion was injected intramuscularly into both thighs of the rabbit. Booster injections were given on the 15^{th} day and again on the 4^{th} and 8^{th} week. One week after the priming immunization, the rabbit was ready to deliver antibodies at periodic intervals.

7.17.2 Bleeding:

A gentle diagonal incision was made on the ear vein. Blood was collected by allowing blood to ooze down freely into a clean glass tube. Repeated bleeding was carried out on the 4^{th} – 5^{th} day after every booster injection thereon.

7.17.3 Separation of Serum:

The tube containing the blood was allowed to stand tilted till it was properly clotted (usually 8-10 hr). The serum that got separated out was gently transferred into a clean sterile tube with a micropipette. The traces of red blood cells in the serum were removed by centrifuging at 10,000 x *g* for 10 min at 4 °C. The serum, containing the polyclonal antibody, was aliquoted and stored at -20 °C until use.

7.17.4 Double Immunodiffusion:

The specificity of the antiserum raised was checked by double immunodiffusion technique as described by Ouchterlony (1949). One % agarose solution in normal saline (0.9 % NaCl) containing 0.1 % sodium azide was prepared by heating it to about 70 °C. The solution was poured on a glass slide and allowed to cool at room temperature for

solidification. Three wells were punched 1 cm apart into the gel. The antibody was loaded in the central well while antigen and commercial BSA were loaded on the peripheral wells. Several such slides were prepared which were loaded with serially diluted antibody solution. The samples were allowed to diffuse for about 36 hr at 37 °C in a humid chamber. The slides were observed for the formation of precipitin line.

7.18 CELL VIABILITY ASSAY:

The proportion of dead cells can be estimated easily by using the dye exclusion test, in which trypan blue is used to differentially stain non-viable cells (Phillips, 1973; Wary and Sharan, 1989). For staining, nine parts of the cell suspension in growth medium were mixed with one part of 4 mg ml^{-1} trypan blue in PBS. The numbers of both viable and non-viable cells were counted while viewing them under a phase contrast microscope within 5 min of mixing.

The percent cell viability was calculated by the equation: -

$$\% \text{ cell viability} = \frac{\text{total viable cells (unstained)}}{\text{total cells (stained and unstained)}} \times 100$$

7.19 ACETYLCHOLINE ESTERASE (AchE) ACTIVITY ASSAY:

The methodology followed was based on Ott *et al.* (1975). Equal volume of cell suspension and 1 % Triton X-100 were mixed and incubated on ice for 2 min prior to disruption of the cells. The resulting suspension was used for the enzyme assay. The composition of reaction mixture for the assay is shown below:

200 mM PBS (pH 7.4)	1 ml
6 mM acetylcholine	0.5 ml
0.75 mM DTNB	0.5 ml
Cell sample	1 ml

The enzyme activity was then assayed and calculated as described by Plummer (1989) by measuring the change in absorbence at 412 nm in a double beam Shimadzu spectrophotometer. The following equation was used for the enzyme activity calculation:

$$\text{AchE activity} = (\Delta E \times 1{,}000 \times 3) / (1.36 \times 10{,}000 \times 0.5)$$

ΔE = Extinction change per min

1,000 = Factor to obtain μmoles

3.0 = total volume of reaction mixture (ml)

0.5 = volume of the sample used

1.36 x 10,000 = molar extinction coefficient of chromophore at 412 nm (lit $mol^{-1}cm^{-1}$)

7.20 DATA AND STATISTICAL ANALYSIS:

All data analyzed were plotted using Cricket Graph software (version 1.3). The results are expressed as the average of the means from each experiment, and the standard error or standard deviation (mean ± SE or SD bar). Each experiment had variable number of independent replicates and is shown in the respective figure legends. The student's *t* test for statistical significance between control (non-irradiated) and irradiated samples was performed using StatWorks software version 1.2.

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CHAPTER 8

RESULTS

8.1 QUANTIFICATION OF γ-RADIATION INDUCED DNA STRAND BREAKAGE BY COMET ASSAY:

Comet assay is one of the widely used techniques in measuring DNA strand breaks. The sensitivity and rapidity in analyzing DNA strand breaks in a large number of individual cell makes it a very convenient method. The alkaline version of the assay especially detects SSB and alkali labile damages. Fig. 8.1 shows the appearance of the propidium iodide stained image of human kidney T1 cells as viewed under fluorescence microscope. The images were obtained from non-irradiated (control) cells (A) and after being exposed to 0.5 (B), 1 (C) and 2 Gy (D) of γ-radiation, respectively. The images, which were from comet assay processed after 20-min post irradiation incubation, did not show significant difference in appearance between control and irradiated cells.

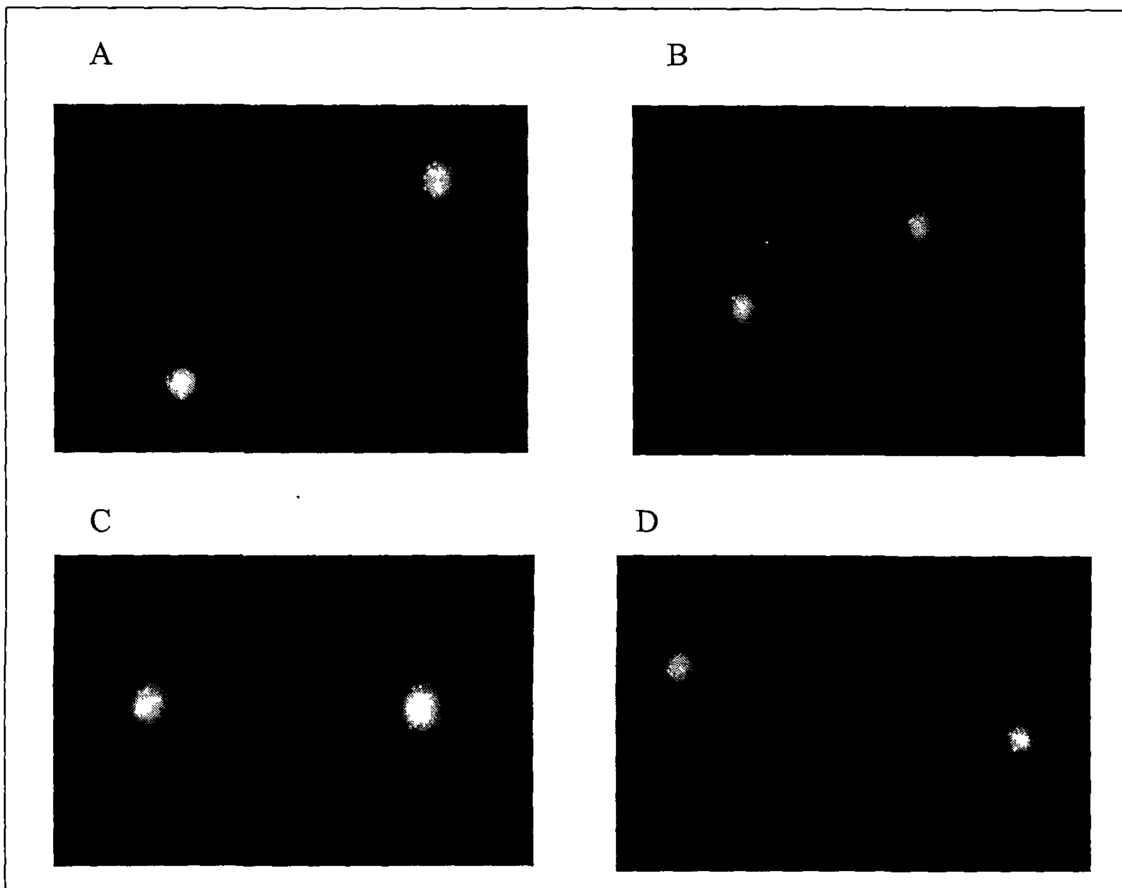


Figure 8.1: Propidium iodide stained image of human kidney T1 cells (alkaline comet assay). The direction of comet migration is from left to right. A is from non-irradiated (control) cells, B, C and D are from cells exposed to 0.5, 1 and 2 Gy of γ-radiation, respectively.

