# INDUCTION OF NUMERICAL CHROMOSOMAL ABERRATIONS BY REACTIVE OXYGEN SPECIES

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THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS OF THE DEGREE OF
MASTER OF SCIENCE
in the School
of
Kinesiology


Simon Fraser University
April 1995

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INDUCTION OF NUMERICAL CHROMOSOMAL ABERRATIONS BY REACTIVE OXYGEN SPECIES

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**ABSTRACT**

Cells exposed to reactive oxygen species will incur a range of genetic aberrations, which may either be structural (e.g. deletion, DNA strand breaks) or numerical (e.g. chromosomal loss/gain). Some studies suggest that chromosome 11 contains loci which prevent the induction of chromosomal aberrations by reactive oxygen species. For example, the insertion of an extra chromosome 11 into bladder carcinoma cells decreases the frequency of micronuclei (index of chromosomal damage) induced by hydrogen peroxide and X-rays.

The development of fluorescence *in situ* hybridization (FISH) facilitates studies exploring the significance of numerical chromosomal aberrations in carcinogenesis. In this study, the hypothesis tested was that chromosome 11 insertion protects against numerical chromosomal changes induced by hydrogen peroxide and X-rays. The level of chromosome loss and gain were assayed with the FISH technique, which allows the determination of chromosome number in interphase cells.

Peroxide treatment induced numerical chromosome alterations, but neither chromosome loss nor gain was significantly different between the parent and hybrid cell cultures. X-irradiating cells at 0-2 Gy did not result in significant chromosomal number changes in either cell cultures, in spite of the fact that a dose of 2 Gy induced large numbers of micronucleated cells. Further investigation exposed parent cells to higher X-ray doses (0-4 Gy) and to a range of time intervals between irradiation and cell harvesting. These variations yielded significant chromosome gains, but not losses. The apparent gain in chromosomes without a corresponding loss points to the possibility of a fragile FISH target region. In summary, the results of this thesis suggest that (1) the insertion of an extra chromosome 11 does not protect against numerical chromosomal aberrations induced by hydrogen peroxide, and (2) levels of X-rays which induce high levels of structural chromosomal damage (micronuclei) fail to induce significant levels of numerical chromosomal aberrations.

## DEDICATION

# To my parents,

**who unceasingly emphasize**
**the importance of education.**

## ACKNOWLEDGEMENTS

I would like to express sincere thanks to my senior supervisor, Dr. Miriam Rosin, to whom I am indebted for the opportunity to learn under her guidance. I greatly appreciate her time and insightful advice in this thesis project. I would also like to extend my gratitude to Dr. Bruce Dunn who patiently and critically appraised this thesis. I treasure his opinions and encouragement. Many thanks also go out to Dr. Shizu Hayashi who graciously agreed to the role of an external examiner. Thanks are also due to Dr. O. Pereira-Smith who presented the cell lines as a gift.

This thesis is also made possible by the support of many caring individuals. I respectfully applaud my parents' unfaltering interest and support in the furtherance of my education. Special thanks go to my husband, Daryl, who spent his evening hours drawing and customizing the indispensable illustrations for this thesis. My earnest gratitude also encompasses those who prayed unceasingly for my well-being in the completion of this thesis.

Most of all, I thank my Heavenly Father for bringing all of you into my life according to His wise timing.

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# 1 INTRODUCTION

## 1.1 Overview

It is becoming increasingly apparent that tissues must accumulate multiple genetic changes for cancer to develop (Flier et al., 1988). Some genetic changes are associated with early stages of cancer development while others occur most commonly with late-stage events that are related to invasion and metastasis (Bishop, 1987; Hittleman et al., 1993). Little is known about the processes that drive the accumulation of these changes. One possibility is that at some point during the early stages of carcinogenesis a change occurs that makes a cell more genetically labile. An example of such a change would be the loss of cellular protection against a class of agents capable of damaging DNA or an alteration in the cell's capacity to repair DNA damage by these agents.

This thesis explores the possibility that one such critical change involves an alteration in a cell's sensitivity to oxidative damage by reactive oxygen species (ROS). All living cells are exposed to ROS. They are generated during normal cell metabolism (e.g. during oxidative phosphorylation in the mitochondria, as products of oxidases such as xanthine oxidase, during the biosynthesis of prostaglandins, etc.), released during inflammation and produced in cells by many xenobiotics. ROS are also at least partially responsible for the genotoxic/cytotoxic effects of agents like radiation or chemical oxidants such as hydrogen peroxide (reviewed in Cerutti, 1985; Halliwell and Aruoma, 1991; Foegh et al., 1990; Fridovich, 1989; Imlay and Fridovich, 1991; Frenkel, 1992). Alteration in a cell's capacity to deal with ROS could lead to genetic instability in a tissue. This hypothesis is supported by data from genetic syndromes such as ataxia-telangiectasia, from studies with pre-malignant lesions and tumours, and from *in vitro* studies with cancer cells. This data will be reviewed in later sections.

There are several indications that chromosome 11 contains loci that prevent the induction of chromosomal aberrations by ROS. Some of

this data come from studies in this laboratory which show that insertion of a normal chromosome 11 into a bladder carcinoma cell line (A1698 termed "parent") protects a cell from chromosomal damage (Ward et al., 1993; Hofseth, unpublished data). More micronuclei are induced in the parent cell line than in the hybrid (A1698 plus chromosome 11 insert) after treatment with conditions that induce oxidative damage: hydrogen peroxide, xanthine and xanthine oxidase mixtures, X-rays and co-incubation with TPA-activated human neutrophils. Micronuclei come from two sources (Rosin, 1992). They can be derived from acentric chromosomal fragments or from entire chromosomes that are left behind when the chromosomes migrate to the spindle poles during mitosis, possibly due to disruption of the spindle apparatus. Micronuclei appear as Feulgen-positive bodies in the cytoplasm of the daughter cells. At least a portion of the micronuclei formed in the aforementioned bladder cell lines after oxidative damage could result from spindle disruption and consequently chromosome loss, thus representing alterations to chromosome number.

Numerical chromosomal alterations represent a type of genetic instability about which little is known. Since the development of the fluorescence *in situ* hybridization (FISH), this area of research has become increasingly active. FISH uses chromosome-specific probes to hybridize to target chromosomes. The bound probe is visualized by reacting it with a fluorescent agent (eg, fluorescein-labelled avidin). This procedure allows researchers to begin to explore the significance of numerical chromosomal alterations to the process of carcinogenesis, to identify agents that induce such damage in cells, and to explore means by which cells can act to protect themselves from such changes. The current study uses FISH to determine whether alterations to chromosome number are occurring in aforementioned bladder carcinoma cells during treatment with oxidative agents. The possibility that hybrid cells are protected from the induction of such an alteration by loci on the normal chromosome 11 is explored. The data obtained from the study will provide more information on the mechanism by which loci on this chromosome protect cells from chromosomal damage. More importantly,

the study will provide data on the ability of oxidative agents to induce alterations in chromosome number.

## 1.2 Mechanisms of aneuploidy

The term aneuploidy usually describes the condition in which parts of chromosomes (segmental) or whole chromosomes (chromosomal) are absent from or present in addition to the normal genome set. This thesis however uses "aneuploidy" to refer to any condition where there is a loss or gain of whole chromosome(s) in the cell karyotype. An agent which is capable of inducing aneuploidy is termed an aneuploidogen (Oshimura et al., 1986). Aneuploidogens that act directly on DNA or chromosomes will likely cause segmental and chromosomal aneuploidy, while those that act on the mitotic apparatus will result in chromosomal aneuploidy only (Figure 1).

Table 1 shows a list of chemicals that induce aneuploidy and the mechanisms by which they act to induce such changes in cells. Briefly, microtubules make up the predominant part of the mitotic apparatus. They are essential in attaching and guiding the chromosomes to the respective poles during mitosis. Microtubules consist of tubulin subunits containing sulfhydryl groups that are important in polymerization. Colchicine and vincristine are examples of chemicals that are capable of blocking these sites, inducing a disruption in the mitotic apparatus, which results in aneuploidy.

At the centromeres (primary constrictions) of all metaphase chromosomes is a trilaminar structure called the kinetochore (Earnshaw et al., 1989). This is the principal point of attachment of chromosomes to the mitotic spindles. Malformed or damaged kinetochores may not connect with the microtubules, giving rise to lagging chromosomes.

Aneuploidy can also result from increased chromosome stickiness. Agents such as actinomycin D and quinacrine that intercalate into DNA induce chromosome stickiness. This phenomenon is due to the

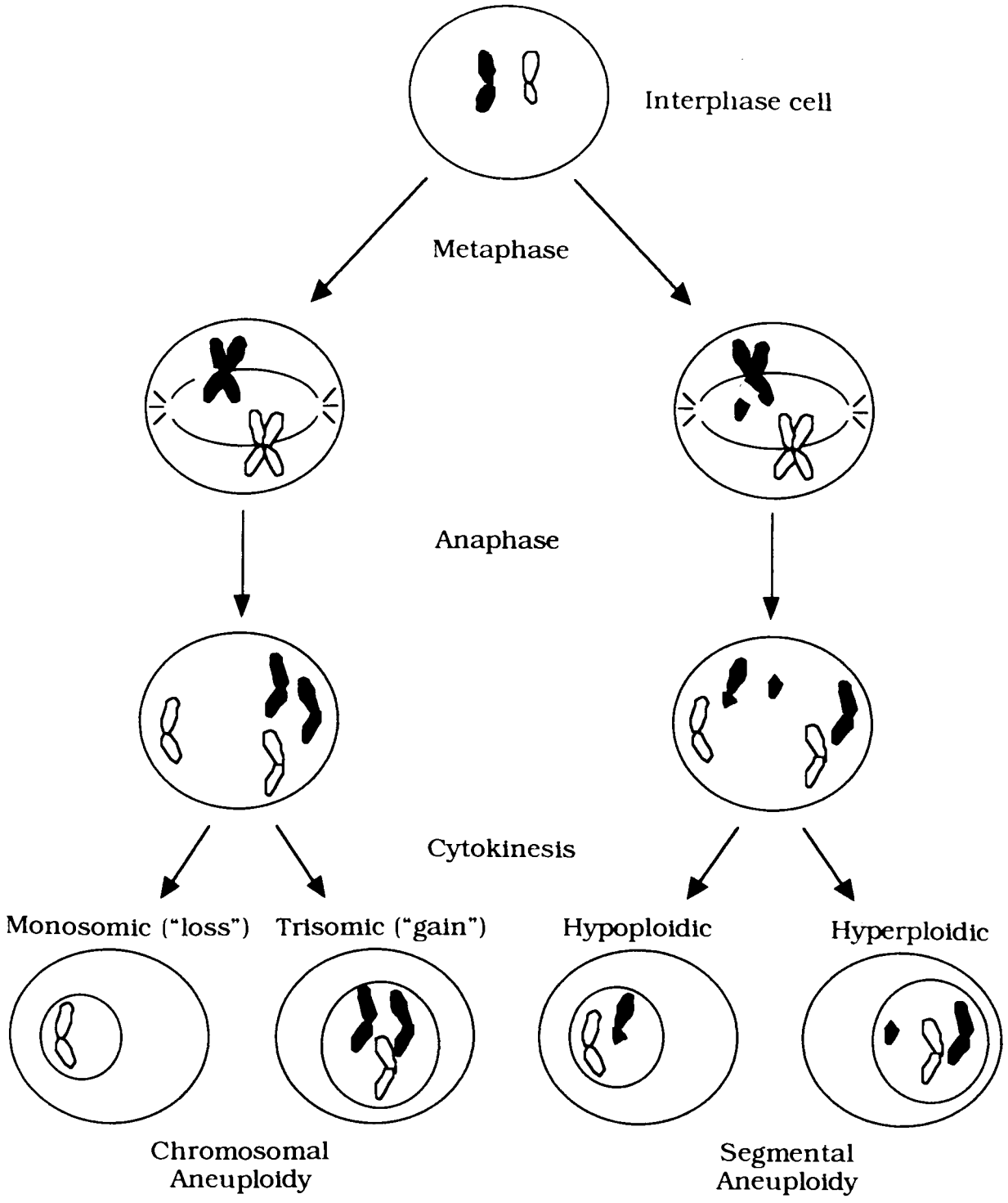



Figure 1: Possible Mitotic events leading to aneuploidy. When an aneuploidogen disrupts the mitotic apparatus, chromosomal aneuploidy occurs with one or more chromosomes being lost or gained. On the other hand, segmental aneuploidy is caused by fragmenting the chromosome arm. (Adapted from Eastmond et al, 1989 and Wagner et al, 1993)

**Table 1: Known aneuploidogens and their effects on targets.**

| Aneuploidogen | Target | Effect |
|---|---|---|
| Colchicine | tubulin | a) inhibits microtubule assembly<br>b) results in mitotic arrest<br>c) alter kinetochore structures (tubulin present in trilaminar structure of kinetochore)<br>d) decreases elongation of daughter centrioles of the division poles |
| Vinblastine<br>Vincristine | tubulin | a) inhibits microtubule assembly<br>b) disassembles preformed MT to spiralised protofilament |
| Thimerosal | tubulin | interacts with sulphydryl groups on tubulin dimers to inhibit microtubule asssembly |
| Mitomycin C | kinetochore | removes kinetochores from chromosomes |
| Actinomycin D<br>Quinacrine<br>Hoechst 33258 | DNA | a) induces chromosome stickiness<br>b) prevents proper chromosome separation at anaphase |
| Chloral hydrate | microtubules | prevents pole-to-pole elongation |
| Hydroquinone | microtubules | prevents tubulin and microtubule assembly |
| Diazepam | centrioles | prevents separation of centrioles. |
| Griseofulvin | a) microtubule-associated-proteins (MAP)<br>b) centrioles<br>c) microtubules | a) causes depolymerisation/inhibition of spindle microtubules<br>b) causes non-separation of centrioles<br>c) prevents micrutubule-associated-proteins from combining with microtubules<br>d) inhibits sliding function of microtubules |
| Caffeine | a) centrosomes<br>b) spindle | a) prevents centosomal separation<br>b) breakdown of prometaphase spindle |

entanglement of chromatin fibers of unrelated chromosomes, preventing proper division at anaphase (Hsu et al., 1979). In less severe situations, tangled chromosomes may be able to separate during anaphase with some rupturing of chromatin fibers. These broken fibers are observed as breaks or rearrangements in the next cell generation (Oshimura et al., 1986). Figure 2 shows a schematic representation of possible targets leading to aneuploidy.