Fig. 8.2 shows the results of quantitative analysis of alkaline comet assay performed on non-irradiated and γ-irradiated T1 cells. The tail moment, which reflects the strand breakage of DNA, behaved differently under the two conditions studied. Under non-permissive conditions for repair or without post-irradiation repair incubation, the assay

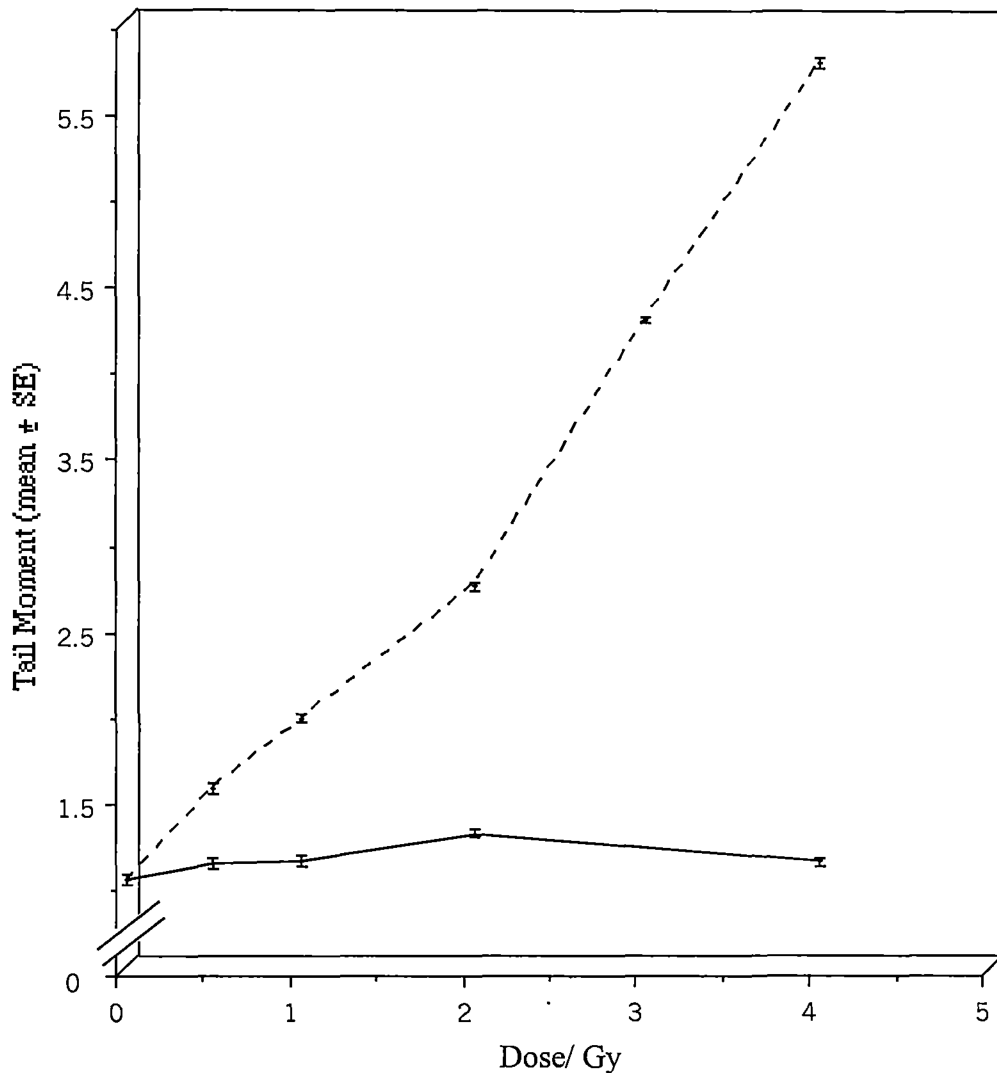


Figure 8.2: Detection of strand breaks in γ-irradiated human kidney T1 cells using alkaline comet assay. Tail moment, a measure of DNA damage is plotted as a function of γ-radiation dose. Broken and solid lines represents tail moment obtained after non-repair and 20 min repair incubation (37 °C), respectively. Each point represents about 400 random comets.

shows almost a linear dose dependent increase in damage. On the other hand, after 20 min incubation at 37 °C, the DNA strand break increased only up to 2 Gy ($p < 0.001$). The

damage at higher dose of 4 Gy, unlike the other case, showed a tendency to fall back to the control level ($p < 0.05$).

8.2 CHROMATIN ACTIVITY ASSAY:

In order to assess the impact of γ-radiation induced SSB on the structural organization of chromatin, the ability of DNase I to fragment chromatin was investigated. Fig. 8.3 shows electropherogram of DNase I fragmentation of non-irradiated and irradiated chromatin isolated from T1 cells. A progressively increasing degradation of the chromatin by DNase I was observed up to the dose of 1 Gy (lanes 3-5). Increase in the dose from 2 to 4 Gy (lanes 6-7), however, did not show any significant increase in the extent of degradation by DNase I. The appearance of high molecular weight DNA band resembling that from non-irradiated (control) digest was observed for the two highest doses (arrow).

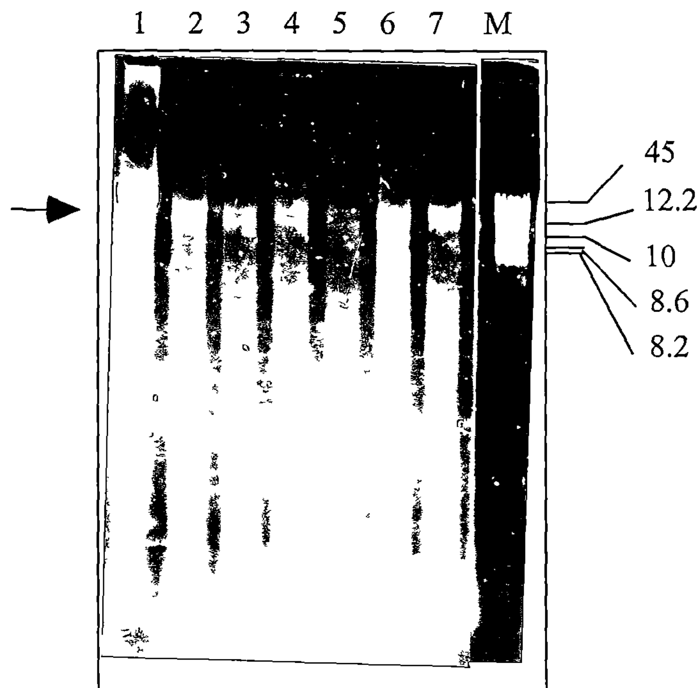


Figure 8.3: DNase I fragmentation pattern of chromatin isolated from T1 cells after 2 min of enzyme action. Lane 1: non-irradiated nuclei without DNase I; Lane 2: non-irradiated nuclei with DNase I; Lane 3-7: γ-irradiated nuclei at doses of 0.25, 0.5, 1.0, 2.0, and 4.0 Gy, respectively with DNase I; Lane 8: standard high molecular weight DNA marker in kbp.

8.3 EFFECT OF γ-RADIATION ON THE INDUCTION OF APOPTOSIS IN *EX-VIVO* MOUSE SPLEEN CELLS:

The induction of apoptosis was measured by immuno-based assay using cell death detection ELISA kit. Since the assay detects generally mono- and oligonucleosomes, the level of detection corresponds to the extent of apoptotic genomic fragmentation generated following irradiation. Fig. 8.4 depicts the apoptotic fragmentation in mouse spleen cells immediately after exposing them to γ-radiation and that after 20-min incubation at 37 °C.

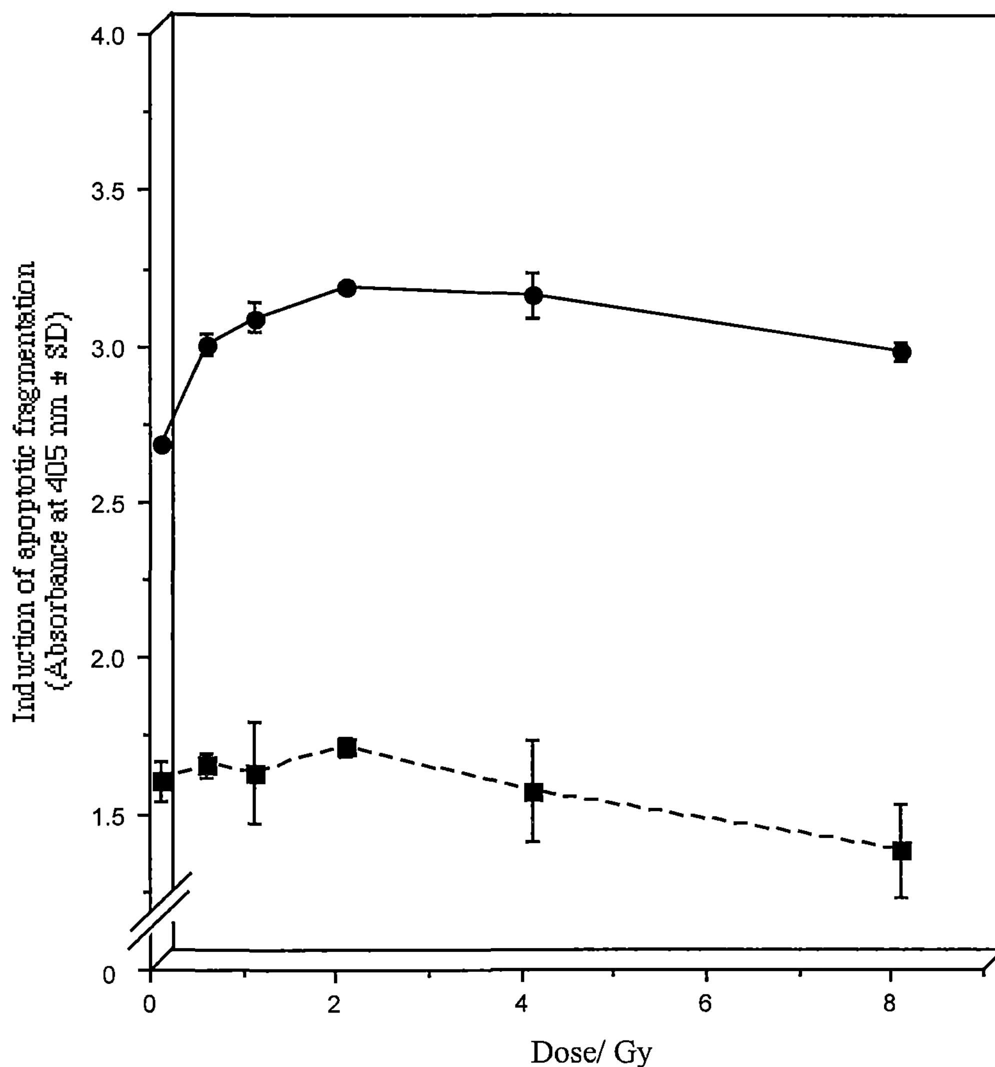


Figure 8.4: Induction of apoptosis following γ-irradiation as measured by Cell Death Detection ELISA kit. Samples were titrated and measured in duplicates for each assay. Solid and broken lines represent apoptotic induction measured with or without 20 min incubation at 37 °C, respectively. Data point represents mean ± SD of two independent assay.

Under repair permissive conditions (20 min post-irradiation incubation at 37 °C), a dose dependent increase in apoptosis was observed up to 2 Gy. After reaching its maximum

at 2 Gy, the curve sloped down to a saturation level even as dose was increased from 4 to 8 Gy. On the other hand, apoptosis was observed to be insignificantly induced in cells when measured immediately after irradiation (without post-irradiation incubation).

8.4 SPECIFICITY OF POLYCLONAL ANTIBODY RAISED AGAINST ADP-RIBOSE POLYMERS:

To test the presence and specificity of the polyclonal antibody against mouse spleen ADP-ribose polymer, Ouchterlony's immunodiffusion assay was carried out. Fig. 8.5 shows the formation of precipitin line between the wells containing the antiserum and the antigen. It reveals that the whole serum, used as antibody, was specific to ADP-ribose polymer. The well where commercial BSA was loaded did not show any precipitation line also confirming the non-cross reactivity of the antiserum.

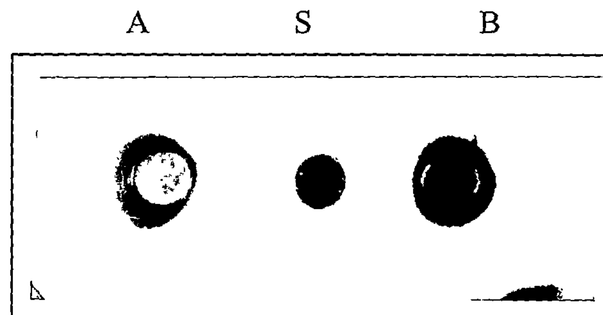


Figure 8.5: Ouchterlony immunodiffusion showing a precipitin line between S and B wells. Well S was loaded with the antiserum; well A and B are with BSA and antigen (ADP-ribose polymer) isolated from normal mouse spleen cells, respectively. After samples were allowed to diffuse for 36 hr at 37 °C in a moist chamber, the slides were washed overnight in saline and stained with 0.25 % Coomassie brilliant blue solution prepared in acetic acid and methanol solution.

8.5 EFFECT OF γ-RADIATION ON THE POLY-ADP-RIBOSYLATION (PAR) OF CELLULAR AND HISTONE PROTEINS IN *EX VIVO* MOUSE SPLEEN CELLS:

Poly-ADP ribosylation (PAR) of certain cellular and nuclear proteins is a well-established process. This process is known to respond to DNA damage induced by several stimuli including ionizing radiation. In an attempt to understand the involvement of PAR in

radiation-induced DNA damage and its influence on chromatin structure, PAR of cellular and individual histone proteins in *ex-vivo* mouse spleen cells was studied. The PAR assay (Slot and Western blotting) were carried out after allowing the non-irradiated (control) and irradiated samples to undergo a 20 min repair incubation at 37 °C. Fig. 8.6 shows video densitometric image of Slot blot membrane used for detection of total cellular PAR.