## 1.3 Involvement of numerical chromosomal alterations in carcinogenesis

Chromosomal aberrations are characterized as being either structural (e.g. deletions, inversions, translocations) or numerical (gain or loss of entire chromosomes). Both of these changes have been shown to play a role in carcinogenesis. For example, chromosome aberrations can be involved in the conversion of normal cellular proto-oncogenes to oncogenes as well as in the loss of tumour suppressor genes.

The advent of molecular biology and recombinant techniques has led to the identification of many examples in which structural chromosomal alterations have been shown to be associated with particular cancers. The most commonly cited example is that of the Philadelphia chromosome ($Ph^1$) (De Klein et al., 1982). This chromosome represents a reciprocal translocation, t(9,22) (q34, q11), that moves the Abelson proto-oncogene, *c-abl*, from chromosome 9 to a position adjacent to *bcr*, a transcription unit on chromosome 22. The *bcr* sequence stimulates the transcription of *c-abl* and a chimeric protein of the bcr-abl fusion gene (p210) is produced that has growth-promoting tyrosine kinase activity (Sayers et al., 1991) as well as the ability to induce leukemia in transgenic mice (Heisterkamp et al., 1990). There are numerous other examples of deletions, translocations and inversions and their relevance to carcinogenesis (Radman et al., 1982; Spriggs, 1974; Mitelman and Levan, 1978; Cairns, 1981). In contrast, we are only beginning to explore the significance of numerical chromosomal alterations to carcinogenesis.

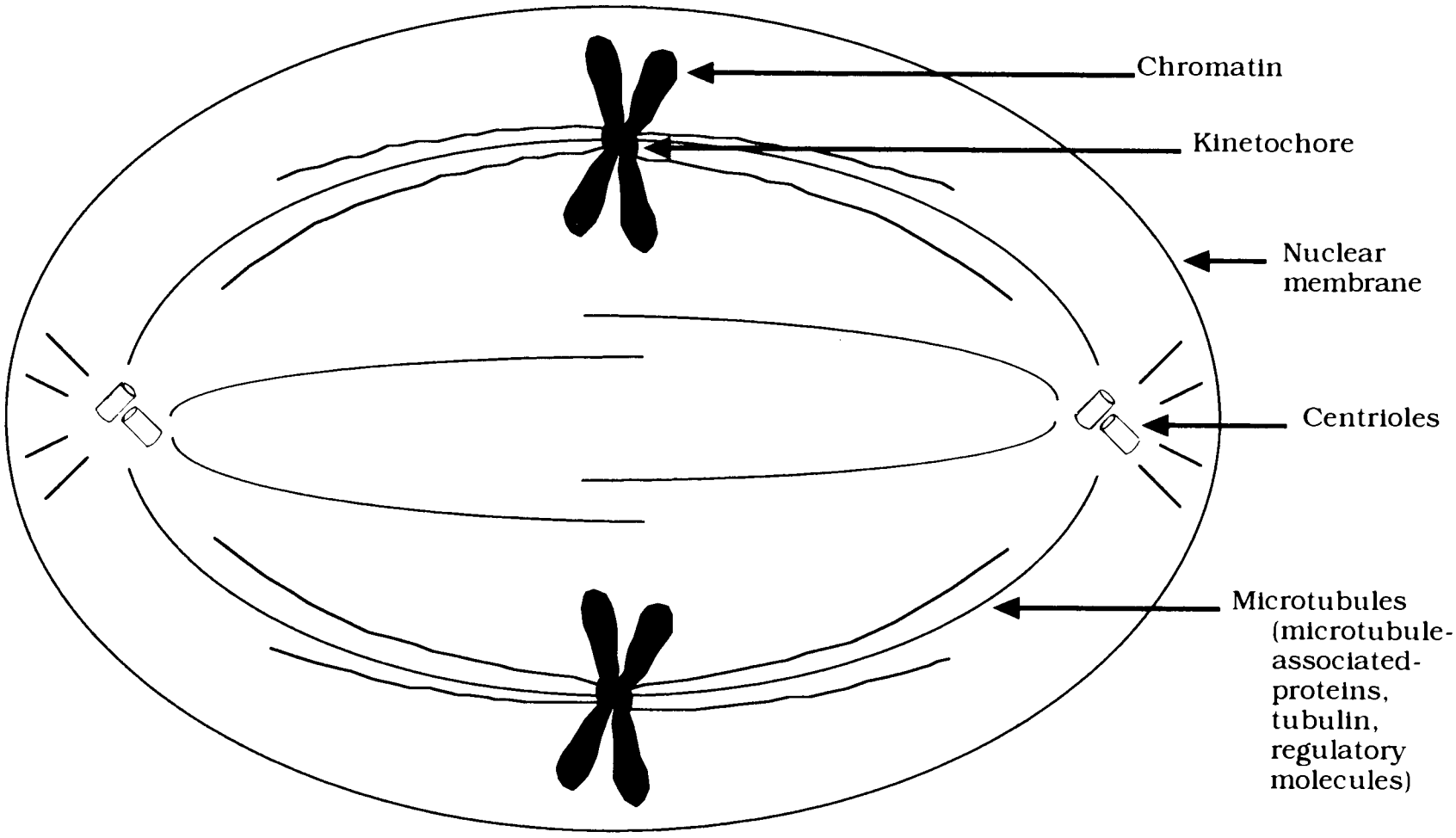



Figure 2: Possible targets for damage leading to aneuploidy

It has long been known that a common characteristic of tumours is alteration in chromosome number. In some cases, there is a duplication of the entire set of chromosomes (termed polyploidy) whereas in other cases a specific chromosome or a subset of chromosomes can be altered in number. Polysomy refers to cells containing 3, 4, or more homologous chromosomes. In this thesis, the term aneuploidy refers to any condition in which there is a loss or gain of whole chromosome(s) in the cell karyotype. Until recently it has been difficult to determine whether numerical chromosome alterations were merely a consequence of the generation of genetically unstable tumour cells that randomly lost or gained chromosomes, or alternatively were unique changes that occurred in pre-malignant cells that were prerequisite to the development of the cancer. For example, the gain of a chromosome with a growth factor receptor on it (e.g. chromosome 7 with epidermal growth factor receptor) could give this cell a selective advantage and lead to its clonal expansion in a tissue (Sidransky and Messing, 1992). Alternatively, if a tumour suppressor gene were present in a tissue in a heterozygous state (gene is normal on one chromosome and mutated on the other), the loss of the chromosome containing the wild-type suppressor gene would also be significant. An example would be a gene such as the retinoblastoma (Rb) gene. Mutation of a single Rb locus would result in a phenotypically normal cell since both loci have to be dysfunctional for the activity of the gene product to be lost (Flier et al., 1988). Loss of the other Rb copy would be required for the suppressor function to be deleted. Among the many ways of losing the normal copy is the loss of the entire chromosome.

## 1.4 Fluorescence *in situ* hybridization: a tool for identifying chromosomal alterations in cells and in tissues

Until recently, studies of the involvement of chromosomal aberrations early in the development of malignancy in tissues have been limited by the difficulty of obtaining metaphases for analysis. Biopsies had to be obtained from tissues and cells stimulated into mitosis. This difficulty was removed with the development of a new method, fluorescence *in situ*

hybridization (FISH). This procedure allows one to detect numerical and structural chromosomal changes in interphase nuclei and metaphase spreads (Gray and Pinkel, 1992). The procedure uses *in situ* hybridization with DNA probes specific for blocks of repetitive DNA sequences on defined regions of chromosomes. The probe is often biotinylated, thus allowing the bound probe to be identified using a second treatment that involves the use of non-radioactive fluorescent avidin such as fluorescein isothiocynate conjugated avidin (FITC-avidin). It is possible to quantify the number of specific chromosomes that are present in a cell by using probes that hybridize to the centromeric regions of that chromosome. This determination involves counting the number of fluorescent hybridization signals in a cell nucleus.

Some of the more recent data obtained with this approach suggest that alterations in chromosome number are occurring early in carcinogenesis. For example, Hittleman and co-workers (1993) used *in situ* chromosome hybridization with centromeric probes to chromosomes 7 and 17 to explore paraffin sections of head and neck tumours harbouring premalignant lesions. They found that the frequency of polysomies of chromosome 7 and 17 increased as tissue progressed from "normal"-appearing tissue adjacent to tumours (33% of biopsies examined) to hyperplasia (67%) to dysplasia (95%) to squamous cell carcinoma (96%). Biopsies obtained from controls that had normal oral epithelium showed no chromosomal polysomy. These workers also tried to determine whether the rate of polysomy for these 2 chromosomes could predict whether an individual with a pre-malignant lesion, such as an oral leukoplakia, would go on to develop cancer. They found that 3 of 5 patients who had polysomies in their leukoplakia biopsies later developed oral cancer compared to 1 out of 8 individuals who showed no polysomy for these 2 chromosomes in the leukoplakia sample.

The current study makes use of the FISH technique to evaluate the rate of chromosome loss and gain in cell cultures treated with oxidative agents. In this case the advantage of the technique is that it allows an examination of large numbers of cells quickly and accurately for such

changes. This is important since the rate of such changes can be quite low, and analysis of at least 1000 cells may be necessary to obtain a reliable estimate of the frequency of such events. Traditional metaphase analyses are not amenable to analysis of such large numbers of cells.

## 1.5 Reactive oxygen species and carcinogenesis

Reactive oxygen species (ROS) are a class of oxygen-centered molecules with one or more unpaired electrons. ROS include an array of radicals such as superoxide anion, hydroxyl radicals, and numerous products of lipid peroxidation. ROS are highly unstable and reactive. In many cases, they are directly mutagenic and capable of producing DNA strand breaks, chromosomal deletions, chromosomal rearrangements and altered expression of proto-oncogenes and tumour suppressor genes (Goldstein et al., 1990). Hence ROS are biologically significant and a central role has been proposed for their involvement in carcinogenesis in tumour initiation, promotion and progression. ROS are responsible for the most frequent DNA damage in living cells, particularly the reactive hydroxyl radical (·OH) which induces a large percentage of strand breakages and damage to nucleic acid moieties (Ward, 1975; Hagen, 1986; Hutchinson, 1985; Teoule, 1987). Hydrogen peroxide and X-irradiation are two agents studied in this project which are capable of producing reactive oxygen species. The following is a brief description of their activities.

### 1.5.1 Hydrogen peroxide

In itself, hydrogen peroxide has limited chemical reactivity. The most popular mechanism that is proposed for its DNA-damaging activity is that the hydrogen peroxide passes through the cytoplasmic and nuclear membranes to regions adjacent to the DNA (Frenkel, 1992). Here it reacts with chromatin-bound metal ions such as iron to generate hydroxyl radicals via a Fenton-type reaction or, if superoxide is present also, a Haber-Weiss reaction:

$Fe(III) + O_2^- \longrightarrow Fe(II) + O_2$

$Fe(II) + H_2O_2 \longrightarrow \cdot OH + OH^- + Fe(III)$ (Fenton reaction)

$\cdot O_2^- + H_2O_2 \longrightarrow \cdot OH + OH^- + O_2$ (Haber-Weiss reaction)

The resultant hydroxyl ion is very reactive towards organic compounds. If it is formed in close proximity to DNA, it may interact with the genetic material to form DNA adducts, such as 8-hydroxyguanine, or it can cause DNA strand breakage (Halliwell and Aruoma, 1991; Frenkel, 1992). When the ion is in close proximity to membrane lipids, lipid peroxidation may occur. This can also lead to the formation of mutagenic and clastogenic compounds, such as malondialdehyde, 4-hydroxynonenal, and lipid hydroperoxides (Halliwell and Aruoma, 1991). Finally, structural and enzymatic proteins can also be damaged and could have significant biological effects in a cell. The latter effect of ROS on proteins is of particular significance to this thesis since among the proteins that could be affected are the microtubular proteins. Microtubules are the main structural component of the mitotic apparatus. They are responsible for guiding the chromosomes to the appropriate poles of the cell during division. One way of inducing aneuploidy in a cell is to disrupt the proper functioning of the mitotic spindles, resulting in lagging chromosomes. For example, the aneuploidy-inducer colchicine acts by attaching itself to the sulfhydryl groups of the microtubular proteins and preventing microtubule assembly. In their tubulin polymerization studies, Davison and co-workers demonstrated that hydrogen peroxide treatment can inhibit the polymerization of microtubules (Davison et al., 1986).

### 1.5.2 Nuclease activation

Oxidative agents can induce genetic damage in cells by activating nuclease enzymes which cleave the DNA backbone. It has been suggested that oxidative stress induces an increase in intracellular free calcium by (a) inactivation of calcium-binding by endoplasmic reticulum, (b) inhibition of plasma membrane calcium-extrusion systems, and (c) release of calcium from mitochondria. These events increase the

intracellular free calcium which activates calcium-dependent endonucleases that in turn induce strand breakage in the DNA (Halliwell and Aruoma, 1991).

### 1.5.3 X-Rays

*In vitro* and *in vivo* studies have shown that X-rays induce a variety of chromosomal aberrations, including DNA strand breaks, translocations, inversions and crosslinks (Armitage et al., 1991; Stich and Rosin, 1983; Cleaver, 1986). The damage may be produced by a direct hit on the DNA structure by the X-rays, leading to the extraction of an electron or hydrogen atom from the DNA and the creation of a carbon-centered free radical that reacts with the surroundings. Alternatively, the X-rays may induce DNA damage indirectly by generating hydroxyl radicals by ionizing cellular water (Hill, 1987). The latter reaction would probably lead to DNA changes similar to those observed with hydrogen peroxide treatment: strand-breakage, oxidized bases, and DNA-protein crosslinks (Cleaver, 1986). Sonntag and co-workers (1981) have estimated that the latter mechanism was responsible for the bulk of the chromosomal aberrations, with 33% of strand breakages attributed to direct X-ray interaction with the DNA structures.

## 1.6 Cellular defence mechanisms against ROS damage

An array of cellular defences have evolved to prevent damage to the cell by ROS. These defences include antioxidant defences and DNA repair systems. When these defences are overwhelmed, a condition termed "oxidative stress" is induced and damage to the cell occurs (Sies, 1985).

Cellular anti-oxidant defences include enzymatic and non-enzymatic mechanisms. The three major enzymes responsible for preventing oxidative stress are superoxide dismutase, catalase, and glutathione peroxidase. Superoxide dismutase catalyses the reduction of superoxide anions to oxygen and hydrogen peroxide (Bannister et. al., 1987), whereas catalase and the glutathione redox cycle reduce hydrogen

peroxide to oxygen and water (Harris et al., 1992). Non-enzymatic scavengers of toxic oxidants are also of great importance in protecting against damage. Vitamin E, ascorbate, and beta-carotene are examples of such scavengers (Reilly et al., 1991). Another form of "scavenging" involves metal chelators. These chelators bind to the metal ions preventing them from participating in the acceleration of free radical reactions (Halliwell and Aruoma, 1992). These defences act together to diminish the production of ROS and to reduce the availability of ROS to interact with cellular components.

In the event of oxidative DNA damage, DNA repair enzymes are activated to correct the defects. There are multiple pathways of repair of oxidative DNA damage (Zhang et al., 1992). These processes are best understood in bacteria, although there is an increasing amount of data becoming available for humans. The latter information comes mainly from studies with DNA repair syndromes such as xeroderma pigmentosum, ataxia-telangiectasia, or Bloom's syndrome. Of particular interest are the ataxia-telangiectasia cell cultures which are sensitive to the chromosome-damaging action of X-rays and to oxidants such as hydrogen peroxide or ROS-generating mixtures like xanthine and xanthine oxidase (Ward et al., 1994). The AT locus is at 11q22-23 (Gatti, 1993). Although the molecular mechanism underlying the AT defect is unresolved, data suggest that the defect lies in the processing of DNA damage by these cells (Thacker, 1989; Powell et al., 1993; Ganesh et al., 1993).

## 1.7 Host factors controlling susceptibility to reactive oxygen species: the involvement of chromosome 11

We have been focusing our attention on the role of loci on chromosome 11 in protecting cells against chromosomal damage by ROS. Our choice of this chromosome for study was initially based on the aforementioned observations made on ataxia-telangiectasia cultures. Our interest in this chromosome was further increased by the work of Sanford and colleagues on tumour cells (Parshad et al., 1992). These

workers reported an abnormally high frequency of chromatid breaks and gaps when human tumours are X-irradiated during the $G_2$ phase of the cell cycle. This effect was seen in tumours of different tissue origin and/or histopathology. Insertion of normal chromosome 11 into these tumour cell lines resulted in a reduction of radiation-induced damage to the level observed in normal cells. These workers hypothesized that this effect was linked to the presence of a defective DNA repair process and suggested that this defect occurs in many tumours as a critical early event in tumourigenesis. This event would increase the rate at which genetic changes accumulate in the tissue.

In order to further test this hypothesis a model system has been set up in the laboratory. This model includes a bladder tumour cell line, A1698 (termed "parent"), and its microcell hybrid (termed "hybrid") containing a der11 chromosome insert. As mentioned in the overview, the hybrid cell line has been shown to be protected against micronuclei induction (an index of chromosomal breakage) by hydrogen peroxide ($H_2O_2$), xanthine/xanthine oxidase (X/XO), and X-irradiation. Micronucleus frequencies after any of these treatments were significantly lower in hybrid cultures after treatment than in parent (Ward et al., 1993; Hofseth, unpublished data). The mechanism underlying this protection is unknown. Two possibilities are currently being explored by colleagues in this laboratory: 1) a restoration of normal repair process and 2) altered antioxidant status.

This bladder model is an appropriate system in which to begin to study numerical chromosomal alterations resulting from oxidative stress, and their significance to tumourigenesis. Numerical chromosomal alteration are common events in bladder tumourigenesis (Sandberg, 1993; Waldman et al., 1991; Poddighe et al., 1991). Moreover, alterations to chromosome 11 occur in 60-70% of bladder tumours and seem to occur as an early event (Hopman et al., 1991). It is possible to hypothesize that this alteration could be driving genetic instability in bladder tissues and increasing the rate at which chromosomal change occurs. Although micronuclei induction in response to oxidative stress

has been looked at in our bladder model, there is no data on the response of these cells with respect to numerical chromosomal aberrations. This aspect is the major focus of research in this thesis.

## 2 EXPERIMENTAL APPROACH

### 2.1 Hypotheses

1) The insertion of a normal chromosome 11 will not affect the spontaneous rate of numerical chromosomal alteration in the bladder carcinoma cell lines.

2) Chromosome 11 insertion will protect cells against numerical chromosomal alteration induced by hydrogen peroxide and X-rays.

3) Protection by chromosome 11 against numerical chromosomal aberrations will be less for X-rays than for hydrogen peroxide, as only a portion of damage from X-irradiation is due to reactive oxygen species.

### 2.2 Approach

#### 2.2.1 Cell cultures

This study used two bladder cell lines: 1) a bladder-carcinoma cell line A1698 termed "parent", and 2) its microcell hybrid clone, A1698 + der(11) clone 1, termed "hybrid". The hybrid contains a chromosome insert that contains most of chromosome 11 (loci proximal to q23) (Ning et al., 1991).

The parent and hybrid cells were exposed to hydrogen peroxide and X-rays as described in Materials and Procedures. After treatment, FISH was used to quantitate the number of chromosome 9 centromeric regions (hybridization signals) present in cells in the treated cultures.

Chromosome number changes were assessed throughout a range of doses and a comparison made of the responses of the parent and hybrid cell cultures.

### 2.2.2 Fluoresence *in situ* hybridization

The chromosome 9 probe was chosen because its probe signals are large and are easily distinguishable from background staining. This probe hybridizes to the short repeats related to AATGG in "classical" satellites found in the pericentric heterochromatin defined by the centromere and q12 on chromosome 9. Chromosome 9 is one of the commonly altered chromosomes in bladder carcinomas (Hopman et al., 1991; Tsai et al., 1990). In this project, we quantified numerical aberrations induced by hydrogen peroxide and X-rays as the loss and gain of chromosome 9 copies.

### 2.2.3 Genotoxic agents

Hydrogen peroxide and X-rays were previously shown to induce micronuclei in both the parent and hybrid cell lines. Although a portion of the DNA damage induced in cells by X-irradiation is due to reactive oxygen species, other types of damage appear to result from direct action of X-rays on the DNA. Preliminary data from this laboratory suggest that a larger protective effect is observed in hybrid cultures against micronuclei induction by hydrogen peroxide treatment than by X-irradiation (Hofseth, unpublished data). This is consistent with the hypothesis that the protection conferred by an extra chromosome 11 is specific to damage caused by oxidative species.

## 3 MATERIAL AND PROCEDURES

### 3.1 Cell lines and media

The bladder carcinoma cell line (A1698/Parent) and its microcell hybrid (A1698 + der(11) clone 1/Hybrid) were received as a gift from Dr. O. Pereira-Smith, Baylor College of Medicine, Houston, Texas. The parent cell line has a near-triploid chromosome complement with 72-76 chromosomes per cell. Results using karyotype analysis on metaphases and FISH analysis of interphase cells suggest that the parent and the hybrid cell line each contains 4 copies of chromosome 9. However, the parent has 3 copies of chromosome 11, while the hybrid has 4 copies. The hybrid has inserted into it an intact der(11) from a normal donor containing a balanced X;11 translocation [der(11)t(x;11)(q25;q23)]. The der(11) consists of a major part of chromosome 11 and a minor portion of distal Xq with a HPRT locus attachment (Scott et. al., 1979; Ning et. al., 1991).

This study also used a normal fibroblast cell line (GM 5757) as an internal control for the FISH procedure. This cell line was obtained from the National Institute of General Medical Sciences, Human Genetic Cell Repository (NIGMS, Camden, NJ).

The cell lines were cultured in Dulbecco's modified Eagle's media (D-MEM, Flow Laboratories, McLean, VA) supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco Laboratories, Grand Island, NY), streptomycin (100 μg/ml) and penicillin (100 units/ml). The hybrid cell line was further supplemented with HAT (hypoxanthine 10 x $10^{-4}$ M, aminopterin 5 x $10^{-7}$ M, and thymidine 10 x $10^{-5}$ M) as a selective pressure to ensure that the hybrid culture maintained the der(11) chromosome. The cells were propagated in 75 $cm^2$ culture flask in a humidified water jacket incubator at 37°C with 5% $CO_2$ and 95% air.

### 3.2 Hydrogen peroxide treatment

The cells were seeded onto 22 x 22 mm glass coverslips in 10 x 35 mm tissue culture dishes and allowed to grow for 24 hours. The cell density at seeding was 1.5 x $10^5$ cells per dish. Prior to beginning the treatment, the medium was removed from the dishes by suction and the cells were washed once with phosphate-buffered saline (PBS, constituents per litre: 8 g sodium chloride, 1.15 g anhydrous dibasic sodium phosphate, 0.2 g potassium chloride and 0.2 g anhydrous monobasic potassium phosphate, pH 7.0). Cultures were exposed for 1 hour at 37°C to hydrogen peroxide prepared in PBS. A PBS control was used in each experiment consisting of a coverslip culture that was washed with PBS and left in the buffered salt solution for 1 hour. As an additional control, one sample was left undisturbed in the DME medium. Following exposure, the hydrogen peroxide solution was suctioned off, the coverslips were rinsed once with PBS and then with wash DME (no serum). Fresh DME with 10% serum was added to the dishes and then they were returned to the incubator for another 24 hours before harvesting.

### 3.3 X-ray treatment

The cells were seeded onto 75 x 25 mm glass slides placed in 90 mm square tissue culture dishes with 25 ml of DME with 10% serum each. The seeding density was 3.0 x $10^6$ cell per dish. After 24 hours, the cells were exposed to doses (1-4 Gy) of 250 kV (0.5 mm Cu) X-rays at the rate of 2.222 Gy/min. Controls were left undisturbed. Following irradiation, the dishes were returned to the incubator for another 24 hours at 37°C before harvesting.

### 3.4 Harvesting and analysing cells

Cells were harvested by dipping the coverslips into PBS for 5 seconds and air-drying. The coverslips were fixed in Carnoy's solution (3:1 methanol:glacial acetic acid) for 20 minutes and air-dried. They were

then placed into plastic bags, nitrogen gas was passed over them to remove air, and the bags were sealed. The bags were stored in a freezer at -20°C.

### 3.4.1 Micronucleus assay

Following exposure, cytochalasin B (final concentration of 2 µg/ml) was added to the cultures and incubated until harvest 24 hours later. The cells were harvested, fixed and stored as described above.

Nuclei were visualized by staining with the Feulgen reaction: 10 seconds in room temperature 1N HCL; 10 minutes in 63°C 1N HCL; 10 seconds in room temperature 1N HCL; 15 seconds in double distilled water; 120 minutes in Schiff's reagent; 15 minutes in running lukewarm tap water. Counterstaining was provided with Fast Green (0.5% in 95% ethanol): 6-10 seconds in aged (3 months) fast green solution; 5 seconds in 70% ethanol; 5 seconds in 90% ethanol; 1 minute each in two washes of n-butanol; 1 minute each in two washes of n-butanol/xylene (1:1); 1 minute each in two washes of xylene. The dehydrated preparations were mounted in Permount.

Prior to scoring, the slides were randomised and coded. Scoring was done on a microscope with a10x ocular and a100x oil immersion objective lens. A minimum of 500 binucleated cells were scored for micronuclei on each slide. Nuclear division index (NDI) is used as a measurement of toxicity and/or inhibition of cell growth. NDI is defined as the number of binucleated cells with micronuclei divided by the total number of binucleated cells scored.

Micronuclei were observed as Feulgen stained domains within the cellular cytoplasm that were distinctly separated from the main nucleus. Other criteria included similar staining pattern and chromatin texture as the main nucleus (Rosin, 1992).

### 3.4.2 Fluorescence *in situ* hybridization (FISH)

The biotinylated human chromosome 9-specific pericentromeric probe (classical satellite D9Z1) was purchased from Oncor Inc., Gaithersburg, MD. FITC conjugated avidin and biotinylated goat anti-avidin antibody were supplied by Vector, Burlingame, CA. Propidium iodide, formamide, dextran sulfate, colchicine, and p-phenylenediamine dihydrochloride (anti-fade) were supplied by Sigma Co., St. Louis, MO.

The *in situ* hybridization procedure used in this study was a modification of that described by Eastmond and Pinkel (1990). The coverslip cultures to be probed were placed on a hot plate for 1 hour at 60°C to ensure that the cells adhered throughout this procedure. The coverslips were then placed into a denaturing solution of 70% formamide prepared in 2 x SSC (1 x SSC was made with 1.5 x $10^{-2}$ M sodium chloride and 1.5 x $10^{-2}$ M sodium citrate, pH 7]) at 75°C for 2 minutes. Care was taken to maintain the temperature. Immediately after this treatment, the slides were taken through a series of ice-cold alcohol washes of 70%, 85% and 100% (2 minutes each) and were air-dried. To aid probe penetration, cells were then warmed to 37°C and digested with pepsin (Sigma Co., St. Louis, MO., prepared at 200 µg/ml in 0.2 N HCL) for 5 minutes at 37°C.

The slides were again taken through the series of ice-cold alcohols, air-dried and warmed to 37°C. Meanwhile, the probe mixture was prepared as follows (volumes shown are amounts required for each slide): 7.0 µl MasterMix 2.1 solution (55% formamide/1 x SSC/10% dextran sulfate), 0.5 µl carrier DNA (1 mg/ml Herring sperm DNA), 2.0 µl double-distilled water, and 0.5 µl probe. This probe mixture was heated to 75°C for 5 minutes and immediately chilled on ice. 10 µl of the probe mixture was placed onto a fresh slide and the coverslip was inverted (cells facing downward) onto this solution. The edges of the coverslip were sealed with rubber cement to prevent evaporation of the hybridization mixture. The

slides were incubated overnight (16 hours) in a humidified chamber at 37°C.

Following incubation, the rubber cement was removed with forceps and the coverslips were left submerged in a sufficient volume of 50% formamide/2 x SSC at 45°C for 10 minutes. The wash was repeated two more times with fresh solution each time and the fourth wash was carried out similarly in 2 x SSC. The coverslips were then transferred into 2 x SSC at room temperature for 10 minutes before placement of a preblock solution (4 x SSC/0.1% BSA, room temperature) on the sample for 5 minutes. Excess preblock was drained off the sample and an aliquot of FITC-avidin solution (0.25% FITC-avidin in 4 x SSC/0.1% BSA) was added to each slide, parafilm was placed over the solution to spread it evenly, and the sample was incubated at room temperature for 30 minutes in the dark. The coverslip was washed 3 times, once in 4 x SSC, then 4 x SSC/0.1% Triton X-100 (t-Octylphenoxypolyethanol), and finally in 4 x SSC at room temperature, with each wash being 10 minutes in duration. To amplify the hybridization signal, pre-blocking solution (4 x SSC/0.1% BSA) was added again to the sample for 5 minutes followed by 0.1% biotinylated goat anti-avidin prepared in PN milk buffer (5% Carnation Milk/0.1% sodium azide prepared in PN buffer). PN buffer was prepared by mixing 0.1 M sodium phosphate dibasic heptahydrate/0.1% Nonipet-40 (nonionic detergent) with 0.1M sodium phosphate monobasic hydrate/0.1% Nonipet-40 at a ratio of 1 : 0.05 and adjusting the pH to 8. Parafilm was placed over the solution and the sample was incubated at room temperature for 30 minutes in the dark. The washing was repeated with a fresh change of solutions (4 x SSC, 4 x SSC/0.1% Triton X-100, and 4 x SSC) at 10 minutes each. FITC-avidin was introduced again (0.25% FITC-avidin in 4 x SSC/0.1% BSA) for 30 minutes. The coverslips were taken through the series of 3 washes a final time, rinsed in double-distilled water and air-dried. 9.0 ml of propidium iodide prepared in Antifade (Sigma) was dropped onto a fresh slide and the coverslip culture was inverted on this drop and sealed with rubber cement. The final slides were either analysed immediately or stored at 4°C in the dark (Figure 3).