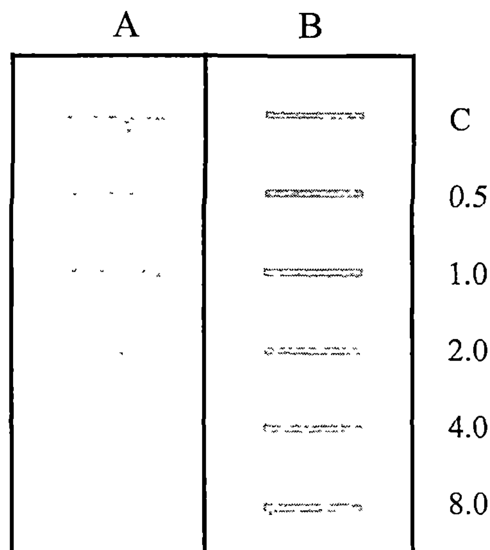


Figure 8.6: Densitometric scan image of Slot blot membrane. Each slots were loaded with 100 μl crude protein extract (mouse spleen cells), equivalent to 0.1 x 10^6 cells. Slots from top to bottom contains samples of non-irradiated (control), 0.5, 1, 2, 4 and 8 Gy of γ -irradiation, respectively. Panel A is stained with rotring ink, while panel B is the immnuodetected membrane.

Panel A shows the total protein sample on nitrocellulose (NC) membrane stained with Rotring ink, while panel B shows immunodetected NC membrane. Slots from top to bottom correspond to non-irradiated (control), 0.5, 1, 2, 4 and 8 Gy of γ-irradiation, respectively. Fig. 8.7 shows a similar image of Western blot membrane that was used for the detection of PAR of individual histone proteins. Panel A show ink stained of crude protein extract (mouse spleen cells), that were separated on SDS-PAGE and electroblotted on to NC membrane. Panel B is its replica after immunodetection. Samples from lanes a-f correspond to non-irradiated (control), 0.5, 1, 2, 4 and 8 Gy of γ-irradiation, respectively. The immunodetected membrane shows ADP-ribosylated H1 (arrow 1) and of core histone proteins (arrow 2).

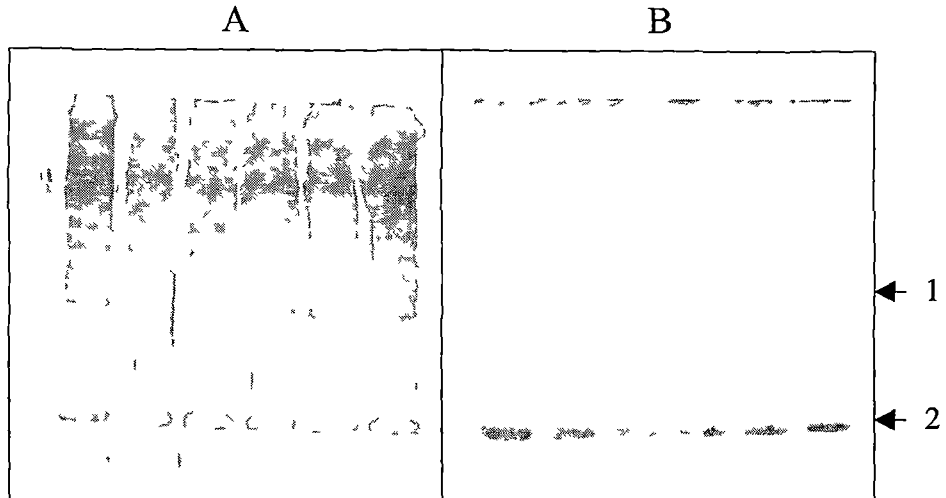
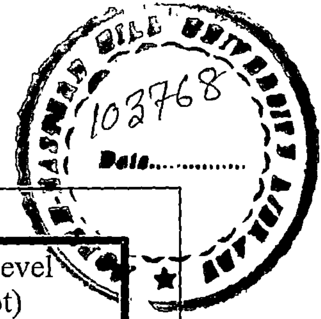


Figure 8.7: Densitometric scan image of Western blot membrane. 12 µl crude protein extract (mouse spleen cells), equivalent to 0.5 x 10^6 cells were loaded into each well Lanes a-f represent samples of non-irradiated (control), 0.5, 1, 2, 4 and 8 Gy of γ-irradiation, respectively. Panel A is stained with rotring ink, while panel B is the immnuodetected membrane Arrow 1 indicates immunodetected signals from H1 while 2 indicates signals from core histone proteins.

Fig. 8.8 shows densitometric quantification of immunodetected signals in Fig. 8.6 and Fig. 8.7. It depicts the change in the level of PAR following irradiation. Significant lowering of PAR level, with respect to the normal (control) level, was observed up to 4 Gy in case of H1 as well as core histone proteins. While an increase to 8 Gy did not show any significant change in the case of core histone in its level of PAR, a tendency to rise was observed for H1. The level of total cellular PAR, on the other hand, showed an initial depression at 1 Gy. It, however, tended to recover back to normal level as dose was increased from 2 to 8 Gy.