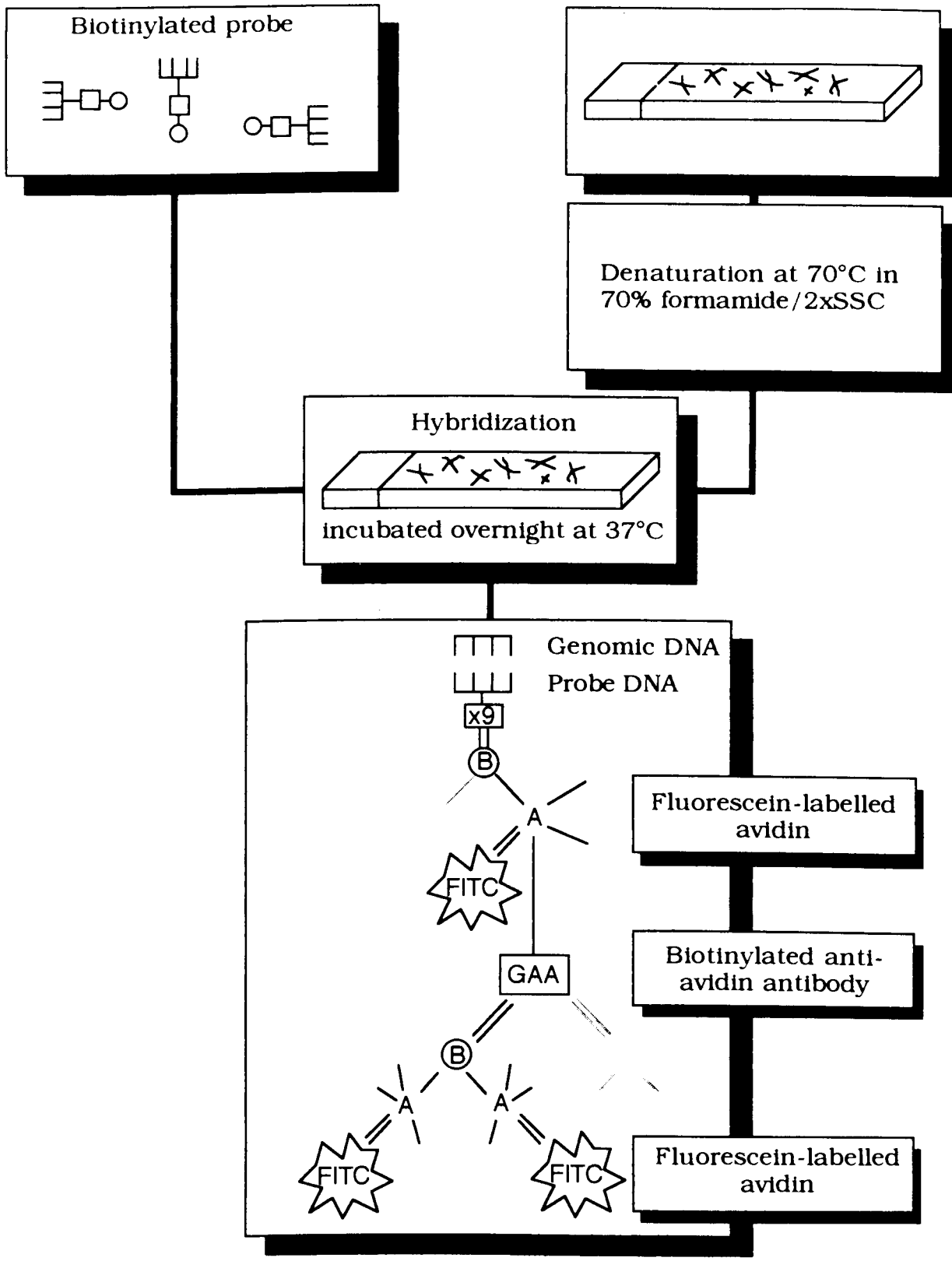



Figure 3: Schematic representation of fluorescence *in situ* hybridization (FISH) technique (Adapted from Zhang, 1993)

Scoring was done using an Olympus microscope equipped with epifluorescent illumination, under a 10x ocular and a 100x oil immersion objective lens with an Interference Blue filter (excitation at 450-490 nm, dichroic at 510 nm, emission at 500 nm). The hybridization domains appeared green-yellow against the red nucleus (appendix 1). The slides were randomized and coded prior to scoring. For each slide, at least 1000 cells were scored and the number of hybridization signals present in the nuclei, eg: 1, 2, 3, 4, 5 and >5 were recorded (appendix 2).

The scoring criteria used in this study were published by Zhang et al. (1993). They included the following: 1) only intact and isolated nuclei were scored, 2) hybridization domains had to appear bright and have similar intensity, 3) spots were examined by changing the focus and two overlapping spots or split spots were scored as one if they did not separate with this change, and 4) all spots within a cell had to have the same size and staining intensity to be scored as separate spots.

### 3.5 Statistical analysis

Most experiments were conducted three times. Spontaneous chromosome loss or gain of chromosomes was compared in parent and hybrid lines and the effect of solvent on these frequencies was examined, looking at the proportion of cells in three categories (2 or less hybridization domains, 3 and 4 hybridization domains, and 5 or more hybridization domains) using the Chi-square test. The effect of treatment with either X-rays or hydrogen peroxide on chromosome loss or gain was analyzed using the analysis of covariance (Ancova) with the cell line as a categoric variable and the dose as a continuous variable. The micronuclei data was analysed using the analysis of variance (Anova). Raw count data was used for all analysis. The P value chosen for significance in these studies was 0.05.

# 4 RESULTS

## 4.1 Validation of technique

A series of preliminary experiments was done to standardize the use of the FISH technique on coverslip cultures prior to beginning the central studies in this thesis.

### 4.1.1 Probe penetration

One of our concerns with respect to this procedure was to ensure adequate and reproducible probe penetration into the cells. In initial studies, when the FISH procedure was performed directly on coverslip cultures, a significant portion of the cells (20 - 50%) showed no hybridization signals. Pepsin digestion was deemed necessary for probe penetration. In order to determine an appropriate digestion time, replicate coverslips were exposed to pepsin (200 μg/ml in 0.2 N HCL) for 5, 10 or 20 minutes and cells were analyzed for the number of hybridization signals. As shown in Table 2, after pepsin digestion all cells had at least one hybridization signal. There were significant differences in the hybridization patterns between the three digestion times ($p \leq 0.05$), but there was no consistent trends which would suggest the superiority of any one time. A 5-minute treatment was chosen for subsequent experiments.

### 4.1.2 Scoring validation

A second concern was whether the scoring criteria used in this laboratory was similar to that in other laboratories. A normal diploid fibroblast culture (GM 5757) was grown, harvested, pretreated with pepsin for 5 minutes, and then hybridized with the chromosome 9 probe. Cells were assayed for numbers of hybridization domains. As shown in Table 3, the distribution of domains was very similar for the fibroblast culture and for 2 other cell lines that have been studied in other laboratories. This confirms that the FISH technique used in our

**Table 2: Hybridization of the chromosome 9 probe to cultures pretreated with pepsin (200 μg/ml) for indicated times**

| Cell line | Disgestion time[b] (minutes) | Number of hybridization domains[a] | | | | |
|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | ≥5 |
| Parent | 5 | 3.7 | 35.3 | 328.4 | 607.6 | 25.1 |
| | 10 | 2.8 | 37.6 | 337.9 | 613.6 | 8.2 |
| | 20 | 1.6 | 34.5 | 311.7 | 643.2 | 9.0 |
| Hybrid | 5 | 9.3 | 55.8 | 353.5 | 576.7 | 4.7 |
| | 10 | 4.0 | 101.3 | 364.2 | 488.5 | 5.7 |
| | 20 | 4.4 | 37.3 | 306.1 | 645.7 | 6.6 |

[a] Number of cells per 1000 showing the indicated number of hybridization spots.
[b] There is a significant difference between the hybridization patterns of all the digestion times when parent ($p=0.021$) and hybrid ($p\leq0.001$) lines are examined separately.

**Table 3: Comparison of hybridization results with chromosome 9 in studies of diploid cell lines**

| Cell line | Number of hybridization domains[a] | | | | | |
|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | >5 |
| GM 5757 | 0.0 | 95.9 | 885.8 | 10.8 | 7.5 | 0 |
| Lymphocytes[b] | 1.5 | 91.9 | 903.5 | 2.5 | 1.5 | ND[d] |
| HL60[c] | 0.2 | 99.2 | 893.4 | 6.3 | 0.9 | 0 |

[a] Number of cells per 1000 showing the indicated number of hybridization domains

[b] Eastmond and Pinkel, 1990

[c] Zhang et al., 1993

[d] ND, not done

laboratory gives results comparable to those of other laboratories.

A well known phenomenon in the FISH technique is signal overlap. In diploid cell cultures, signal overlap results in an elevated number of cells with one rather than the expected two hybridization spots. The actual rate of monosomy in diploid cells as determined in metaphase spreads is less than 1%. FISH analysis of interphase cells shows 9-10% of these cells with only one signal. (Table 3).

### 4.1.3 Sensitivity of assay

A third concern was whether or not the FISH technique performed in our laboratory would be sensitive enough to pick up alterations in chromosome number in treated cells. Colchicine is known to interact with microtubule proteins and to disrupt spindle fibers during mitosis. This disruption prevents chromosomes with damaged spindles from migrating to the spindle poles and leads to daughter cells that have either lost or gained chromosomes. Therefore, we examined the effect of colchicine on bladder carcinoma cells by using the FISH technique to assay for numerical chromosomal changes. The parent and hybrid cells were grown for 24 hours, exposed to colchicine (0.5 μM) for 1 hour, washed, fresh medium added, and incubated for 24 hours prior to harvest.

Table 4 shows the number of hybridization regions per 1000 cells for untreated and colchicine-exposed cultures. Replicate data from two such experiments involving the parent culture are shown in Figure 4.

Without any treatment, about 60% of the interphase cells in both cell lines had 4 hybridization spots and about 30% had 3 spots. Since karyotype analysis of these cultures has shown that 4 copies of chromosome 9 are present in both the parent and hybrid cultures, the large number of cells showing 3 hybridization spots is probably due to overlap of signals rather than to aneuploidy.

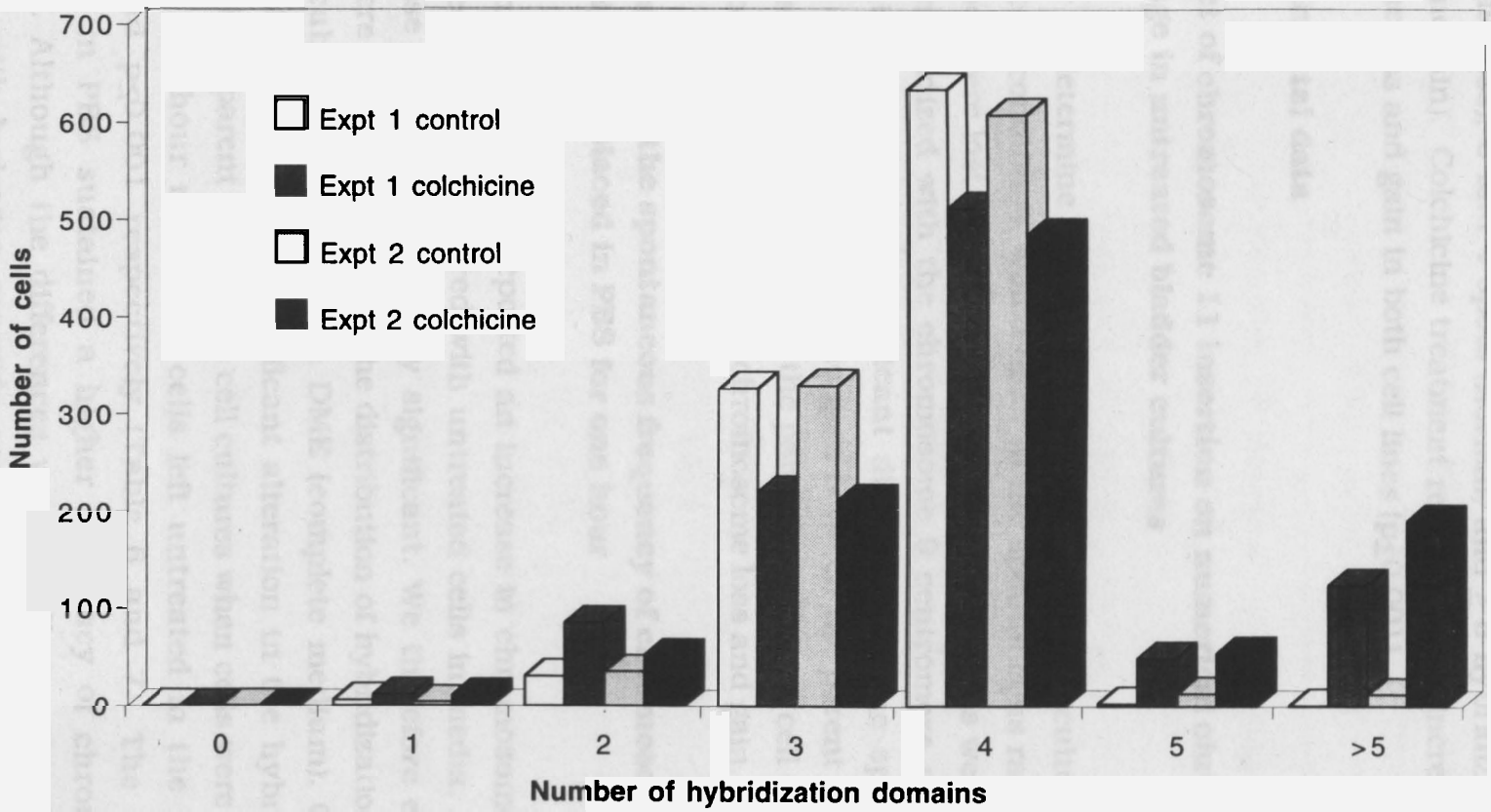
**Table 4: Alteration in chromosome number in cell cultures treated with colchicine (0.5 μM)**

| Cell line | Number of expt | Treatment[b] | Number of hybridization domains[a] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | 4 | 5 | >5 |
| Parent | 1 | Control | 0.0 | 5.9 | 30.5 | 325.8 | 633.9 | 3.0 | 1.0 |
| | | Colchicine | 0.0 | 11.0 | 85.2 | 219.2 | 511.0 | 48.9 | 124.6 |
| | 2 | Control | 0.0 | 3.7 | 35.3 | 328.4 | 607.6 | 13.0 | 12.1 |
| | | Colchicine | 0.0 | 10.4 | 51.8 | 210.4 | 483.7 | 54.2 | 189.6 |
| Hybrid | 1 | Control | 0.0 | 1.8 | 32.6 | 311.0 | 650.1 | 1.8 | 2.7 |
| | | Colchicine | 0.0 | 12.3 | 61.6 | 232.4 | 593.3 | 32.6 | 67.8 |

[a] Number of cells per 1000 showing the indicated number of hybridization spots.

[b] There is a significant difference between the hybridization patterns of the treated and untreated cells in both parent ($p \leq 0.001$) and hybrid ($p \leq 0.001$)

**Figure 4: Positive controls using parent cells exposed to colchicine (0.5 μM) compared to DME controls.**



To look for the effect of treatment on chromosome number, an analysis was made of alterations in the proportion of cells with $\leq 2$ spots (chromosome loss), 3 and 4 spots (normal) and $\geq 5$ hybridization spots (chromosome gain). Colchicine treatment resulted in an increase in both chromosome loss and gain in both cell lines ($p \leq 0.001$).

## 4.2 Experimental data

### 4.2.1 Effect of chromosome 11 insertion on numerical chromosomal change in untreated bladder cultures

In order to determine whether the parent and hybrid cultures, under normal culture conditions, would differ in the spontaneous rate at which chromosomes were lost or gained, cells from both cultures were grown in DME and hybridized with the chromosome 9 centromere probe. The results (Table 5) show a significant difference in the spontaneous frequencies of chromosome loss and gain between the parent and hybrid cell cultures ($p \leq 0.001$). Relative to the parent, the hybrid cell culture has a higher spontaneous frequency of chromosome loss and gain.

### 4.2.2 Alteration of the spontaneous frequency of chromosome loss or gain in cells placed in PBS for one hour

Zhang et al. (1993) reported an increase in chromosome gain in cells exposed to PBS compared with untreated cells in media, although this increase was not statistically significant. We therefore examined whether there was a difference in the distribution of hybridization signals in cells incubated in PBS and in DME (complete medium). Our data suggested that there was a significant alteration in the hybridization patterns in both parent and hybrid cell cultures when cells were removed to PBS for one hour relative to cells left untreated in the medium, $p \leq 0.005$ and $p \leq 0.001$ respectively (Table 6 and 7). The cultures maintained in PBS sustained a higher frequency of chromosomal aberrations. Although the differences were small, in all subsequent experiments with hydrogen peroxide control cultures in PBS were used

Table 5: Spontaneous frequencies of chromosome loss or gain in cell lines maintained in DME[a]

| Cell lines | Number of expt | Number of hybridization domains | | | |
|---|---|---|---|---|---|
| | | Normal distribution | | Loss | Gain |
| | | 3 domains | 4 domains | ≤2 domains | ≥5 domains |
| Parent | 1 | 397.1 | 584.0 | 15.1 | 3.8 |
| | 2 | 343.8 | 604.3 | 43.0 | 9.0 |
| | 3 | 409.5 | 556.0 | 30.9 | 3.6 |
| | average | **383.5 (34.91)** | **581.4 (24.3)** | **29.7 (14.0)** | **5.5 (3.0)** |
| Hybrid | 1 | 324.4 | 603.5 | 53.0 | 19.3 |
| | 2 | 307.7 | 656.7 | 26.9 | 8.7 |
| | 3 | 352.1 | 609.9 | 27.3 | 10.7 |
| | average | **328.0 (22.4)** | **623.4 (29.1)** | **35.7 (14.9)** | **12.9 (5.6)** |

[a] Number of cells per 1000 (S.D.)
There is a significant difference between the hybridization patterns of parent and hybrid cell lines ($p \leq 0.001$)

**Table 6: Effect of PBS on the spontaneous frequencies of chromosome loss or gain in parent cell line[a]**

| Medium | Number of expt | Number of hybridization domains | | | |
|---|---|---|---|---|---|
| | | Normal distribution | | Loss | Gain |
| | | 3 domains | 4 domains | ≤2 domains | ≥5 domains |
| PBS | 1 | 348.4 | 608.0 | 34.7 | 8.9 |
| | 2 | 346.1 | 613.8 | 30.8 | 9.3 |
| | 3 | 343.9 | 603.2 | 40.9 | 12.1 |
| | **average** | **346.1 (2.3)** | **608.3 (5.3)** | **35.5 (5.1)** | **10.1 (1.7)** |
| DME | 1 | 397.1 | 584.0 | 15.1 | 3.8 |
| | 2 | 343.8 | 604.3 | 43.0 | 9.0 |
| | 3 | 409.5 | 556.0 | 30.9 | 3.6 |
| | **average** | **383.5 (34.9)** | **581.4 (24.3)** | **29.7 (14.0)** | **5.5 (3.0)** |

[a] Number of cells per 1000 (S.D.)
There is a significant difference between the hybridization patterns of PBS and DME in the parent cell line ($p \leq 0.005$)

Table 7: Effect of PBS on the spontaneous frequencies of chromosome loss or gain in hybrid cell line[a]

| Medium | Number of expt | Number of hybridization domains | | | |
|---|---|---|---|---|---|
| | | Normal distribution | | Loss | Gain |
| | | 3 domains | 4 domains | ≤2 domains | ≥5 domains |
| PBS | 1 | 337.0 | 599.5 | 51.8 | 11.8 |
| | 2 | 361.4 | 594.7 | 36.3 | 7.7 |
| | 3 | 352.3 | 581.1 | 61.8 | 4.8 |
| | average | **350.2 (12.3)** | **591.7 (9.5)** | **44.0 (12.8)** | **8.1 (3.5)** |
| DME | 1 | 324.4 | 603.5 | 52.9 | 19.3 |
| | 2 | 307.7 | 656.7 | 26.9 | 8.7 |
| | 3 | 352.1 | 609.9 | 27.3 | 10.7 |
| | average | **328.0 (22.4)** | **623.4 (29.1)** | **35.7 (14.9)** | **12.9 (5.6)** |

[a] Number of cells per 1000 (S.D.)
There is a significant difference between the hybridization patterns of PBS and DME in the hybrid cell line ($p \leq 0.001$)

to minimize confounding effects of antioxidants such as glutathione and ascorbate in the serum and complete medium.

### 4.2.3 Effect of hydrogen peroxide treatment on numerical chromosomal aberrations

Parent and hybrid cultures were exposed to a range of hydrogen peroxide treatments known to induce micronuclei (Hofseth, unpublished data) and then were harvested and assayed for numerical chromosomal changes. The frequency of chromosome loss and chromosome gain in the treated cultures is shown in Tables 8 and 9 respectively. The parent cell culture had a higher rate of chromosome loss per unit change in dose than the hybrid but this difference was insignificant ($p \geq 0.05$). The peroxide treatment induced significant chromosome losses, considering both cultures together ($p \leq 0.01$). Without treatment, the frequency of chromosome loss was higher for hybrid than for parent but this difference was insignificant ($p \geq 0.05$). With regards to the gain of chromosomes, the parent cell culture had a higher rate of chromosome gain per unit change in dose than the hybrid. However, the difference was insignificant ($p \geq 0.05$). Hydrogen peroxide did induce significant chromosome gains considering both cell cultures together ($p \leq 0.05$). Without treatment the parent had a higher frequency of chromosome gain than hybrid but this was insignificant ($p \geq 0.05$).

### 4.2.4 Effect of X-ray treatment on numerical chromosomal aberrations

In a series of micronuclei experiments, the hybrid showed a lower level of chromosomal damage than the parent culture when exposed to X-rays (Hofseth, unpublished data). Using the same dose range and similar protocol, both cell cultures were exposed to X-rays and assayed for numerical chromosomal aberrations. Tables 10 and 11 display the frequency of chromosomal loss and gain respectively. Although the rate of chromosome loss per unit change in dose was higher for the hybrid than the parent cell culture, this difference was insignificant ($p \geq 0.05$).

**Table 8: Chromosome loss[a] in parent and hybrid cell lines exposed to hydrogen peroxide.**

| Cell lines[b] | Number of expt. | $H_2O_2$ concentration[c] (μM) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0.0[d] | 20.6 | 23.5 | 26.5 | 29.4 | 32.3 |
| Parent | 1 | 34.6 | 35.6 | 57.0 | 36.0 | U.S.[e] | 79.9 |
| | 2 | 30.8 | 54.4 | 49.0 | 75.5 | 67.3 | 76.4 |
| | 3 | 40.9 | 41.0 | 73.7 | 110.7 | 72.2 | 56.2 |
| | average | **35.5 (5.1)** | **43.7 (9.7)** | **59.9 (12.6)** | **74.1 (37.4)** | **69.7 (3.5)** | **70.8 (12.8)** |
| Hybrid | 1 | 51.8 | 75.3 | 50.7 | 75.3 | 58.9 | 98.7 |
| | 2 | 36.3 | 43.6 | 34.3 | 73.9 | 61.2 | 56.2 |
| | 3 | 61.8 | 38.1 | 60.6 | 38.2 | 63.0 | 43.0 |
| | average | **44.0 (12.8)** | **52.3 (20.1)** | **48.5 (13.3)** | **62.5 (21.0)** | **61.1 (2.1)** | **66.0 (29.1)** |

[a] Number of cells per 1000 with 1 or 2 hybridization domains (S.D.)

[b] The parent cell culture had a higher rate of chromosome loss per unit change in dose than the hybrid but it was insignificant, p=0.17

[c] Hydrogen peroxide induced significant chromosome loss when considering parent and hybrid together, p=0.005

[d] Without treatment, the frequency of chromosome loss in parent and hybrid were not significantly different, p=0.27

[e] Unscorable due to technical difficulties

**Table 9: Chromosome gain[a] in parent and hybrid cell lines exposed to hydrogen peroxide**

| Cell lines[b] | Number of expt. | $H_2O_2$ concentration[c] (μM) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0.0[d] | 20.6 | 23.5 | 26.5 | 29.4 | 32.3 |
| Parent | 1 | 8.9 | 18.23 | 22.47 | 27.00 | U.S.[e] | 23.49 |
| | 2 | 9.3 | 15.00 | 15.33 | 19.69 | 14.75 | 9.89 |
| | 3 | 12.1 | 11.97 | 19.40 | 14.52 | 20.89 | 17.02 |
| | **average** | **10.1 (1.7)** | **15.06 (3.13)** | **19.07 (3.58)** | **20.40 (6.27)** | **17.82 (4.35)** | **16.80 (6.80)** |
| Hybrid | 1 | 11.8 | 11.89 | 5.63 | 5.31 | 20.89 | 11.85 |
| | 2 | 7.6 | 5.81 | 2.94 | 8.87 | 9.56 | 17.16 |
| | 3 | 4.8 | 7.09 | 12.31 | 6.69 | 4.85 | 7.65 |
| | **average** | **8.1 (3.5)** | **8.27 (3.21)** | **6.96 (4.82)** | **6.96 (1.79)** | **11.77 (8.25)** | **12.22 (4.77)** |

[a] Number of cells per 1000 with 5 or >5 hybridization domains (S.D.)

[b] The parent cell culture had a higher rate of chromosome gain per unit change in dose than the hybrid but this difference was insignificant, p=0.24

[c] Hydrogen peroxide induced significant chromosome gain when considering parent and hybrid together, p=0.02

[d] Without treatment, the frequency of chromosome gain in parent and hybrid were not significantly different, p=0.30

[e] Unscorable due to technical difficulties

**Table 10: Chromosome loss[a] in parent and hybrid cell lines exposed to x-ray.**

| Cell lines[b] | Number of expt. | x-ray doses[c] (Gy) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 0.00[d] | 0.00[d] | 0.125 | 0.25 | 0.50 | 1.00 | 2.00 |
| Parent | 1 | 63.1 | 54.8 | 55.0 | 89.8 | 72.3 | 66.2 | 55.9 |
| | 2 | 68.6 | 61.8 | 57.9 | 67.8 | 77.4 | 52.6 | 77.3 |
| | 3 | 59.1 | 59.5 | 50.5 | 47.0 | 52.5 | 52.1 | 49.9 |
| | average | **63.6 (4.8)** | **58.7 (3.6)** | **54.4 (3.7)** | **68.2 (21.4)** | **67.4 (13.1)** | **57.0 (8.0)** | **61.0 (14.4)** |
| Hybrid | 1 | 77.1 | 63.1 | 52.4 | 51.7 | 60.4 | 51.0 | 39.0 |
| | 2 | 65.5 | 77.1 | 128.9 | 115.4 | 82.9 | 88.9 | 90.3 |
| | 3 | 57.9 | 85.6 | 92.6 | 79.8 | 60.4 | 77.7 | 60.8 |
| | average | **66.9 (9.7)** | **75.3 (11.4)** | **91.3 (38.3)** | **82.3 (31.9)** | **67.9 (13.0)** | **72.5 (19.5)** | **63.3 (25.7)** |

[a] Number of cells per 1000 with 1 or 2 hybridization domains (SD)

[b] The hybrid cell culture had a higher rate of chromosome loss per unit change in dose than the parent but this difference was insignificant, p=0.44

[c] X-rays did not induce significant chromosome loss when considering parent and hybrid together, p=0.34

[d] Without treatment, the hybrid had a significantly higher frequency of chromosome loss than parent, p=0.03

**Table 11: Chromosome gain[a] in hybrid and parent cell lines exposed to x-ray.**

| Cell lines[b] | Number of expt | x-ray doses[c] (Gy) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 0.00[d] | 0.00[d] | 0.125 | 0.25 | 0.50 | 1.00 | 2.00 |
| Parent | 1 | 9.7 | 13.0 | 3.9 | 3.9 | 12.2 | 12.1 | 12.3 |
| | 2 | 7.8 | 6.8 | 5.8 | 0.0 | 7.8 | 9.6 | 15.5 |
| | 3 | 7.9 | 2.0 | 2.7 | 4.0 | 4.5 | 2.6 | 2.9 |
| | average | 8.5 (1.1) | 7.2 (5.5) | 4.1 (1.6) | 2.6 (2.3) | 8.2 (3.9) | 8.1 (5.0) | 10.2 (6.5) |
| Hybrid | 1 | 10.5 | 7.6 | 10.7 | 11.7 | 12.1 | 9.1 | 13.7 |
| | 2 | 16.9 | 8.8 | 9.7 | 13.6 | 8.9 | 19.5 | 10.9 |
| | 3 | 6.8 | 14.8 | 7.1 | 12.2 | 7.0 | 8.7 | 8.7 |
| | average | 11.4 (5.1) | 10.4 (3.9) | 9.2 (1.8) | 12.5 (1.0) | 9.3 (2.6) | 12.5 (6.1) | 11.1 (2.5) |

[a] Number of cells per 1000 with 5 or more hybridization domains (SD).