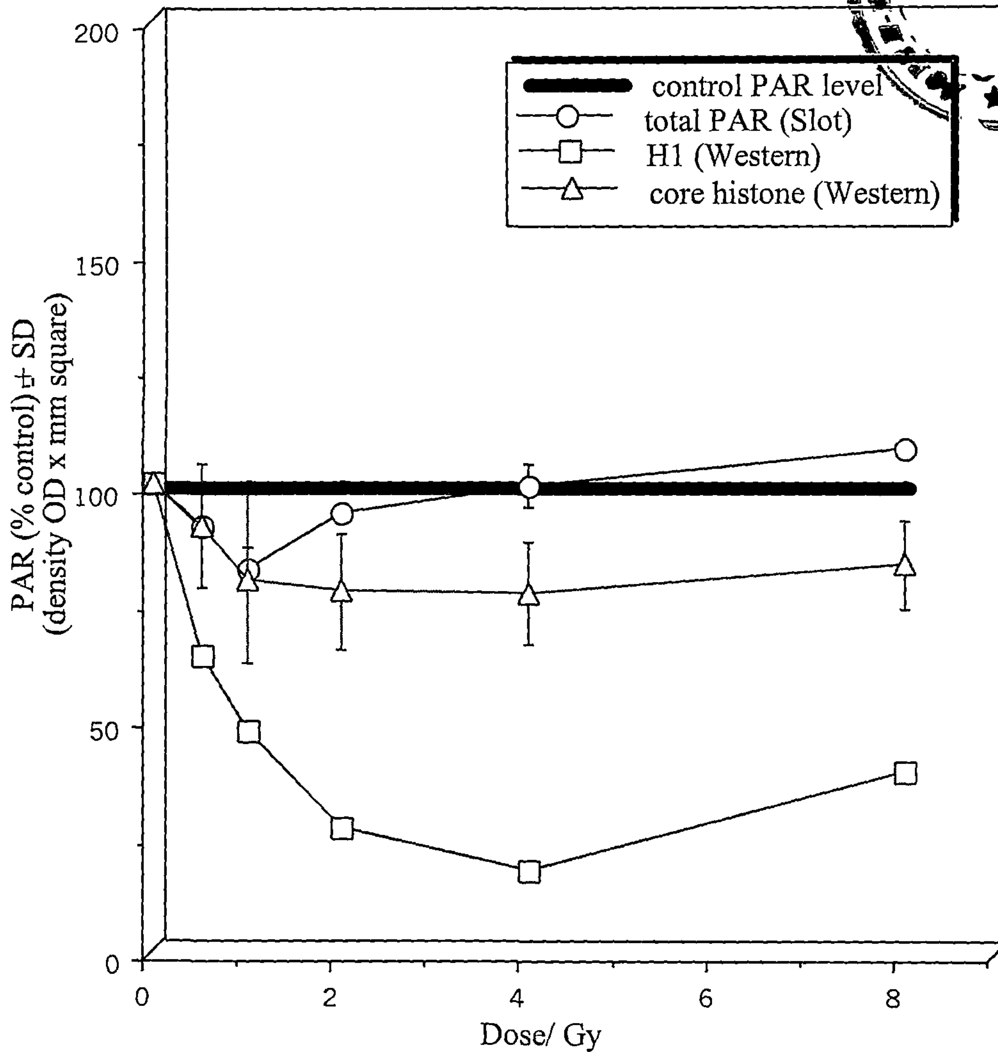


Figure 8.8: Changes in the level of PAR following γ-irradiation as measured by Slot and Western blotting using rabbit polyclonal antibody raised against mouse ADP-ribose. Assay were carried out after 20 min incubation at 37 °C. Non-irradiated (control) PAR level normalized to 100 % is indicated by shaded fat line. The data for total PAR and core histone were obtained from three independent assay and represents mean ± SD. Point for H1 represents data from a single assay.

8.6 EFFECTS OF ^{11}B PARTICLE IRRADIATION ON CELL VIABILITY OF T1, HG AND R1H CELL LINES:

Fig. 8.9 shows the percent cell survival of HG, R1H and T1 cell lines after ^{11}B particle irradiation. The three cell lines showed a varied cell survival curves as measured by dye exclusion test. Human T1 cells was observed to be the most effected as viability

reduced from approximately 95% to 60%. While the other human cell line (HG) reduced from 87% to 70%, rat cell (R1H) was reduced from 93% to 81%.

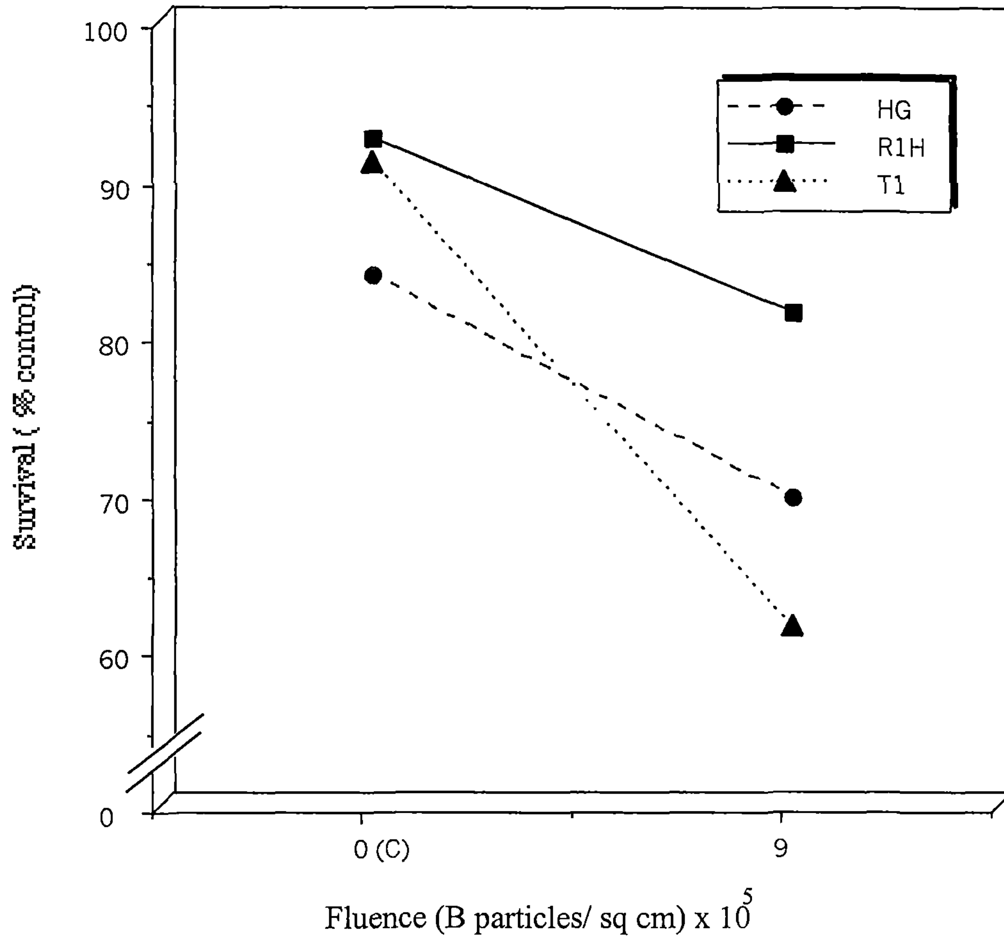


Figure 8.9: Cell viability as a function of ^{11}B particle irradiation in three cell lines. Each point, measured as % cell survival, represents mean from four independent sets. SD values have not been indicated.

8.7 EFFECTS OF ^{11}B PARTICLE IRRADIATION ON THE ACTIVITY OF ACETYLCHOLINE ESTERASE IN T1, HG AND R1H CELL LINES:

Fig. 8.10 shows the AchE activity of the three different cell lines after ^{11}B particle irradiation. AchE activity in T1 was significantly reduced ($p < 0.0001$) following irradiation, while in HG it showed a tendency of reduction ($p < 0.022$). On the other hand,

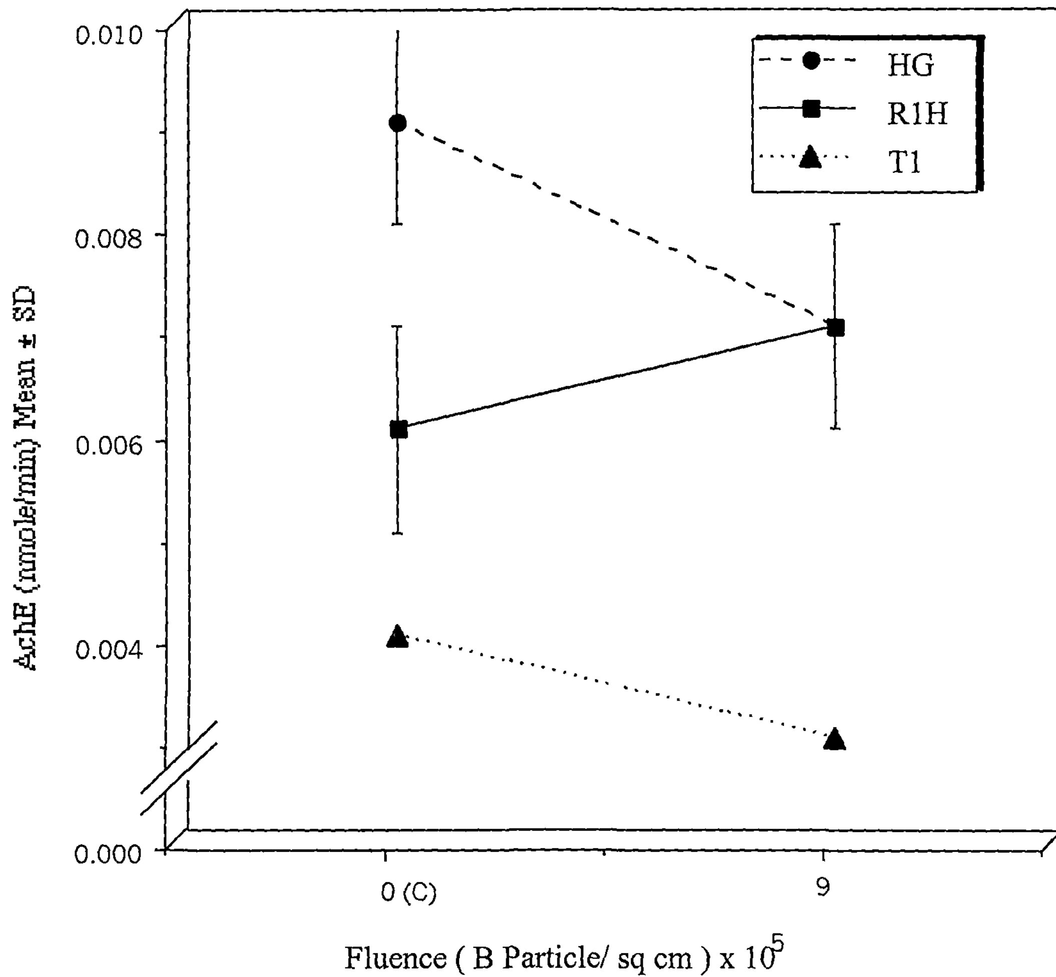


Figure 8.10: AchE activity as a function of fluence of ^{11}B particle irradiation in three different cell lines. Each point, obtained from 4-5 independent sets, shows mean ± SD.

AchE level in R1H, unlike the human cell lines, remained unchanged ($p < 0.272$) after 9 x 10^5 particles cm^{-2} of ^{11}B irradiation.

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CHAPTER 9

DISCUSSION

It is generally accepted that ionizing radiation kills eukaryotic cells by damaging the structure and function of genomic DNA and membrane. The cellular endpoints following irradiation are believed to be primarily due to induction, processing and manifestation of DNA damage. In this, role of membrane can not be ignored since membrane integrity is vital to regulation and maintenance of cellular microenvironment. Much efforts has been focused on understanding the relationship between DNA damage and cells response (Szumiel, 1998; Woudstra *et al.,* 1998; Nunez *et al.,* 1998). Studies from different radiosensitive cell lines show distinct dose-response correlation for DNA damage (Taylor *et al.,* 1991; Malyapa *et al.,* 1996; Woudstra *et al.*, 1996). The effects are known to be influenced by both the presence of DNA-bound proteins and the higher order chromatin structural organization (Warters and Lyons, 1990; Ljungman, 1991). These reports suggest that cellular radiosensitivity may be partially dependent on the fragility of chromatin structure. In this context, it becomes pertinent to study the status of chromatin organization in relation to DNA strand breaks during or after radiation assault. Evaluation of biochemical events in relation to DNA damage in mammalian cell system has an important bearing on the understanding of the biochemical and molecular basis of sensitivity to ionizing radiation. Besides shedding light on the complexities of cellular responses, it is likely to provide information in the understanding of carcinogenesis, susceptibility to cancer and patients response to radiotherapy.

In the alkaline comet assay employed in this investigation, cells are embedded in low-melting-point agarose and layered on microscope slides. The cells are lysed, electrophoresed, stained with fluorescence dye and examined under fluorescence microscope. In normal undamaged cells, a bright fluorescence core known as "head" is seen with a less intense edge of fluorescence, facing the anode, called as "tail". However, when DNA strand breaks are present, fluorescence appears in a dose dependent extension of the tail from the core to the anode. The assay essentially reflects the displacement of fluorescence from the head to tail in damaged cells. The tail moment, a product of the percentage of DNA in the tail and the tail length of the comet is, therefore, considered and used as a valuable quantification parameter of analyzing DNA strand breaks (Singh *et al.*, 1988; Olive *et al.,* 1992). Double strand breaks can be measured under neutral conditions (Olive *et al.,* 1991).

The linear increase in tail moment (Fig. 8.2) indicates that increasing DNA damage is being induced in human kidney T1 cell lines with increasing dose of γ-rays when analyzed immediately after irradiation. The alkaline comet assay detects mostly SSB and alkali labile sites with DSB constituting only about 5 % of total measured damage (Singh *et al.*, 1988; Olive *et al.*, 1990a). The dose-dependent increase in the tail moment may, therefore, indicate that progressively more SSB were induced as cells were exposed to 0,

0.5, 1, 2 and 4 Gy of γ-radiation. This is in line with results of Pouget *et al.* (1999) wherein SSB was studied after exposure to 0-8 Gy of γ-radiation in a neoplastic monolytic cell line, THP-1. Upon incubation at 37 °C for 20 min, on the other hand, the nature of curve for tail moment was different (Fig. 8.2). The extent of dose dependent increase in tail moment was significantly less (signifying less SSB) with a plateau at 2 Gy and marginal decrease at 4 Gy. It suggests that during the 20-min repair incubation, the radiation induced SSB may have been at least partially, repaired. The rate of strand breaks rejoining is known to influence the fluorescence of DNA stained with propidium iodide (Olive *et al.,* 1994). During repair process, the fluorescence gradually returns to normal as strand breaks were rejoined (Olive, 1999). The time of recovery has been observed to be linearly related to dose, where complete recovery requires about 15 min per Gy. From the kinetics of tail moment curve (Fig. 8.2) and microphotograph of cells DNA (Fig. 8.1), it appears that the rate of SSB repair must have been fast under the experimental condition. Under similar conditions, the chromatin activity assay in intact nuclei isolated from T1 cells demonstrated a progressive degradation by DNase I up to 1 Gy. However, at the higher doses of 2-4 Gy, the chromatin digestion started to show a similar pattern as that of the control (Fig. 8.3). This coincides with dose when SSB seemed to undergo partial rejoining as detected by the comet assay (Fig. 8.2). It is likely that rejoining would possibly favor recoiling of the DNA superstructure which in turn would partially protect the chromatin from DNase I action (Fig. 8.3).

Radiation are known to induce PCD or apoptosis (Soldatenkov *et al.*, 1995; Vral *et al.*, 1998; Radford and Aldridge, 1999). During this process certain nucleases cleave the double strand DNA at most accessible inter-nucleosome regions. The production of DNA fragments that are detected as "DNA ladders" on electrophoresis is considered classically a biochemical feature of apoptosis (Wyllie and Morris, 1982). Poly-ADP-ribosylation (PAR), a post-translational modification of chromosomal proteins, has been known to be an important molecular event effecting organization of DNA in chromatin. Since the accessibility of inter-nucleosome is greatly dependent on chromatin organization, it is likely that PAR also influences PCD. This becomes even more probable because radiation is also a known inducer of PAR. The PAR metabolizing enzyme PARP is known to be activated by radiation induced DNA strand breaks (Althaus and Richter, 1987; Boulikas, 1989; Weinfeld *et al.*, 1997). It also influences their repair by affecting structural organization of chromosomal DNA (Poirier *et al.,* 1982; Neidergang *et al.,* 1985; De Murcia *et al.,* 1988; Schneeweiss *et al.*, 1995; Sharan *et al.*, 1998b). Thus, it is evident that PAR is a potent key factor influencing the processing of radiation induced damage, i.e. repair or PCD. To understand the molecular intricacies after cellular injury *in vivo* it is imperative to have clear evaluation on the level of PAR. For this purpose the changes in the levels of induction of apoptosis and PAR of total cellular and chromosomal protein were

studied.