[b] The hybrid cell culture had a higher rate of chromosome gain per unit change in dose than the parent but this difference was insignificant, p=0.31

[c] X-rays did not induce significant chromosome gain when considering parent and hybrid together, p=0.14

[d] Without treatment, the hybrid had a significantly higher frequency of chromosome gain than parent, p=0.003

Our analysis showed that X-rays did not induce significant chromosome losses considering both cell cultures together ($p \geq 0.05$). Without treatment, the frequency of chromosome loss in both cell cultures was significantly different, with the hybrid having a higher frequency than the parent ($p \leq 0.05$). As for chromosome gain, the parent had a greater rate of gain per unit change in dose than the hybrid but this difference was insignificant ($p \geq 0.05$). X-rays did not induce significant chromosome gain considering both cell cultures together ($p \geq 0.05$). Without treatment, the frequency of chromosome gain in both cell cultures was significantly different ($p \leq 0.01$), with the hybrid having a higher frequency than the parent.

### 4.2.5 Effect of higher X-ray doses and extended incubation time prior to harvest on numerical chromosomal aberrations

When the parent and hybrid were irradiated, neither cultures yielded numerical chromosome aberrations at X-ray doses up to 2 Gy, even though identical treatments induced large numbers of micronuclei (Hofseth, unpublished data). To explore whether higher X-ray doses and/or longer incubation periods could induce chromosomal number changes, further experiments were conducted with the parent cell culture. The cells were exposed to X-rays from 0-4 Gy, harvested at 24, 36, and 48 hours following irradiation, and assayed for chromosome loss and gain with the FISH technique. As a check for X-ray induced damage, a set of dishes were designated as micronuclei controls. Some of these dishes were irradiated at 2 Gy and others left untreated. All micronuclei controls were treated with cytochalasin B and incubated until harvest at 24, 36, and 48 hours. The cells were then assayed for micronuclei as described in Materials and Procedures.

Table 12 shows that X-rays induced a significant increase in micronuclei levels ($p \leq 0.05$). Prolonging harvest time did not cause a significant impact on the percentage of cytokinesis-blocked cells with micronuclei ($p \geq 0.05$). The nuclear division indices (NDI) for 0 and 2 Gy at 24 hour were significantly different ($p \leq 0.001$) with treated cells

**Table 12: Micronuclei induction in the parent cell line exposed to X-rays.**

| Harvest time[b] | X-ray dose[a] (Gy) | % cytokinesis-blocked cells with MN | Nuclear Division Index[c] |
|---|---|---|---|
| **24 hours** | 0 | 4.18% | 51.72% |
| | 0 | 2.68% | 51.94% |
| | 2 | 19.31% | 23.90% |
| | 2 | 24.53% | 28.60% |
| **36 hours** | 0 | 3.81% | 60.48% |
| | 2 | 35.78% | 68.92% |
| **48 hours** | 0 | 3.33% | 63.08% |
| | 2 | 33.38% | 57.05% |

[a] X-rays induced significant increase in micronuclei when all the harvest times were considered, $p=0.02$

[b] Extended harvest time did not cause a significant difference in the percentage of cytokinesis-blocked cells with micronuclei, $p=0.48$

[c] The nuclear division index for 24 hours was significantly different for 0 Gy and 2 Gy, $p \leq 0.001$

showing a reduction in nuclear division index. Table 13 shows the frequencies of chromosome loss and gain assayed by the FISH technique. The rate of chromosome loss per unit change in dose was increased insignificantly as harvest times were prolonged ($p \geq 0.05$). Our analysis showed that X-rays did not induce significant chromosome loss considering all harvest times together ($p \geq 0.05$). Without treatment, the frequencies of chromosome loss for all the harvest times were not significantly different ($p \geq 0.05$). As for the addition of chromosomes, X-rays induced significant chromosome gain for each of the harvest times, with 36 hours showing the highest rate of gain and 24 hours the lowest ($p \leq 0.05$). Without treatment, the frequencies of chromosome gain for all the harvest times was not different ($p \geq 0.05$).

**Table 13: Induction of numerical chromosomal aberrations in parent cell line with higher x-ray doses and extended incubation time prior to harvest.**

| Harvest Time | Chromosomal alterations | x-ray doses (Gy) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0.0 | 0.0 | 0.0 | 0.0 | 1.0 | 2.0 | 3.0 | 4.0 |
| 24 hr | Loss[a] | 64.5 | 64.1 | 67.7 | 34.09 | 44.3 | 45.0 | 48.7 | 70.6 |
| | Gain[b] | 4.7 | 6.5 | 3.7 | 4.73 | 19.5 | 15.3 | 15.6 | 8.3 |
| 36 hr | Loss[a] | 55.1 | 47.6 | 68.0 | 44.8 | 50.0 | 27.1 | 45.9 | 41.6 |
| | Gain[b] | 2.9 | 0.9 | 3.9 | 10.7 | 11.1 | 10.8 | 33.9 | 29.3 |
| 48 hr | Loss[a] | 46.0 | 63.8 | 81.8 | 85.9 | 49.0 | 79.1 | 79.3 | 75.3 |
| | Gain[b] | 1.8 | 0.0 | 1.9 | 5.7 | 6.7 | 19.0 | 21.5 | 31.2 |

[a] Number of cells per 1000 with 2 or less hybridization domains.
The rate of chromosome loss per unit change in dose increased for each harvest time but this increase was insignificant, p=0.32.
X-rays did not induce significant chromosome loss when considering all the harvest times together, p=0.995
Without treatment, the frequencies of chromosome loss for all harvest times were not significantly different, p=0.23

[b] Number of cells per 1000 with 5 or more hybridization domains.
The rate of chromosome gain per unit change in dose was significantly different for each harvest time, p=0.04.
X-rays did induce significant chromosome gain when considering all the harvest times together, p=0.0001
Without treatment, the frequencies of chromosome gain for all harvest times were not significantly different, p=0.46

## 5 Discussion

Several studies have demonstrated that the insertion of a normal chromosome 11 into a tumour cell line offers protection against structural chromosomal aberrations. The parent cells (bladder carcinoma cells) consistently yielded more micronuclei than the hybrid cells (with chromosome 11 insert) when treated with hydrogen peroxide, xanthine/xanthine oxidase and X-rays (Parshad et al., 1992; Ward et al., 1993; Hofseth, unpublished data).

In this project, cells were treated with oxidative agents and assayed for numerical chromosomal aberrations. Numerical chromosomal changes result from a loss and/or gain of whole chromosomes. Hydrogen peroxide induced significant chromosome loss ($p \leq 0.01$) and gain ($p \leq 0.05$) in the parent and hybrid cell cultures. However, the response of parent and hybrid cell cultures to the treatment was not significantly different for either chromosome loss or gain ($p \geq 0.05$). Though insignificant, it was noteworthy that the parent had a higher rate of chromosome loss and gain per unit change in dose than the hybrid cell culture (with chromosome 11 insert). This observation was consistent with the demonstrated protective effect of chromosome 11 insert in other studies (Ward et al., 1993; Parshad et al., 1992; Hofseth, unpublished data). One plausible explanation for the reduced sensitivity to oxidative stress in the hybrid cell culture, is the restoration of a defective repair process. After irradiating human tumour cells, Parshad and co-workers (1992) observed an abnormally high frequency of chromatid breaks and gaps resulting from deficient DNA repair. The insertion of chromosome 11 into these cell lines reduced the amount of radiation-induced DNA damage to the level sustained by normal cells. This protective effect exerted by the chromosome 11 insert is attributed to the restoration of a defective repair process. In another study, ataxia-telangiectasia (AT) fibroblast cultures were reported to be sensitive to ionizing radiation and oxidative agents, such as hydrogen peroxide (Shilol et al., 1983). Peroxide treatment induced a higher frequency of micronuclei in the AT fibroblasts when compared to the normal culture (Yi et al., 1990). By inserting a

chromosome 11, Komatsu and co-workers (1990) demonstrated that radiation resistance was restored in the AT cells. Genetic linkage analyses have suggested that the AT gene lies in the region of 11q22-23 (Sanal et al., 1990). In this thesis, the chromosome 11 insert in the hybrid cell line contains nearly the whole of chromosome 11 with a break occurring proximal to 11q23. It is not known if the AT gene is included in the insert and, thereby, is responsible for the reduced level of DNA damage in the hybrid compared to the parent cell culture. With regards to numerical chromosomal aberrations, our results indicated that chromosome 11 insert cannot protect treated cells against whole chromosome loss and gain induced by hydrogen peroxide.

With the catalase gene localized to 11p13, it is proposed that the presence of chromosome 11 insert would increase the level of catalase in the cell and thereby alter its sensitivity to oxidative stress. The catalase would reduce hydrogen peroxide to water and oxygen, decreasing the availability of hydrogen peroxide to cause DNA damage. Catalase activity, defined as the rate of hydrogen peroxide decomposition in the presence of catalase, has been studied in the parent and hybrid cell cultures and found to be a third higher in the hybrid (Hofseth, unpublished data). If the catalase hypothesis is true, we would expect the hybrid (with chromosome 11 insert) to sustain significantly less numerical chromosomal aberrations than the parent. Since our results indicated that hydrogen peroxide induced significant chromosome loss and gain in the hybrid and parent cell cultures but that the parent and hybrid cell cultures did not differ significantly in their response to hydrogen peroxide, we conclude that the additional catalase present in the hybrid cells is insufficient to prevent numerical chromosome loss and gain in the hybrid. Ward and co-workers (1993) tested the catalase hypothesis by exposing the parent and hybrid cell cultures to xanthine/xanthine oxidase and measuring the level of single strand DNA breakage. Their experimental outcome indicated that the amount of single strand DNA damage in the parent and hybrid was not significantly different. Experiments to study the expression of the catalase gene in these cell cultures have also demonstrated no significant differences.

The second agent used in this project was X-rays. X-rays have been shown to produce a variety of chromosomal aberrations including DNA strand breaks (Armitage et al., 1991; Stich and Rosin, 1983). In a series of X-ray induced micronuclei experiments, the hybrid cell culture was observed to sustain less DNA damage than the parent (Hofseth, unpublished data). To investigate whether the same X-ray doses could alter chromosomal number, both cell cultures were exposed to 0-2 Gy of X-rays. Our analysis indicated that X-rays did not cause a significant alteration in chromosomal numbers in the parent and hybrid cell cultures ($p \geq 0.05$). Although the hybrid had a higher rate of chromosome loss and gain than the parent, the response of both cell cultures to irradiation was not significantly different ($p \geq 0.05$). The difference in hybridization patterns between the two cultures could be explained by their spontaneous frequencies. Comparatively, the hybrid had significantly higher spontaneous chromosomal aberrations than the parent. Ward et al. (1993) also reported similar observations but the significance diminished as more experiments were analysed. The higher spontaneous frequencies in hybrid could be due to the presence of an extra chromosome 11, as Kodama et al. (1992) reported that the introduction of chromosome 11 insert into AT cells increased spontaneous chromosome aberrations. Therefore, the difference in the rate of chromosome loss and gain between the parent and hybrid was probably due to the spontaneous frequencies.

This suggested that the X-ray doses that were effective in inducing micronuclei were not causing numerical chromosomal changes. Numerical chromosomal aberrations and micronuclei represent two forms of DNA damage. The former results from the loss and gain of whole chromosomes. While this process may be involved in the formation of micronuclei, these (micronuclei) can also be formed by acentric chromosome fragmentation. In light of the observed differences in using X-rays to induce micronuclei and numerical chromosomal aberrations, we lack evidence to support our hypothesis that chromosome 11 insert protects against numerical chromosomal aberrations caused by X-rays.

The observations that X-ray at 2 Gy induced micronuclei but not numerical chromosomal changes were consistent with those of Gudi and co-workers (1990). In their experiments, the method of kinetochore staining was applied to micronuclei to distinguish between the clastogenic and aneuploidy-inducing effects of X-rays and vincristin sulfate. Micronuclei stained positive for kinetochores are assumed to contain intact chromosomes resulting from aneuploidy, while unstained micronuclei contain acentric chromosome fragments which originate from clastogenic activities. Both agents tested were capable of inducing micronuclei in a dose-dependent manner. However, the micronuclei induced by X-rays (1 Gy to 2.5 Gy) were negative and those induced by vincristine sulfate were positive for kinetochore proteins (Gudi et al, 1990). These data suggest that X-rays may not be capable of disrupting chromosome segregation to result in aneuploidy.

In the X-ray experiments assaying for numerical chromosomal changes in treated bladder carcinoma cells at a dose of 2 Gy, no effect of the agent was observed. We therefore investigated whether a higher X-ray dose range and/or longer incubation time prior to harvest could induce numerical chromosomal aberrations. The parent cell culture was exposed to 0-4 Gy of X-ray and harvested at 24, 36 and 48 hours following irradiation. For the purpose of this experiment, the parent cell line was chosen over the hybrid due to the possible protective effect of chromosome 11 insert masking any damage sustained by the latter.

Judging from the levels of micronuclei obtained, the treated cells received sufficient irradiation to induce DNA damage. At 2 Gy, the level of micronuclei generated was significantly higher than that at 0 Gy ($p=0.02$). This implied that an adequate proportion of cells had survived irradiation and divided to express DNA damage. The prolonged harvest times did not have a significant impact on the percentage of cytokinesis-blocked cells with micronuclei.

The FISH assay indicated that neither the extended harvest times nor the higher X-ray doses had a significant influence on the loss of

chromosomes. On the other hand, chromosome gains increased with X-rays, with the extent of gain being influenced by harvest times ($p=0.04$). This apparent gain in chromosome number unaccompanied by a corresponding increase in the loss of chromosome was puzzling. It may be speculated that X-rays induced a split in the target hybridization region, thereby creating two signals, mimicking a gain. This could be possible if the hybridization target region is large relative to the entire length of the chromosome arm. To test this hypothesis, cells could be X-irradiated and harvested as metaphase spreads. We could then apply the FISH technique to highlight chromosome 9. If this hypothesis were true, we would expect to see spreads with three whole chromosome 9 with hybridization signals and a fourth chromosome 9 split into two or more fragments with hybridization signals (Figure 5).