Fig. 8.4 indicates the induction of apoptosis following γ-irradiation, while Fig. 8.8 depicts the changes in the level of PAR of total cellular and histone proteins under similar conditions. The PAR, which was measured after 20-min repair incubation, indicated a differential pattern in its PAR response for different proteins. A general inhibition of PAR was evident (Fig. 8.8). The PAR of total cellular protein was inhibited initially at 1 Gy after which the level increased gradually reaching to that of the control. In histone proteins the inhibition of PAR was observed up to 2-4 Gy. Under identical experimental conditions maximum induction of apoptosis was measured at about 2 Gy which coincides with the dose at which PAR was markedly inhibited. Even though the drastic reduction of PAR in H1 (Fig. 8.8) is not fully clear, one possible reason for the general inhibition of PAR could be the inactivation of PARP by caspases and simultaneous activation of the ADP-ribose polymer-degrading enzyme, PAR-glycohydrolase. PARP, the enzyme which is responsible for poly-ADP-ribosylation, has lately been implicated in apoptosis (Lazebnik *et al.,* 1994; Nicholson *et al.,* 1995; Tewari *et al.,* 1995; Simbulan-Rosenthal, 1999b). It has been suggested that during the initial execution phase of apoptosis the enzyme PARP gets cleaved into two fragments - 29 and 85 kDa subunits, by caspase mediated proteolysis (Kaufmann *et al.,* 1993; Shah *et al.,* 1996; D'Amours *et al.,* 1998). The degradation of PARP during the initial stages of apoptosis accompanied by the rapid removal of poly-ADP-ribose chains from the target proteins due to the action of constitutively expressed PAR-glycohydrolase have been suggested (Khodarev *et al.,* 1998). It is, therefore, likely that PARP underwent degradation as apoptosis was initiated by γ-radiation (Fig. 8.4) by inducing caspases mediated cleavage. Cleavage of PARP by caspases and activation of glycohydrolase would, thereby, reduce the PAR level significantly. As dose was increased from 4-8 Gy, the level of PAR detected from H1 and core histone remained unchanged, while in total cellular protein it showed a tendency to recover back to control level. It suggests that at higher dose of γ-radiation, PAR-glycohydrolase may also be inactivated by radiation. Radiation induced inactivation of PAR enzyme in T1 cells has been shown (Sharan *et al.,* 1996). The activities of poly (ADP-ribose) glycohydrolase or ADP-ribosyl protein lyase were suggested to be stimulated up to 1.5 Gy of γ-rays which resulted in decrease of PAR. Above 1.5 Gy of γ-rays these PAR degrading enzymes appeared to be affected, thereby, reversed the levels of cellular PAR.

Rosenthal *et al.* (1997) have reported that PAR of nuclear proteins occur prior to commitment to apoptosis and that cleavage and inactivation of PARP begin shortly thereafter, resulting to very little PAR *per se* during the later stages of apoptosis. Using mouse spleen *ex-vivo* cells, the results demonstrate that inactivation of PAR of nuclear proteins occurs during the initiation of radiation induced apoptosis, despite the presence of

a very large number of expected DNA strand breaks. Decreased level of PARP and PAR have also been observed in intact human osteosarcoma cells undergoing spontaneous apoptosis (Simbulan-Rosenthal *et al.*, 1999a). The results from *ex-vivo* mouse spleen cells also support the theory of PARP cleavage by caspases during the execution phase of apoptosis or PCD.

Radiobiological behavior after accelerated heavy particle irradiation is widely pursued because of its potential relevancy in tumor radiotherapy. Heavy charged particle upon interaction with matter produces a track of well-defined diameter and energy deposition. The energy deposition is determined by the energy loss of the particle, which depends on the squared atomic number, whereas the track diameter depends on the velocity of the particle. Its good depth-dose distribution characterized by a low dose plateau in the entrance channel and a sharp peak (Bragg peak) near the end of its track gives an excellent physical dose distribution. These biophysical characteristics make heavy ions especially useful in radiotherapy as compared to conventional methods such as by γ- or x-rays. However, different biological systems respond differently to heavy charged particle irradiation. Particle size, charge and linear energy transfer (LET) have been reported to influence the impact on biological systems. Despite results demonstrating promising effects, the lack of proper understanding of the mechanism of damage and its consequences in biological system obscures the successful application of accelerated charged particle to clinical use. Understandably, basic biological investigations to clarify the significance of molecular and cellular damages induced by heavy charged particles have been of global interest. This part of the work was aimed to study the immediate cellular response on three mammalian cell lines, viz., human kidney (T1), human glioblastoma (86HG39) and rat primary rhabdomurosarcoma (R1H), after exposing to accelerated boron (^{11}B) beam. Acetylcholine esterase (AchE) enzyme activity and cell survival were the chosen parameters for the study.

Figs. 8.9 and 8.10 show the cell viability and of AchE activity of the three different cell lines after ^{11}B particle irradiation, respectively. AchE activity in T1 was significantly reduced following irradiation, while in HG it showed a tendency of reduction. On the other hand, AchE levels measured in R1H, unlike T1 and HG cell lines, remained unchanged after 9 x 10^5 particles cm^{-2} ^{11}B irradiation. The difference in the extent of enzyme inactivation may also reflect the radiosensitivity of the three cell lines. The cell viabilities of the three cell lines exhibited a similar pattern. T1 showed significant reduction as compared to the HG and R1H cell lines. The observations indicate that human cell lines (T1 and HG) were responsive to ^{11}B particle irradiation unlike the rat cell line (R1H). A parallel between AchE activity and cell viability for the three cell lines used in this investigation corroborate the observations. Thus, basing on the two biochemical

parameters, the cell membrane, which is the most exposed component of the cell, seems to be effected differently to ^{11}B ion immediately following the exposure. It is, however, not clear whether these observations were only physiological changes or the effects extended to molecular level(s). More work is required to understand the intricacies of heavy charged particle induced biological response.

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CHAPTER 10

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SECTION D

GENERAL CONCLUSION

⇒ **Low-LET ^{60}Co γ-radiation causes predominantly DNA SSB than DSB in pMTa4 plasmid. Since plasmid DNA was irradiated in aqueous solution, •OH seems to be the major determinant in the production of SSB.**

⇒ The formation of defined extra fragments from irradiated pMTa4 DNA by *Hae II* and *Nci I* (but not by others 7 RE that were used in the investigation) suggest a non-random manifestation of effect by radiation.

⇒ **Within the conditions and parameter studied it suggest that GC-rich nucleotides were being more affected or chemically modified upon exposure to ^{60}Co γ-radiation. It may be speculated that non-GC-motif were not affected by ^{60}Co γ-radiation due to which it did not afford any resistance to certain RE (*Acc I, Bgl I, Bgl II, Dra I, Hinf I, Ksp I* and *Pvu II*).**

⇒ Unlike in low-LET, high-LET ^{7}Li particle radiation induced more DSB than SSB in pMTa4. High-LET radiation lead to a denser deposition of ionization than γ-rays and this could explain the increase in the yield of DSB.

⇒ **Even though high-LET ^{7}Li particle radiation caused more DSB than SSB the nature of damage on pMTa4, as revealed by RE approach, appeared to be similar to that observed for low-LET ^{60}Co γ-radiation. RE fragmentation analysis indicates that, though the extent and impact of the damage induced by low- and high-LET may differ, the molecular basis of damage may likely follow a similar mechanism.**

⇒ The study suggests the likely possibility that radiomodified GC nucleotides would form important premutagenic lesions. This indication also points that clusters of GC in the DNA molecule may very likely form hotspots for radiation induced damages.

⇒ **While further detailed investigation would be required, it opens up the likely possibility that inherent radiosensitivity and genome instability may be at least partly determined by the GC-richness of nucleotide sequence in the DNA.**

⇒ Since in majority of eukaryotic genomes, especially human genome, there exists "CpG islands", our results suggest a likely possibility that "genes" are more radiosensitive than "non-gene" components of a genome.

⇒ **RE can be used in the partial characterization of radiation induced specific nucleotide damage in small defined DNA molecules. This approach may also be applicable in study of damages induced by other agents than radiation.**

⇒ RE approach does not reveal any apparent modification induced by radiation in *E. coli* genomic DNA. Due to the large number of undefined fragments produced

as compared to the plasmid (pMTa4), the qualitative fragmentation analysis was not revealing. This makes RE approach and analysis by agarose gel electrophoresis non-sensitive for studying specific NT damage in a large and highly complex DNA molecule.

⇒ **The alkaline comet assay was able to detect ^{137}Cs γ-radiation induced SSB in human kidney T1 cells. Under these conditions, SSB favored the relaxation or decondensation of chromatin.**

⇒ SSB, however, got partially repaired or rejoined during repair permissive conditions (20-min incubation at 37 °C). Under these conditions, repair or rejoining of SSB appeared to allow the chromatin reorganize its structural configuration favoring a re-condensation.

⇒ **Using a novel immuno-blot assay (Slot and Western blot), a general inhibition of PAR in *ex-vivo* mouse spleen cells upon ^{60}Co γ-irradiation was observed.**

⇒ The PAR of total cellular protein was inhibited initially at 1 Gy after which the level increased gradually reaching the control level. For histone proteins, the inhibition was observed up to 2-4 Gy.

⇒ **Under identical experimental conditions maximum induction of apoptosis or PCD was measured at about 2 Gy in *ex-vivo* mouse spleen cells upon ^{60}Co γ-irradiation. It is suggested that reduction in the level of PAR of nuclear proteins occur during the initiation of radiation induced apoptosis.**

⇒ The lowering of PAR can be due to the loss of PARP activity. The results from *ex vivo* mouse spleen cells thus support the theory of PARP cleavage by caspases during the execution phase of apoptosis. It can be inferred that PAR degrading enzymes such as, poly-ADP-ribose glycohydrolase may also be inactivated by ^{60}Co γ-radiation.

⇒ **The lowering of PAR may be proposed as a biomarker during early stages of radiation induced apoptosis. Thus, the immuno-blot assay of PAR can potentially be used as a predictive assay for monitoring progression of radiotherapy.**

⇒ The study with ^{11}B charged particle on three cell lines (T1, R1H and HG) led to loss or inactivation of acetylcholine esterase enzyme as well as cell death. These cell membrane related biochemical parameters suggest that the accelerated charged ^{11}B particle effected cell membrane variantly in different cell types *in vitro*. Further investigations are necessary to see if these changes were only physiological or extended to molecular level.

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Curriculum Vitae

Name : J. Orenthung Humtsoe
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Academic Qualifications:

DEGREE	AWARDING BOARD/ UNIV.	YEAR
High School Leaving Certificate	Nagaland Board of School Education, Kohima	1988
Pre-University	North-Eastern Hill University, Shillong	1990
B. Sc. (pass course)	- do -	1992
B. Sc. (Hons. in Zoology)	- do -	1993
M. Sc. (Biochemistry)	- do -	1995

Techniques familiar with:

In house animal handling; ADP-ribose polymer isolation from mouse spleen and bone marrow cells, column chromatography, antibody raising; bacterial cell cultures; *in vitro* mammalian cell cultures; plasmids DNA and genomic DNA isolations from bacteria and chromatin from mammalian cells; restriction analysis using diffferent restriction endonucleases; histones isolation; handling of irradiation source (^{60}Co and ^{137}Cs); acquaintance with accelerator (15 UD Pelletron, Nuclear Science Center, New Delhi); agarose gel electrophoresis; SDS-PAGE electrophoresis, comet assay; dot/slot blotting; Western blotting; ELISA; densitometric analysis of gel/photographics scans using Molecular Analyst software; handling of common equipments like: ultra centrifuge, standard refregirated centrifuges, UV/visible spectrophotometers etc. and other standard biochemical assays.

Fellowship awarded (UGC-NEHU) :

Junior research fellow (1996-98)
Senior research fellow (continuing since 1998)

* Visited Institute of Medicine, Research Center, Juelich, Germany (3 months), under Indo-German bilateral co-operation programme (1999).

Publications/ Reports:

Humtsoe, J. O., Schneeweiss, F. H. A., Srivastava, A., Sarma, A. and Sharan, R. N. (2000) Biological effects induced by boron and lithium accelerated charged particle irradiation of human and rat cell lines and plasmid DNA *in vitro. Proceedings of the Workshop on New Frontiers in Heavy Ion Radiation Biology*, pp. 31-36, Nuclear Science Center, New Delhi.

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and acetylcholine esterase activity in human and rat cell lines *in vitro. Annual Report,* 5.4.2, 114-116, Nuclear Science Center.

Awards:

* Joint-recepient "*Dr. A. R. Gopal-Ayengar-Best Poster Award*" at the **International Conference on Radiation Biology, RADIOBIOLOGY 2000**, held at Regional Cancer Centre, Triruvananthapuram, Kerala, February, 2000.

Conference/ workshop:

Participated with oral paper presentation on the workshop "**New Frontiers in Heavy Ion Radiation Biology**" held at Nuclear Science Centre, New Delhi, May, 2000.

* Participated with oral and poster paper presentation at the **International Conference on Radiation Biology, RADIOBIOLOGY 2000**, held at Regional Cancer Centre, Triruvananthapuram, Kerala, February, 2000.

Participated awareness programme on "**Application of Radioisotopes and Radiation Technology**" held at Shillong, November, 1999.

* Participated "**NSC School on Accelerator Based Science and Technology**" held at Nuclear Science Centre, New-Delhi, April, 1998.

* Participated workshop on "**Molecular Genetics of Human Cancer**" held at Chittaranjan National Cancer Institute, Calcutta, January, 1998.

* Participated with poster paper presentation at the **International Conference on Radiation Biology: DNA Damage, Repair and Carcinogenesis and Indo-German Satellite Symposium on Molecular Biology of Radiation Damage and Repair** held at Department of Biochemistry, North-Eastern Hill University, Shillong, April, 1998.

* Participated with poster paper presentation at the "**17th annual convention of Indian Association for Cancer Research**" at Science City, Calcutta. January, 1998.

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