Not only may the target region be split randomly, it may be particularly susceptible to breakage. Fragile sites are defined as specific locations on the chromosome that are less stable than the rest of the chromosome. Therefore, these sites are more prone to breakage (Burck et al., 1988). The target hybridization region is the pericentric heterochromatin bounded by the centromere and q12 on chromosome 9. The presence of a fragile site in the target area might result in two or more fragments that could hybridize with the probe, giving multiple signals. Luke and co-workers (1992) have observed pericentric inversions in human chromosome 9 involving the centrometric region and postulated that this region is "breakage-prone". Studies of 9:1 translocation breakpoint in patients with myeloproliferative disease indicated that the majority of cases involved breakpoints in the centromeric regions of both chromosomes (Dal Cin et al., 1991). Chromosome 9 has also been found to carry a common fragile site at q12 which can be induced chemically (Povey et al., 1991). Using the technique of G-banding, Kucerova and Polivkova (1976) have reported pronounced chromosome breakage in the region of 9q12 after X-irradiation. This is reinforced by Eastmond and Pinkel (1990) who recorded statistically significant increases in the number of hyperploid cells after radiation treatment of up to 4 Gy.

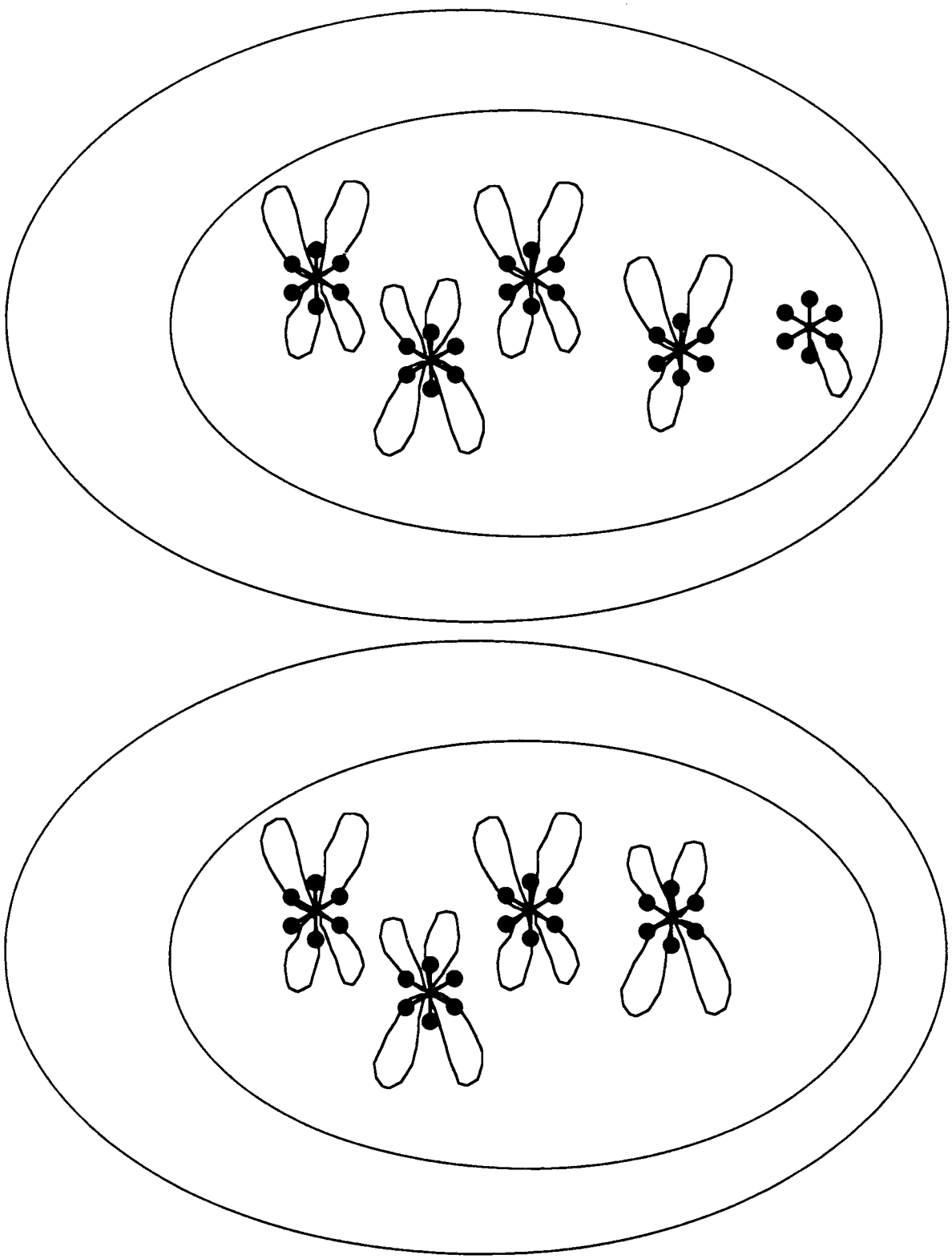


Figure 5: Schematic representation of two cells with four chromosome 9's each. In the first cell, the target hybridization region has been split, yielding an apparent gain without a corresponding loss.

It is not known if fragmentation of the target hybridization region will result in signals that appear visibly smaller than whole target signals. Arguably, the fragments would contain lesser amounts of DNA necessary for hybridization. However, the broken or frayed ends of the split target region may have more surface area exposed and available for hybridization with the probe, resulting in signals that are not visibly smaller or dimmer than intact targets. Scoring of hybridization signals require the aid of a microscope at 10 x 100 magnification. The clarity of a signal can be affected by the amount of cytoplasm on top of it and the extent of probe penetration during assay. Under such visual limitations, a slight change in the size or the brightness of a signal is difficult to detect.

In addition to harbouring a fragile site at the target hybridization region, chromosome 9 is also prone to alterations in bladder cancer. Extensive studies of human bladder cancer tumours revealed frequent structural and numerical aberrations in chromosomes 1, 7, 9 and 11 (Knowles et al., 1994; Hopman et al., 1991; Tsai et al., 1990). Such chromosome 9 associated instabilities may contribute to high spontaneous frequencies of numerical chromosomal changes observed in the A1698 lines. Assuming that a more stable chromosome would have lower spontaneous frequencies of numerical aberrations, it would be interesting to assay for this chromosome in addition to chromosome 9. We may expect to see a greater extent of peroxide-induced numerical aberrations against a background of low spontaneous frequencies relative to a background of high spontaneous frequencies. Nevertheless, if the amount of numerical aberrations is similar, we may conclude that the induction of aneuploidy by peroxide is not specific to chromosome 9.

The chromosome 9 probe used in this thesis hybridizes to a large target region spanning the pericentric heterochromatin up to q12. Many studies have reported that this region is breakage-prone (Luke et al., 1992; Dal Cin et al., 1991; Povey et al., 1991; Kucerova and Polivkova,1976). To avoid false positives for hyperploidy, a different and smaller chromosome 9 centromeric probe may be used. Its target region is

restricted to the centromere, not reaching the area of fragility.

In the hydrogen peroxide-induced micronuclei experiment, the parent cell culture sustained a significantly higher amount of DNA damage than the hybrid (Ward et al., 1993; Hofseth, unpublished data). In a comparative experiment where the peroxide damage was assayed as numerical chromosomal aberrations, a similar but insignificant trend was observed. It may be enlightening to delineate the mechanisms of DNA damage leading to the production of micronuclei. The technique of anti-kinetochore staining makes use of antibodies that recognize the kinetochore proteins. Micronuclei which contain whole chromosomes will be stained kinetochore-positive, while micronuclei with acentric DNA fragments will be kinetochore-negative. This technique will allow us to distinguish whether the route of micronuclei production lies predominately with damage to the mitotic apparatus or with fragmentation of the chromosome arms.

Agents which act on the mitotic apparatus can cause genetic alterations that ultimately lead to carcinogenesis. The association between aneuploidy and carcinogenesis was observed by Hittleman and co-workers (1993). They reported increased numerical chromosomal abnormalities in progressive stages of head and neck tumours. One of the essential components responsible for proper chromosome segregation is microtubules. Microtubules are formed from the polymerization of tubulin subunits. Davidson and co-workers (1986) observed the inhibition of tubulin polymerization in the presence of hydrogen peroxide and 6-hydroxydopamine (6-OHDA). 6-OHDA is unstable and forms superoxide anions, hydroxyl radicals and hydrogen peroxide (ROS) in the presence of molecular oxygen. This is supported by Zhang's work on 1,2,4-benzenetriol induced aneuploidy in HL60 cells (Zhang et al., 1993). 1,2,4-benzenetriol (BT) is one of the products of benzene metabolism. Like 6-OHDA, BT is unstable in molecular oxygen and easily oxidises to quinones and ROS (Bandy et al., 1990). These ROS in turn cause damage to DNA and cellular macromolecules (Halliwell and Aruoma, 1991), such as by interacting with the nucleophilic sulfhydryl groups in tubulin

subunits resulting mitotic disruptions which lead to aneuploidy. Anti-tubulin staining in the BT treated HL60 cells revealed disruptions to the microtubule organisation (Zhang et al., 1993). All living cells are exposed to reactive oxygen species released during normal metabolism, inflammation and bioactivation of xenobiotics. Under constant assaults from ROS and with insufficient defences, cells may stand a higher chance of incurring aneuploidy. Although aneuploidy is not the only event leading to carcinogenesis, recent chromosomal localizations of tumour suppressor genes, oncogenes and growth factors implicates the importance of chromosome loss and gain in the neoplastic process (Eastmond, 1993).

In summary, the results of this thesis suggested that chromosome 11 insert did not exert a significant protective effect against numerical chromosomal aberrations induced by hydrogen peroxide. X-rays did not cause a significant chromosomal number change and thus it was not possible for us to observe any protection offered by the chromosome insert. The effect of higher X-ray doses and prolonged harvest times were investigated but were found to have no significant impact on chromosome loss. On the other hand, chromosome gain was significantly increased in the parent cell culture. Since chromosome gain was not accompanied by a corresponding loss, we speculated that this chromosome gain was a result of the fragility of the target hybridization region.

## 6 REFERENCES


Armitage, M.P., P.E. Bryant and A.C. Riches (1991) Cytogenetic responses of human uroepithelial cell lines and a malignant bladder carcinoma cell line to X-ray, Mutagenesis, 6(6), 515-518.

Bandy, B., J. Moon and A.J. Davison (1990) Multiple actions of superoxide dismutase: why can it both inhibit and stimulate reduction of oxygen by hydroquinones?, Free Radical Biology and Medicine, 9, 143-148.

Bannister, J.V., W.H. Bannister and G. Rotilio (1987) Aspects of the structure, function and applications of superoxide dismutase, CRC Critical Review in Biochemistry, 22, 111-180.

Bishop, J.M. (1987) The molecular genetics of cancer, Science, 235, 305-311.

Burck, K.B., E.T. Liu and J.W. Larrick (1988) Potential diagnostic uses of oncogenes. In: Oncogenes, Springer-Verlag Inc., New York, 256-257.

Cairns, J. (1981) The origin of human cancers, Nature, 289, 353-357.

Cerutti, P.A. (1985) Pro-oxidant states and tumour promotion, Science, 227, 375-381.

Cleaver, J.E. (1986) DNA damage and repair. In: Upton, A.C., R.E. Albert, F.J. Burns and R.E. Shore (Eds) Radiation Carcinogenesis, Elsevier Science Publishing Co., N.Y., 43-55.

Dal Cin, P.S., G. Rege-Cambrin, F. Speleman, N. Van Roy, S. Kerim, P. Scaravaglio, F. Carozzi, J.L. Michaux, F. Offner, G. Saglio and H. Van den Berghe (1991) More precise localization of breakpoints involved in the derivative chromosome (1q9p), associated with

myeloproliferative disorders, Cytogenetics and Cell Genetics 58(2), 1852.

Davison A.J., N.A. Legault and D.W. Steele (1986) Effect of 6-hydroxydopamine on polymerization of tubulin, Biochemical Pharmacology, 35(9), 1411-1417.

De Klein, A., A.H.M. Geurts, G. van Kessel, G. Grosveld, C.R. Bartram, A. Hagemeiier, D. Bootsma, N.K. Spurr, N. Heisterkamp, J. Groffen and J.R. Stephenson (1982) A cellular oncogene is translocated to the Philadelphia chromosome in chronic myelocytic leukemia, Nature, 300, 765-767.

Earnshaw, W.C. ans J.B. Rattner (1989) A map of the centromere (primary constriction) in vertebrate chromosomes at metaphase, Progress in clinical and Biological Research, 318, 33-42.

Eastmond, D.A. (1993) Induction of micronuclei and aneuploidy by the quinone-forming agents benzene and *o*-phenylphenol, Toxicology Letters, 67, 105-118.

Eastmond, D.A. and D. Pinkel (1990) Detection of aneuploidy and aneuploidy-inducing agents in human lymphocytes using fluorescence in-situ hybridization with chromosome-specific DNA probes, Mutation Research, 234, 303-318.

Flier, J.S., L.H. Underhill, S.H. Friend, T.P. Dryja and R.H. Weinberg (1988) Oncogenes and tumour-suppressor genes, New England Journal of Medicine, 318(10), 618-622.

Foegh, M.L., G. Thomas. and P.W. Ramwell (1990) Free radicals, arachidonic acid metabolites, and nutrition, Journal of Parenteral and Enteral Nutrition, 14(5), 218S-222S.

Frenkel, K. (1992) Carcinogen-mediated oxidant formation and oxidative

Frenkel, K. (1992) Carcinogen-mediated oxidant formation and oxidative DNA damage, Pharmacotherapy, 53, 127-166.

Fridovich, I. (1989) Superoxide dismutase: an adaptation to a paramagnetic gas, Journal of Biological Chemistry, 264, 7761-7763.

Ganesh, A., P. North and J. Thacker (1993) Repair and misrepair of site-specific DNA double-strand breaks by human cell extracts, Mutation Research, 299, 251-259.

Gatti, R.A. (1993) Candidates for the Molecular Defect in Ataxia Telangiectasia, Advances in Neurology, 61, 127-132.

Goldstein, B.D and G. Witz (1990) Free radicals and carcinogenesis, Free Radical Research Communications, 11(3), 3-10.

Gray, J. W. and D. Pinkel (1992) Molecular cytogenetics in human cancer diagnosis, Cancer, 69, 1536-1542.

Gudi, R., S.S. Sandhu and R.S. Athwal (1990) Kinetochore identification in micronuclei in mouse bone-marrow erythrocytes: An assay for the detection of aneuploidy-inducing agents, Mutation Research, 234, 263-268.

Hagen, U. (1986) Current aspects on the radiation-induced base damage to DNA, Radiation and Environmental Biophysics, 25, 261-271.

Halliwell, B. and O.I. Aruoma (1991) DNA damage and oxygen-derived species: its mechanism and measurement in mammalian system, Federation of Europe Biochemical Societies, 281, 9-91.

Halliwell, B. and O.I. Aruoma (1992) DNA damage by oxygen-derived species: Its mechanism, and measurement using chromatographic method, In: Molecular Biology of Free Radical Scavenging Systems,

Scandalios, J.C. (Ed), Cold Spring Harbour Laboratory Press, 47-67.

Harris, M.L., H.J. Schiller, P.M. Reilly, M. Donowitz, M.B. Grisham and G.B. Bulkley (1992) Free radicals and other reactive oxygen metabolites in inflammatory bowel disease: cause, consequence or epiphenomenon? Pharmacological Therapy, 53, 375-408.

Heisterkamp, N., G. Jenster, J.T. Hoeve, D. Zovich, P.K. Pattengate and J. Groffen (1990) Acute leukaemia in *bcr/abl* transgenic mice, Nature, 344, 251-253.

Hill R., (1987) Cellular basis of radiotherapy, In: The Basic Science of oncology, Tannock I.F. and R.P. Hill, Pergamon Press,237-255.

Hittelman, W.N., N. Voravud, D.M. Shin, J.S. Lee, J.Y. Ro and W.K. Hong (1993) Early genetic changes during upper aerodigestive tract tumourogenesis, Journal of Cell Biochemistry Supp 17F, 233-236.

Hopman, A.H.N., O. Moesker, A.W.G.B. Smeets, R.P.E. Pauwels, G.P. Vooijs and F.C.S. Ramaekers (1991) Numerical chromosome 1, 7, 9, and 11 aberrations in bladder cancer detected by *in situ* hybridization, Cancer Research 51, 644-651.

Hsu, T.C. and W. Au (1979) Effects of chemical mutagens on chromosomes, In: Effects of drugs on the cell nucleus, Busch, H., S.t. Cooke and Y. Daskal, Academic Press, 315-329.

Hutchinson, F. (1985) Chemical changes induced in DNA by ionising radiation, Progress in Nucleic Acid Research and Molecular Biology, 32, 115-154.

Imlay, J.A. and I. Fridovich (1991) Assays of metabolic superoxide production in *Escherichia coli*, Journal of Biological Chemistry, 266, 19328-19333.

Kodama, S., K. Komatsu, Y. Okumura and M. Oshimura (1992) Suppression of X-ray-induced chromosome aberrations in ataxia telangiectasia cells by introduction of a normal human chromosome 11, Mutation Research, 293, 31-37.

Komatsu, K., S. Kodama, Y. Okumura, M. Koi and M. Oshimura (1990) Restoration of radiation resistance in ataxia-telangiectasia cells by the introduction of normal human chromosome 11, Mutation Research, 235, 59-63.

Kucerova, M. and Z. Polivkova (1976) Banding technique used for the detection of chromosomal aberrations induced by radiation and alkylating agents TEPA and Epichlorohydrin, Mutation Research, 34, 279-290.

Luke S., R.S. Verma, R.A. Conte and T. Mathews (1992) Molecular Characterization of the secondary constriction region (qh) of chromosome 9 with pericentric inversion, Journal of Cell Science, 103, 919-923.

Mitelman, F. and G. Levan (1978) Clustering of aberrations to specific chromosomes in human neoplasms, III. Incidence and geographic distribution of chromosome aberrations in 856 cases, Hereditas, 89, 207-232.

Ning, Y., J.W. Shay, M. Lovell, L. Taylor, D.H. Ledbetter and O.M. Pereira-Smith (1991) Tumour suppression by chromosome 11 is not due to cellular senescence, Experimental Cell Research, 192, 220-226.

Oshimura, M. and J.C. Burrett (1986) Chemically induced aneuploidy in mammalian cells: Mechanisms and biological significance in cancer, Environmental Mutagenesis, 8,129-159.

Parshad, R., F.M. Price, M. Oshimura, J.C. Barrett, H. Satoh, B.E.

Weissman, E.J. Stanbridge and K.K. Sanford (1992) Complementation of DNA repair deficiency in six human tumour cell lines by chromosome 11, Human Genetics, 88, 524-528.

Poddighe, P.J., O. Moesker, D. Smeets, B.H. Awwad, F.C.S. Ramaekers and A.H.N. Hopman (1991) Interphase Cytogenetics of Hematological Cancer: Comparison of Classical Karyotyping and *in Situ* Hybridization Using a Panel of Eleven Chromosome Specific DNA Probes, Cancer Research, 51, 1959-1967.

Povey, S., C. Falk and M. Smith (1991) Chromosome 9, Cytogenetics and Cell Genetics, 58(1), 403-427.

Powell, S., S. Whitaker, J. Peacock and T. McMillan (1993) Ataxia telangiectasia: An investigation of the repair defect in the cell line AT5BIVA by plasmid reconstruction, Mutation Research, 294, 9-20.

Radman M., P. Jeggo and R. Wagner (1982) Chromosomal rearrangement and carcinogenesis, Mutation Research, 98, 249-264.

Reilly, P.M., H.J. Schiller and G.B. Bulkley (1991) Pharmocologic approach to tissue injury mediated by free radicals and other reactive oxygen metabolites, American Journal of Surgery, 161, 488-503.

Rosin, M.P. (1992) The use of the micronucleus test on exfoliated cells to identify anti-clastogenic action in humans: a biological marker for the efficacy of chemopreventative agents, Mutation Research, 267, 265-276.

Sanal, O., E. Lange, M. Telatar, E. Sobel, J. Salazar-Novak, F. Ersoy, A. Morrison, P. Concannon, A. Tolun and R.A. Gatti (1992) Ataxia-telangiectasia: Linkage anlysis of chromosome 11q22-23 markers in Turkish families, Federation of Europe Biochemical Societies, 6, 2848-2852.

Sandberg, A.A. (1992) Chromosome Changes in Early Bladder Neoplasms. Journal of Cellular Biochemistry, Supplement 161, 76-79.

Sayers, C.L., C.T. Denny and O.N. Witte (1991) Leukemia and the disruption of normal hematopoiesis, Cell, 64, 337-350.

Scott, A.F., J.A. Philips and B.R. Migeon (1979) DNA restriction endonuclease analysis for localization of human and alpha-globin genes on chromosome 11, Proceedings of National Academy of Science (USA), 76, 4563-4565.

Shiloh, Y., E. Tabor and Y. Becker (1983) Abnormal response of ataxia-telangiectasia cells to agents that break the deoxyribose moiety of DNA via a targeted free radical mechanism, Carcinogenesis, 4, 1317-1322.

Sidransky, Y.R. and E. Messing (1992) Molecular genetics and biochemical mechanisms in bladder cancer, Urologic Clinics of North America, 19(4), 629-639.

Sies, H. (1985) Oxidants and Antioxidants, In: Oxidative Stress, Sies, H. (Ed.), Academic Press, New York, 1-7.

Sonntag, C. von, U. Hagen, A. Schon-Bopp and D. Schulte-Frohlinde (1981) Radiation-induced strand breakages in DNA: chemical and enzymatic analysis of end groups and mechanistic aspects, In: Advances in Radiation Biology, Latt J.T. and H. Adler (Eds), Academic Press, Philadelphia, PA.,109-142.

Spriggs, A.I. (1974) Cytogenetics of cancer and precancerous states of the cervix uteri, In: Chromosomes and Cancer, German, J. (Ed.), Wiley, New York, 423-450.

Stich, H.F. and M.P. Rosin (1983) Micronuclei in exfoliated human cells as an internal dosimeter for exposures to carcinogens. In:

Carcinogens and Mutagens in the Environment, Volume II, Naturally occurring compounds: endogenous formation and mutation, Sitch, H.F. (Ed.), CRC Press, Boca Raton, Florida, 17-25

Teoule, R. (1987) Radiation-induced DNA damage and its repair, International Journal of Radiology Biology, 51, 573-589.

Thacker, J. (1989) The use of integrated DNA vectors to analyse the molecular defect in ionizing radiation-sensitive mutants of mammalian cells including ataxia telangiectasia, Mutation Research, 220, 187-204.

Tsai, Y.C., P.W. Nicholas, A.L. Hiti, Z. Williams, D.G. Skinner and P.A. Jones (1990) Allelic losses of chromosomes 9, 11, and 17 in human bladder cancer, Cancer Research, 50, 44-47.

Wagner, R.P., M.P. Maguire and R.L. Stalling (1993) Variation in chromatin organisation and amount, In Chromosome: A Synthesis, Wiley-Liss, New York, 183-289.

Waldman, F.M., P.R. Carroll, R. Kerschmann, M.B. Cohen, F.G. Field and B.H. Mayall (1991) Centromeric copy number of chromosome 7 is strongly correlated with tumour grade and labeling index in human bladder cancer, Cancer Research, 51, 3807-3813.

Ward, A.J., P.L. Olive, A.H. Burr and M.P. Rosin (1993) A sensitivity to oxidative stress is linked to chromosome 11 but is not due to a difference in single strand DNA breakage or repair, Mutation Research, 294, 299-308.

Ward, A.J., P.L. Olive, A.H. Burr and M.P. Rosin (1994) Response of fibroblast cultures from ataxia-telangiectasia patients to reactive oxygen species generated during inflammation reactions, Environmental and Molecular Mutagenesis, 24, 103-111.

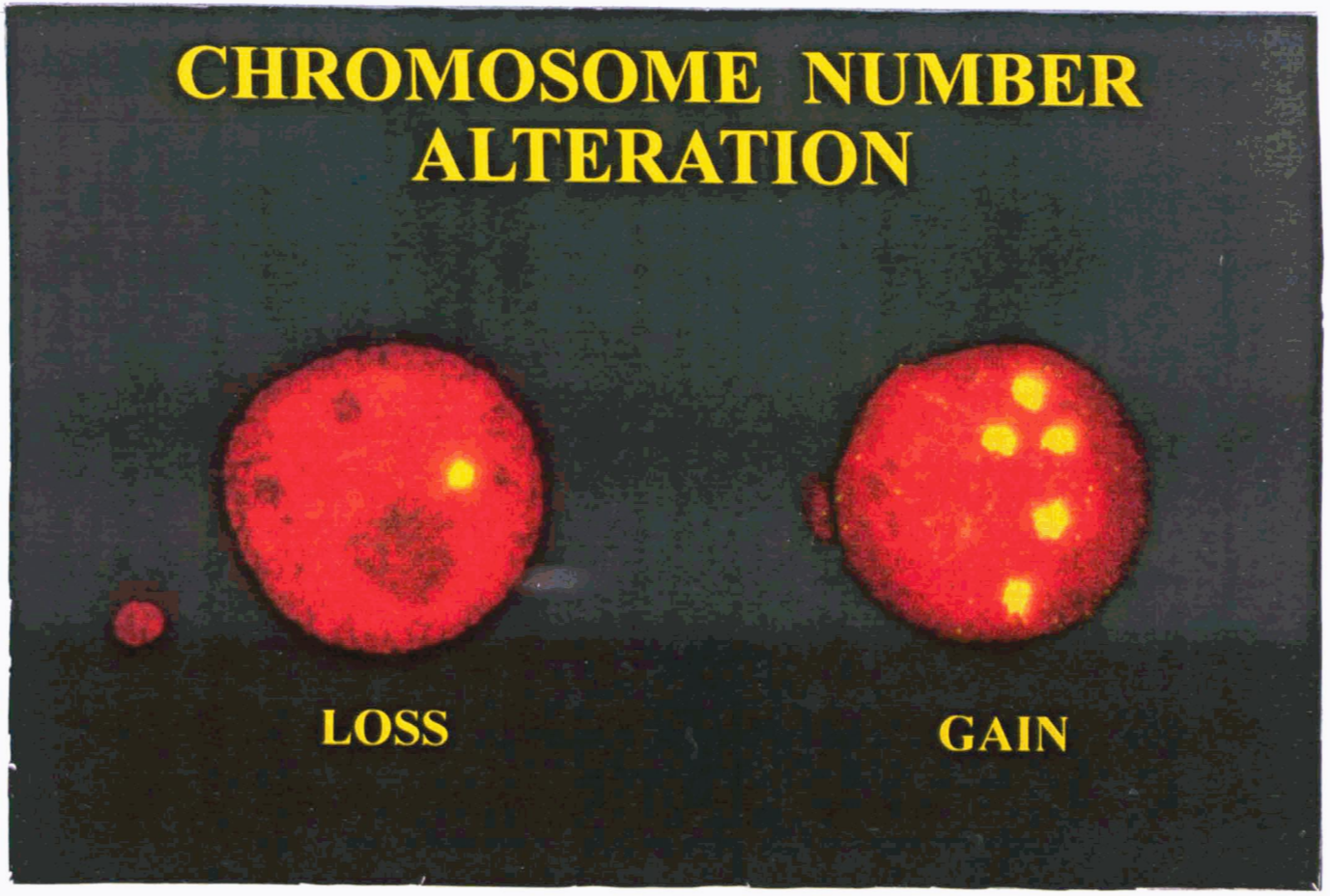
Ward, J.F. (1975) Molecular mechanism of radiation-induced damage to nucleic acids, Advance in Radiology Biology, 5, 181-239.

Yi, M., M.P. Rosin and C.K. Anderson (1990) Response of fibroblast cultures fron ataxia-telangiectasia patients to oxidative stress, Cancer Letters, 54, 43-50.

Zhang, Q.,M., S. Yonei and M. Kato (1992) Multiple Pathways for Repair of Oxidative DNA Damages Caused by X Rays and Hydrogen Peroxide in *Escherichia coli*, Radiation Research, 132, 334-338.

Zhang L., M.L. Robertson, P. Kolachana, A.J. Davison and M.T. Smith (1993) Benzene metabolite, 1,2,4-benzenetriol, induces micronuclei and oxidative DNA damage in human lymphocytes and HL 60 cells, Environmental and Molecular Mutagenesis, 21(4), 339-348.

# APPENDICES




Fluorescence *in situ* hybridization staining of chromosome 9 in A1698 cells

Appendix 2: Score sheet

Cell line: _____ Agent: _____ Probe: _____
Cell density: _____ Dose: _____ Date: _____
Date: _____ Score date: _____

| 0 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| | | | | |
| | | | | |

| 5 | 6 | 7 | 8 | >8 |
|---|---|---|---|---|
| | | | | |
| | | | | |

Total cells scored: _____
Comments: