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GENE EXPRESSION ANALYSIS OF RAT TRACHEAL EPITHELIAL CELLS FOLLOWING EXPOSURE TO IONIZING RADIATION

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee

Jon Brooks Boroughs
May, 1995

DEDICATION

This Thesis is dedicated to my parents

Mr. Gerald Brooks Boroughs

and

Mrs. Linda Gayle Boroughs

who have given me invaluable love and support.

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I am grateful to many people at the University of Tennessee and Oak Ridge National Laboratory for their help during my graduate school experience. I have benefitted from the patient guidance of my major advisor, Stephen Kennel, in particular. The other members of my committee, Peggy Terzaghi-Howe, John Cook, and Dabney Johnson, have also been extremely helpful during the development of this thesis, and as instructors during my three years as a graduate student.

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ABSTRACT

This study involved the use of differential display (DD) to investigate gene expression in rat tracheal epithelial cells following exposure to ionizing radiation. Previous work in X ray response research has provided useful information using cell lines and classical molecular biology techniques. However, the specific molecular mechanisms of the cellular response to ionizing radiation remain unclear. The development of DD (Liang and Pardee, 1992) provided a new means for studying changes in gene expression. In addition, the use of primary rat tracheal epithelial cells in X ray induced transformation studies (Terzaghi-Howe, 1990) provided a model cell population that more closely resembles *in vivo* conditions than do cell lines.

DD was used to analyze gene expression in rat tracheal epithelial cells, revealing six potential X ray responsive gene fragments. Sequence analysis revealed that four of the six cDNA clones had significant sequence identity to known genes involved in growth and differentiation. These include a embryogenic preimplantation gene (Temeles *et al.*, 1994), a human interferon related gene (Tirone *et al.*, 1989), a mouse zinc finger mRNA (Mardon *et al.*, 1990), and elongation factor 1δ (Sanders *et al.*, 1993). Enhanced expression was not detect by northern blot analysis using the cDNA fragments as probes.

Two independent clones were isolated from 2 the original DD cDNA fragments to check the efficiency of reamplification and TA cloning. The secondary clones produced distinct sequences and Northern blot patterns. These clones showed significant identity to cyclin A and rat repetitive mRNA. Despite the inconsistencies, DD remains promising as a means for studying the molecular response of cells to ionizing radiation.

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LIST OF ABBREVIATIONS

Ab	Antibody
bFGF	Basic fibroblast growth factor
cDNA	Complementary DNA
CsCl	Cesium chloride
DD	Differential display
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
EBV	Epstein Barr virus
EF-1δ	Elongation factor 1 δ
EDTA	Ethylenediaminetetraacetic acid
EtBR	Ethidium bromide
EtOH	Ethanol
GITC	Guanine isothiocyanate
HAP	Hydroxyapatite
IL-1	Interleukin 1
MAP	Mitogen activated protein
MgAc	Magnesium acetate
$MgCl_2$	Magnesium chloride
mRNA	messenger RNA
Na_2HPO_4	Sodium phosphate

NaOH	Sodium hydoxide
Nf-κB	Nuclear factor κB
OD	Optical Densitometry
PCR	Polymerase chain reaction
PAGE	Polyacrylamide gel electrophoresis
PDGF	Platelet derived growth factor
RNA	ribonucleic acid
RT	reverse transcription
RTEC	Rat tracheal epithelial cells
S-PBS	*Sans*-phosphate buffered saline
t-PA	Tissue type plasminogen activator
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TB	Terrific broth
tRNA	Transfer RNA
XIP	X ray induced protein
XP	Xeroderma pigmentosum
Zfx	Zinc finger

CHAPTER 1

INTRODUCTION

The response of cells to ionizing radiation has captured the attention of scientists for many years. Knowledge of how cells react to radiation has applications in mutagenesis research, cancer treatment, and the basic understanding of cellular mechanisms. Despite the intense interest, little is known about the specific molecular mechanisms used by cells to cope with the mutagenic or destructive effects of ionizing radiation. As recently as 1989, no genes were directly implicated in the molecular mechanisms of the cellular response to ionizing radiation. During recent years, numerous genes have been identified that are activated by X rays (Weichselbaum *et al.* , 1991). The focus of this study was to search for genes that are specifically involved in the cellular response to ionizing radiation. A new investigative approach in molecular biology was utilized to conduct a rapid search and identification of genes that displayed enhanced expression after irradiation.

Biological effects of X rays

X rays are a type of electromagnetic radiation that was discovered by Wilhem Roentgen in 1895, for which he earned the first Nobel prize for physics in 1901. Radiation is composed of photons, which are packets of energy that move at the speed of light and display properties of waves. Radiation biologists are particularly interested in the harmful effects of ionizing radiation, including X rays. Ionizing radiation is defined as follows: "When the energy per photon is greater than the binding energy of electrons in the target, ejection of an electron from the atomic orbit in the target occurs resulting in

ionization" (Tannock, 1987).

One of the primary sites of damage induced by X rays in mammalian cells is the DNA. Although damage occurs throughout the cell, damage to the DNA molecule is thought to have the greatest effect. Mutations caused by X rays can be the result of direct damage to the DNA or indirect damage through the formation of free radicals in the target cells. DNA damage from radiation includes altered bases in the DNA molecule, loss of bases, single strand breaks in the DNA, and double strand breaks. High doses of ionizing radiation may cause death of cells by reproductive lethality or apoptosis. Reproductive lethality is death of cells due to damage to the cellular machinery, thereby preventing proliferation, while apoptosis is programmed cell death (Alberts, 1989). X-irradiated human lymphoblastic tumor cell lines have been shown to undergo DNA fragmentation and morphological changes consistent with apoptosis (Nakano and Shinohara, 1994).

Following exposure to X rays, DNA repair mechanisms work to reverse the damage. Furthermore, other changes in gene expression may occur leading to transformation of the normal cells into cancerous cells. Finally, the changes in the DNA caused by radiation may not be repaired prior to DNA replication, leading to a heritable change in the genome of the organism. Recent experimental evidence suggests ionizing radiation causes a prolonged instability of the genome, often leading to delayed mutations, and increased incorporation of nonlethal mutations (Kronenberg, 1994).

X-ray exposure can lead to transformation of normal cells into cancerous cells. However, damage to DNA caused by X rays can often be corrected by normal DNA repair mechanisms within cells (Tannock, 1987). Transformation frequency is the highest

within a range X ray doses. The optimum dose for transformation is between levels that would cause cell killing and tolerable levels. When dose levels exceed the optimum intensity to cause changes in gene expression leading to cancer, the transformation frequency decreases. This is primarily due to irreparable damage to genes in the DNA that code for essential functions, and cell survival drops substantially. Figure 1 gives a schematic representation of the relationship between X ray dose, transformation frequency, and cell survival.

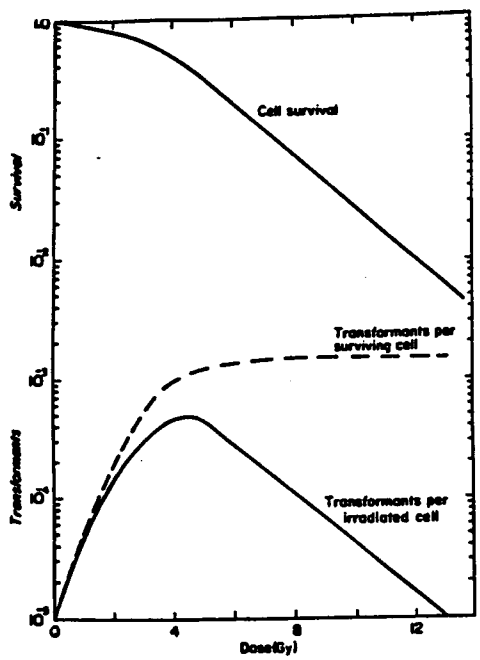


Figure 1. Schematic representation of the relationship between X rays, transformation frequency, and cell survival (Tannock, 1987).

The medical implications of X rays are significant. The extensive use of X rays as a means of diagnosis in medicine is only one example. The link between exposure to X rays and increased frequency of malignant and benign tumors has been well established.

In addition, ionizing radiation is used as a treatment for certain types of tumors (Lighter and Lawrence, 1995). There has been extensive progress in the understanding of radiation biology during the past several decades (Weichselbaum *et al.*, 1994). However, the specific molecular aspects of the radiation response are still unclear. These genetic responses are the primary focus of this work.

Advances in molecular biology relating to gene expression research

It is generally accepted that exposure to X rays leads to an increased expression of a wide variety of genes (Weichselbaum *et al.*, 1991). Most of these, such as the heat shock protein genes, are also activated by other cellular insults. Heat shock proteins, or stress induced proteins, are activated by elevated temperature (hyperthermia), cytotoxins, heavy metals, oxidants, and infection (Morimoto, 1993).

The search for genes that are expressed, or induced, in response to ionizing radiation has intensified during the last several years due to improvements in technology and a better understanding of the cellular mechanisms used to cope with stress (Tannock, 1987). Several laboratory techniques have direct relevance to recent advances in X ray response research and this project. The major molecular biology techniques used to identify genes include 2D gel electrophoresis of proteins, subtractive hybridization of cDNA libraries, and differential display analysis of mRNA.

2D gel electrophoresis has been utilized to study changes in gene expression at the protein level (Boothman *et al.*, 1989). It involves the comparison of the protein content of two similar, but distinct cell populations (ie. $\pm$ X rays). Cells are grown with ^{35}S methionine in the medium followed by preparation of whole cell extracts from each

population. Radioactively labeled proteins are separated on a 2D polyacrylamide gel, which separates proteins by isoelectric focusing in one direction and molecular weight in the other direction. The proteins from each 2D gel are compared for differences. Proteins are then identified that are expressed in one cell population but not the other. These proteins can be extracted from the gel, purified, and sequenced to determine their homology to known proteins of biological significance (Boothman *et al.*, 1989).

Although 2D gel electrophoresis has been used successfully in gene expression research, several weaknesses are apparent. First, proteins are easily denatured and difficult to manipulate relative to nucleic acids. In addition, it is difficult to reproduce 2D gel results due to casting techniques and gel warping. Furthermore, the small quantity of protein extracted from the gel makes amino acid sequencing difficult. The follow up experiments include protein purification, Western blots to determine expression, and amino acid sequence analysis. The 2D gel protein results of Boothman's group will be discussed in later sections.

Subtractive hybridization is another technique used to investigate changes in gene expression. Unlike 2D gel electrophoresis, subtractive hybridization selects messenger RNA (mRNA) uniquely expressed in one of two closely related cell populations (ie. $\pm$ X rays). Total RNA is isolated from two parallel cell populations grown in different conditions. A cDNA library from cell population 1 is constructed and radioactively labeled with ^{32}P-dCTP. This labeled cDNA is hybridized to a ten fold excess of mRNA from cell population 2. The majority of the cDNA forms double stranded hybrids with the mRNA because the two cell populations express many of the same genes. However,

because subtle differences in the cell populations exist, there is a small number of genes that are expressed in the cDNA of one population that are not present in the mRNA of the other population. The mixture of single stranded cDNA and double stranded hybrids are separated, often by hydroxyapatite (HAP) column. The double stranded hybrids are bound, but the single stranded cDNA and excess mRNA passes through the column. The hybridization and column are repeated two more times resulting in a set of selected single stranded cDNA that is expressed only in cell population 1. The cDNAs are cloned, their sequence is analyzed, and compared known genes in Genbank. In addition, probes can be made with the cDNA to confirm differential expression with Northern blot analysis (Lee *et al.*, 1991). Figure 2 shows the protocol used in subtractive hybridization.

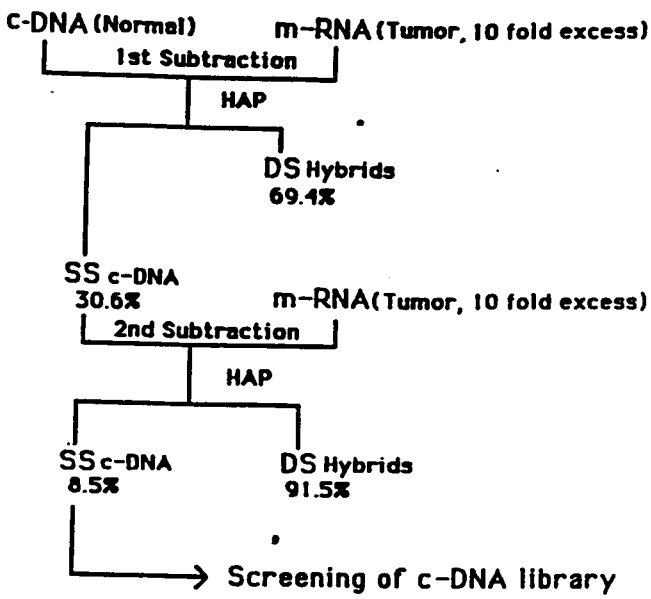


Figure 2. Flow diagram of subtractive hybridization technique (Sager, 1991).

Subtractive hybridization was used to identify potential tumor suppressor genes by comparing normal human 76N cell cDNA with mRNA from tumorigenic human 21MT-2 cells (Lee *et al.*, 1991). This technique used by Sager also showed promise as a means of identifying X ray response genes. However, like 2D gel electrophoresis, subtractive hybridization does have significant weaknesses. The multiple hybridizations and HAP columns lack sensitivity. This lack of sensitivity results in a large number of false positives, requiring further screening of the single stranded cDNA's isolated from the HAP column.

An enhanced version of subtractive hybridization, called differential hybridization, has been used in X ray response research recently (Boothman, 1993). Boothman used total RNA isolated from control and irradiated human melanoma (U1-MEL) cells. The mRNA from each cell population was reverse transcribed into cDNA and libraries were prepared in Lamda Zap II. The cDNA library from the irradiated cell population, which was expected to contain differentially expressed genes, was plated onto XL1-Blue *E. coli* and duplicate plaque lifts were done. This provided two identical sets of membranes containing the plated cDNA libraries. These membranes were then hybridized to ^{32}P-dCTP labeled cDNA from each cell population respectively. Multiple high stringency washes were followed by autoradiography. Colonies were selected that were present only in the irradiated samples. This technique, while extensive and time consuming, has been used effectively to identify cDNA segments of genes differentially expressed in response to ionizing radiation (Boothman *et al.*, 1993). The results of these experiments will be discussed further in the section titled "research focused on identification of novel X ray

response genes". Like the other methods, there are problems with the differential hybridization technique. These include incomplete hybridization and the questionable effectiveness in identifying low copy transcripts.

In 1992 a paper describing a unique approach was published that provided a new direction in gene expression research. This technique, termed differential display (DD) was used to compare mRNA expressed by two parallel sets of cells that had been grown in different conditions (Liang and Pardee, 1992). One difference between DD and the methods described earlier is that DD is based on the polymerase chain reaction (PCR). The amplification results in a sufficient quantity of DNA product to quickly clone, sequence, and identify individual amplified bands. Furthermore, this simplicity and expedience permits a large scale search of mRNA expression.

The first application of DD was to analyze differences in gene expression between normal and tumor cells (Liang and Pardee, 1992). Total RNA was isolated from normal (76N) cells and tumor (BPA31) cells. After treatment of RNA with DNase to remove DNA, the total RNA was converted to cDNA using semispecific oligodT_{11}XX downstream primers with a Moloney murine leukemia virus (MMLV) reverse transcriptase. The oligodT_{11}XX primer was designed to recognize 1/12 of all mRNA expressed in a cell. The series of T residues recognize the polyadenylated tail found at the 3' end of many mRNAs. The two 5' nucleotides, designated as XX, represent two of the four bases A,T,G, and C. Each pair combination recognizes 1/12 of the mRNA rather than 1/16 because a T in the terminal position would contribute to binding of the polyA tail of the mRNA (Liang and Pardee, 1992). The cDNA from the reverse transcriptase

(RT) reaction was then amplified using PCR . The reverse transcribed cDNA served as the template for the PCR reaction, and oligodT_{11}XX downstream primers and arbitrary upstream primers were used. The 10-base (10mer) arbitrary primer was designed to anneal to mRNA transcripts at a frequency to produce 50-100 cDNA fragments of 500 bp or less. The effective binding of a 10 base arbitrary primer at this frequency is part of the DD technology that is not well understood. In theory a 10 base primer, when used with an oligodT_{12}MC primer, should amplify less than 1 mRNA transcript of 500 bases or less (Liang and Pardee, 1992). This is because the number of loci along the DNA molecule where an arbitrary 10 base primer binds is too small to produce amplified 50-100 cDNA less than 500 bases.

The PCR reaction included ^{35}S-dCTP so that labeled, amplified cDNA bands can be identified on a 6% polyacrylamide denaturing gel. The PCR products from each reaction were separated in adjacent gel lanes to compare bands, or gene fragments, that were amplified in one cell type but not the other. Bands of interest were identified by autoradiography and cut from the dried gel for reamplification using the same PCR primers. The reamplified cDNA was cloned using TA cloning. TA cloning exploits the nonspecific addition of an adenine (A) base to the 3' end of PCR products. The cloning vector contains one base thymine (T) overhangs where the amplified DNA is ligated into the vector. ^{32}P-dCTP labeled inserts from the TA clones were used as probes to hybridize Northern blots as a second screening mechanism to check gene expression. Clones were then sequenced by the dideoxy sequencing method (Sanger *et al.*, 1977) and the sequences were inserted into computer files to search databases for known genes that

matched the sequences of the clones.

Liang and Pardee used DD to report the presence of an amplifed band, N1, that was seen only in the normal A31 cells on the DD gel. Northern blotting confirmed that the N1 fragment was expressed in growing and quiescent normal cells , but not in tumorigenic BPA31 cells. The reproducibility and specificity of the technique was also described (Liang and Pardee, 1992).

DD is particularly desirable as a means for studying gene expression because it is relatively simple. It can be used as a rapid screening method, looking at a large number of amplified genes in short period of time. Finally, PCR has the ability to amplify very low copy number mRNA, providing a high level of sensitivity (Liang and Pardee, 1992). A number of refinements have been made to DD including changing from oligodT_{11}XX primers to oligodT_{12}MC downstream primers, where M represents a degenerate combination of the four nucleotides and C represents one of the four nucleotide bases (Liang *et al.*, 1993). The specific protocol of differential display, as it relates to this research project, will be described in detail in chapter two.

Questions remain regarding the effectiveness of DD as a method for studying changes in gene expression. It may not be useful as a means to completely scan the entire spectrum of mRNA expressed in a set of cells. A potential problem may be the efficiency of amplifying low copy number transcripts by the DD method. It is unknown what percentage of the cellular mRNA is reverse transcribed in DD. Despite the drawbacks, DD appears to be useful as a rapid and random search for differences in gene expression between two similar cell populations that have distinct differences.

Historical review of X ray response research

X ray response research has revolved around two basic approaches; looking at the responses of known genes to ionizing radiation and searching for novel genes and mechanisms involved specifically in the response of cells to ionizing radiation. The majority of X ray response research has used known genes and proteins to study the cellular X ray response. Furthermore, genes that respond to ionizing radiation have been categorized as either early (immediate) response genes or late response genes.

Early response genes are either normally not expressed or expressed at low levels. These early response genes are typically turned on within the first hour following irradiation, usually within the first fifteen minutes (Weichselbaum *et al.*, 1991). The increase in mRNA in the cell from these genes can be due to increased transcription of new mRNA molecules or an increased stability of the mRNA already present in the cell (Sherman, 1990). Many of the protein products of the early response genes are involved in regulation of transcription through protein/DNA interactions. The early response genes likely have multiple effects on subsequent changes in gene expression. Examples of early response genes are proto-oncogenes and transcription factors that lead to a cascade of protein synthesis (Weichselbaum *et al.*, 1991). Late response genes to ionizing radiation are expressed several hours after exposure, although the length of time varies for each gene. Many are likely to be secondary effects of the induction of early response genes. Proteins produced by late response genes include growth factors, proteases, and immune system proteins (Weichselbaum *et al.*, 1991).

Known genes and proteins used to study the early response to ionizing radiation

Several proto-oncogenes have been identified as early response genes. Transcription of c-*jun* mRNA in human HL-60 cells exhibited an increase in response to X rays (2,000 rads) within 15 minutes of exposure. In addition, the stability the c-*jun* mRNA increased after exposure (Sherman *et al.*, 1990). The c-*jun* proto-oncogene codes for the nuclear transcription factor AP-1. Monome (1993) showed that c-*jun* expression was directly related to dose level and time of exposure to ionizing radiation. It is noteworthy that an X ray dose of 2,000 rads would be lethal to the majority of cells.

In addition, it was shown that c-*fos* and *jun*-B mRNA expression in HL-60 cells increased in response to X rays. X ray doses above 500 rads caused increased *c-fos* expression. The expression of *jun-B* increased within 3 hours after exposure to 200 rads. The protein products of these proto-oncogenes can form a complex with a leucine zipper DNA binding domain (Sherman *et al.*, 1990). The leucine zipper DNA binding motif is found in DNA binding proteins, many of which are involved in gene regulation.

Another early response gene induced by ionizing radiation is *egr*-1. *Egr*-1 codes for a transcription factor containing a zinc finger DNA binding motif. High doses of X rays (2,000 rads) were used, causing a significant increase in egr-1 transcript within one hour of irradiation. There was an 18 fold increase in egr-1 transcript within three hours followed by a return to baseline levels within twenty four hours (Hallahan *et al.*, 1992). These results were reported as the first direct evidence of activation of a gene that regulates transcription by ionizing radiation. However, this gene has also been shown to respond to stimulation by addition of fresh serum, platelet derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and during differentiation. Furthermore, the 2,000

rad dose required to activate the *egr*-1 gene in human HL-525 cells would be lethal to most cells. These points weaken the possibility that *egr*-1 is a relevant X ray response gene.

A *src*-like tyrosine kinase has also been shown to respond to ionizing radiation. The *lyn* gene codes for two splice variants, p56 and p53. The *src*-like p56/p53 proteins from HL-60 myeloid leukemia cells were shown to increase in expression 2.4 fold within 15 minutes following exposure to 200 rads ionizing radiation (Kharbanda *et al.*, 1994).

The tissue type plasminogen activator (*t-PA*) gene was demonstrated to be an early response gene to ionizing radiation. Immediate increases of the t-PA transcript in U1-MEL cells were observed within 15 minutes of exposure to a X ray dose of 300 rads (Boothman *et al.*, 1991). The increase in mRNA was due to increased stability of the mRNA molecule in the cell. As will be discussed later, *t-PA* was also shown to respond as a late response gene. t-PA is a glycoproteinase involved in the conversion of plasminogen to plasmin. Plasmin is involved in a variety of activities, including dissolving the extracellular matrix, activating latent enzymes, hormone processing, inflammation, tumor cell development, cell migration, and metastasis (Laiho and Keski-Oja, 1989).

Western blot analysis was used to show that the protein kinase C (PKC) ϵ protein, which is normally expressed in brain, was increased substantially within minutes after exposure to low dose (75 rads) ionizing radiation. PKC ϵ returned to normal levels two hours after irradiation (Kim *et al.*, 1992). PKC ϵ activates a cascade of other kinases, leading to enhanced transcription of an assortment of genes involved in cellular growth

and proliferation. It has been reported that elevated levels of PKCs cause a loss of cellular growth control (Housey *et al.*, 1988). A loss of growth control can contribute to cellular transformation and the development of cancer.

An extension of the early response gene research previously mentioned involved mitogen activated protein (MAP) kinases. Stevenson (1994) used NIH3T3 cells to show dose related responses of MAP kinase activity using gel MAP kinase assays. 200 rads ionizing radiation resulted in a 4-fold increase in MAP kinase activity. Furthermore, 1000 rads caused a 6-fold increase in MAP kinase activity (Stevenson *et al.*, 1994). MAP kinases have also been shown to be involved in activation of *c-fos*. These enzymes phosphorylate transcription factor p62TCF, which forms a ternary complex at the *c-fos* promoter (Gille *et al.*, 1992).

Known genes and proteins used to study the late response to ionizing radiation

A variety of known genes, or proteins, have been used to study the late response of cells to ionizing radiation. As mentioned earlier, these late response genes display increased expression levels two to six hours after exposure. These include, among others, tumor necrosis factor α (*TNF*α), interleukin 1 (*IL*-1), platelet derived growth factor (*PDGF*), transforming growth factors (*TGF*s α and β), basic fibroblast growth factor (*bFGF*), epidermal growth factor receptor (*EGF-R*), and cytoskeletal genes coding for actin and tubulin.

TNFα is a protein mediator of the cellular immune response. It's effects include increased adhesion of leukocytes to vascular epithelium through induction of adhesion molecules, free radical formation in cells, DNA fragmentation, and microtubule

destruction (Hallahan *et al.*, 1989). Hallahan (1989) described a 2.5 fold increase of TNFα mRNA levels in human sarcoma cell lines exposed to 500 rads of X rays. This increase, seen at 3 hours post irradiation, returned to baseline levels after 6 hours (Hallahan *et al.*, 1989). TNFα is thought to be involved in host defense to tumor progression. It is reported to have a cytotoxic effect on tumor cells, suggesting the increased expression following irradiation may eventually contribute to cell killing of damaged cells (Hallahan *et al.*, 1989).

Another immune system protein shown to react as a late response gene to X rays is *IL*-1. In general, the IL-1 protein acts as a stimulator of proliferation and differentiation in a variety of cell types through direct action and stimulation of other interleukins and growth factors. Syrian hamster embryo (SHE) fibroblasts were irradiated with low dose X rays (75 rads). Northern blots and densitometric analysis showed an increase in IL-1 mRNA expression. This increase in IL-1 expression began at 1 hour post exposure and reached a maximum level after 7 hours (Woloshak *et al.*, 1990). IL-1 has numerous functions in the immune system and has been implicated as a radioprotector (Morrissey *et al.*, 1988).

Other growth factors implicated in the late response are PDGF and FGF. Following exposure to 2,500 rads of ionizing radiation, porcine aortic endothelial cells (PAEC) displayed a 2.5-fold increase in secretion of PDGF-like peptides and a 2.3-fold increase in FGF-like peptides (Witte *et al.*,1989). Changes in PDGF and FGF levels are connected with increased cellular proliferation and induction of angiogenesis (Laiho and Keski-Oja, 1989).

The enzyme protease, tissue type plasminogen activator (t-PA), which was described earlier as an early response gene, also is expressed as a late response gene. U1-MEL cells showed an increase in t-PA mRNA after exposure to 100 rads ionizing radiation. A maximum increase of t-PA expression of 100 fold was seen at 4 hours post-irradiation with elevated levels persisting over 12 hours later (Boothman *et al.*, 1991). As mentioned earlier, t-PA converts plasminogen into plasmin, leading to a cascade of growth stimulating reactions.

An additional family of late response genes implicated in X ray response research codes for TGF's. TGFα has considerable sequence homology to epidermal growth factor (EGF) and binds to the EGF receptor (EGF-R). It was shown that macrophages isolated from irradiated rabbit lungs displayed enhanced production of TGFα and TGFβ compared to macrophages isolated from normal rabbit lungs (Rubin *et al.*, 1992). TGFs have a wide variety of effects on cells including stimulation of fibroblast proliferation by TGFα and TGFβ, in addition to inducing secretion of extracellular matrix components by TGFβ. TGFs have been described as embryonal growth factors which can be overexpressed in malignant cells (Laiho and Keski-Oja, 1989). Recently, the EGF-R was linked to the cellular response to X rays. MCF-7 cells, which were irradiated with 200 rads, were shown by Northern blot analysis to have a 9 fold increase in EGF-R expression (Schmidt-Ullrich *et al.*, 1994). These results were consistent with the previously described response of TGF to ionizing radiation (Rubin *et al.*, 1992).

Nuclear factor kB (Nf-κB) is a transcription factor that regulates several cytokine genes and other genes coding for immune system proteins. Epstein-Barr virus (EBV)-

transformed lymphoblast cells were exposed to 10-200 rads of ionizing radiation. Nf-κB displayed a 3.9 fold increase in expression after exposure to 50 rads, with a maximum increase seen 8 hours post exposure (Mohan and Meltz, 1994).

Woloschak (1990) examined the expression of genes that code for cytoskeletal proteins actin and tubulin. SHE cells were exposed to X rays (10-200 rads). Alpha tubulin expression increased between 1-3 hours after exposure. In contrast, beta actin expression decreased within an hour after exposure to X rays and remained at low expression levels for 3 hours (Woloschak *et al.*, 1990). These changes in expression of cytoskeletal genes perhaps suggest a role in the radiation response and transformation.

The study of known genes and proteins acting as early and late responses to ionizing radiation has provided significant information about the cellular response to X rays. However, many questions remain about the specific responses of cells to X rays. Research has intensified recently to find specific genes involved in the radiation response.

Research focused on identification of novel X ray response genes

The previous sections represent the wealth of advances in X ray response research. The knowledge gained from studying the response of known genes to ionizing radiation has increased the understanding of the cellular response to X rays. However, fully understanding the complex molecular mechanisms of the X ray response of cells requires uncovering genes that are specifically activated by ionizing radiation. Many of the genes discussed in previous sections are activated by a wide variety of cellular insults. Techniques described in earlier sections such as 2D gel electrophoresis, differential hybridization, and differential display have been used to search for novel genes involved

in the cellular response to ionizing radiation.

Boothman (1989) used 2D gel electrophoresis, described on page 4, to identify 8 proteins that were expressed in U1-MEL cells following exposure to X rays (300 rads). In addition, 2 proteins displayed decreased expression after irradiation. Table 1 lists the X ray induced proteins (XIPs) and the dose response of each.

These proteins were also shown to be specific to X rays. The XIPs were not induced by a variety of other cellular stresses. Among these were 0.1-4.0 uM melphalan, heat shock treatment (44^0-47^0 C for 1 hour), hypoxic treatment ([O_2] less than 100 ppm for 4 hours) , and UV irradiation. None of the XIPs were induced by these cellular insults. Furthermore, the proteins induced by UV radiation were distinct from the proteins stimulated by X rays (Boothman *et al.*, 1989). The proteins were defined as linear induced or plateau induced, referring to the maximum levels of protein seen on western blot relative to X ray dose. The quantity of protein that could be isolated from the 2D gels made sequencing very difficult, preventing identification.

Boothman continued the search for new genes using differential hybridization to uncover 12 X ray induced transcripts in U1-MEL cells and normal, nonfetal human fibroblasts (GM2936B). These clones, designated XIP1-XIP12, were differentially expressed 2-230 fold following exposure to an X ray dose of 300 rads. The 12 cDNA clones were sequenced and compared to known sequences in Genbank (Boothman, 1993). The XIPs isolated, and the inducibility of each are listed in Table 2.

Table 1. X ray induced proteins with changes in expression (Boothman *et al.*, 1989)

X-ray induced protein	Mol. Wt. (Kda)	Increase in expression after 300 rads
Linear induced		
XIP269	269	1.1 fold
XIP145	145	1.4 fold
Plateau induced		
XIP275	275	3.0 fold
XIP147	147	0.9 fold
XIP141	141	2.0 fold
XIP138	138	2.0 fold
XIP135	135	3.0 fold
XIP126	126	1.3 fold

Although some of the XIPs were induced by other cytotoxic treatments, X-irradiation provoked the greatest increase in expression. The 12 XIPs were divided into 4 categories based on cellular function. Clones XIP11 (thymidine kinase); XIP5 (homologous clone to human growth hormone 1 and 2); XIP9 (homologous clone to the proto-oncogene c-*fps/fes)*; and XIP7 [homologous clone to the recombination activating gene 1 (*RAG*-1)] were described as regulatory factors of cellular growth, metabolism, and recombination. *RAG*-1 overexpression has been implicated in the hypersensitivity of mice with severe combined immunodeficiency (*scid*) to ionizing radiation (Bierderman *et al.*, 1991). A second category relating to metastasis included XIP6 (t-PA); and XIP12 (homologous clone a human angiogenesis factor gene). The third category, designated as cellular

defense mechanisms, included two XIPs; XIP1 (a homologous clone to cytochrome p450); and XIP3 (DT diaphorase). Finally, the remainder of XIPs were placed in final category, termed unknown function (Boothman *et al.*, 1993).

The results from this research represented the first random search for specific X ray induced genes which were isolated and identified through sequence analysis. However, the report also portrays one of the shortfalls of X ray induced gene expression research. The large contrast between the results using tumorogenic U1-MEL cells and the normal GM2936B cell line could be due to the genetically damaged state of tumorogenic cell

Table 2. X ray induced transcripts and identities (Boothman *et al.*, 1993).

cDNA clone size (kb)	Inducibilty (fold) U1-MEL/GM2936B	Sequence homology to known sequences
XIP1 (5.0)	50 / 13	67% identity to cytochrome P450
XIP2 (8.0)	230 / 5	No match
XIP3 (4.0)	38 / 3	100% identity to DT diophorase
XIP4 (2.5)	47 / 3	No match
XIP5 (8.0)	30 / 4	47% identity to human growth hormone 1 and 2
XIP6 (3.2)	150 / 5	100% identity to t-PA
XIP7 (7.5)	11 / 3	62% identity to recombinase activating gene 1
XIP8 (7.0)	18 / 3	No match
XIP9 (6.5)	83 / 2	67% identity to c-*fes/fps* proto-oncogene
XIP10 (3.5)	35 / 4	No match
XIP11 (1.2)	8 / 2	100% identity to thymidine kinase
XIP12 (9.0)	9 / 3	37% identity to human angiogenesis factor

lines. The genetic response of the normal cell line, which is also genetically altered to provide immortality, could also be different from similar cell types *in vivo*. The advantages of using cell lines includes the durability of the cells and the immortal phenotype of cell lines, allowing long term culturing. However, the phenotype of cells *in vivo* cannot be studied.

A recent report described the detection of enhanced expression of elongation factor 1δ (EF-1δ) following exposure to ionizing radiation using DD, described on page 9. DD was used to identify a 1-fold increase in expression of EF-1δ in human squamous carcinoma cells (SCC-35) following exposure to ionizing radiation (1000 rads). The maximum increase in EF-1δ was observed 6-8 hours post irradiation. The EF-1 family is thought to be involved in translation inhibition during the M phase of the cell cycle and plays a role as a maturation-promoting factor suggesting a role of EF-1δ in radiosensitivity (Jung, 1994). The dose of X rays (1,000 rads), cell type used (SCC-35), and response (1 fold increase) weaken the conclusions of Jung *et al.*(1994). However, this report represented the first published use of DD in radiation response research.

Purpose of this thesis research project

The goal of this investigation was to analyze changes in gene expression in primary rat tracheal epithelial cells (RTEC) and a rat tracheal epithelial derived cell line, C-18, after exposure to ionizing radiation. A rapid scan of gene expression was done using the newly developed DD technique. A novel aspect of the project was the use of primary RTECs. The bulk of gene expression research, as described in previous sections, has used cell lines because these cells are immortal and can be maintained in culture for extended

generations. This active proliferation is not typical of growth characteristics of cells *in vivo*. The altered growth state and genomic abnormalities, such as modified ploidy of cell lines, complicates interpretation of results of experiments that use cell lines.

A wealth of information has surfaced from experiments using cell lines. However, creating scientific models that resemble *in vivo* conditions is a priority for researchers. One complication of using cells isolated directly from tissues is the mixture of cell types harvested, complicating data interpretation. Epithelial cells extracted from rat tracheas have been used to study the effects of X rays on cell survival and transformation (Terzaghi-Howe, 1990, 1992). These cells were chosen for this project because the genetic makeup resembles cells *in vivo*. Furthermore, the model developed by Terzaghi-Howe selects for a uniform, growth inhibited basal cell population which overcomes the major drawback of mixed cell populations derived from many tissue types.

This study used DD to examine the changes in gene expression in RTECs and C18 cells following exposure to X rays. C-18, a commonly used nontumorigenic cell line, was used as a cell line alternative to the RTECs used in these experiments. DD revealed 2 cDNA bands enhanced in gel lanes containing amplified genes from irradiated RTECs compared to controls. In addition, 4 cDNA bands were identified in DD gel lanes containing amplifed genes from irradiated C18 cells that were absent in control lanes. The identified cDNA bands in lanes from irradiated cells were cut, reamplifed, sequenced analyzed, and used as probes to study expression of each gene by Northern blot analysis. Most of the cDNA gene fragments were identified as coding for a variety of growth related genes including elongation factor 1 delta (EF-1 δ), mouse zinc finger mRNA, and

the cyclin A gene. In addition, other genes including an interferon related gene and a embryonic preimplantation gene, were identified. The DD protocol, results, and implications of each cDNA gene fragment will be discussed in the following chapters.

CHAPTER 2

MATERIALS AND METHODS

Cell culture and X-irradiation of RTECs and C-18 cells

Tracheas were excised from *Fischer* 344 male rats (Taconic Farms, Germantown, NY) and the epithelial cells were harvested as follows. A lengthwise excision was made along the cartilagenous side of the tracheas. The tissue was placed mucosal side up in a Petri dish. Incubation was carried out with 200-300ul of cold pronase type XIV (Sigma, St. Louis, MO) for 14 to 16 hours at 4^0 C. Epithelial cells were then rinsed from the mucosal surface and collected in 4^0 C Ham's F12 plus 2 mg/ml bovine serum albumin (Sigma, St. Louis). In order to disperse cell clumps, cells were slowly passed through an 18 gauge needle approximately 20 times. Cells were counted using a hemocytometer, and checked for cell viability using erythrosin B dye exclusion.

RTECs were center plated in 100 mm culture dishes at a density of 2,000 to 50,000 cells per dish. The cells were cultured in 2XGC medium that consisted of a 1:1 ratio of the following components. The first component of the medium included Ham's F12 (Gibco-BRL, Grand Island, NY), 5% fetal calf serum (Hyclone, Logan, UT), and 50 mg/ml gentamycin (Gibco). The first component was conditioned for 5 days on confluent 3T3J2 cells. The second component of the 2XGC medium contained 2x growth factor Ham's F12 with 0.4% volume mixed sex bovine pituitary extract (Pel Freeze, Pine Bluff, Ark), 20 mg/ml insulin (Sigma), 1 ug/ml hydrocortisone (Sigma), 10 ug/ml transferrin (Sigma), and 50 ug/ml gentamycin (Gibco). Cells were cultured at 37^0 C for 5 days prior

to irradiation, then irradiated with a X ray dose of 450 rads. A Gemini Industrial X ray generator was used delivering 1.5 rads/sec (300 KvP, 3mm Al filter). Previous data, discussed in the introduction, suggests 450 rads is an effective dose to produce a high frequency of changes in gene expression, or transformation, without producing excessive cell killing. After exposure, the irradiated C-18s and RTECs were returned to a 37^0 C incubator for a 3 hour recovery period. X-ray response research suggests late response genes are generally activated by 3 hours post irradiation. In addition, many of the early response genes implicated in the introduction showed increased expression beyond 3 hours.

Extraction and purification of total cellular RNA

Culture medium was removed from the irradiated and control RTECs and C-18 cells respectively, followed by 3 washes with DEPC treated *sans*-phosphate buffered saline (S-PBS). Diethyl pyrocarbonate (DEPC) is an effective inhibitor of RNase and is commonly used in solutions for experiments using RNA. Cells were lysed using guanine isothiocyanate (GITC) reagent. The reagent included 0.4725 g/ml GITC (Gibco), 0.835% 3M sodium acetate (NaAc) (pH 5.2), and 0.835% β-Mercaptoethanol (98%) in H_2O. The GITC reagent was applied directly to the plated cells and cells were subsequently scraped, and the cell lysate from each plate was collected.

The lysates from each cell population, irradiated RTECs, control RTECs, irradiated C-18 cells, and control C-18 cells, were layered over a CsCl gradient in Ultraclear tubes. The CsCl gradient consisted of 0.9597 g/ml CsCl, and 0.83% 3M NaAc (pH 5.2) in H_2O. The lysates were centrifuged at 35,000 rpm over the CsCl gradient overnight at 20^0 C to

separate RNA from DNA and proteins. The supernatant was removed and the tubes were inverted, allowing the pelleted RNA to dry. Using a hot razor blade, the tubes were cut off approximately 1cm from the bottom. The glassy total RNA pellet was resuspended in 1 ml DEPC treated H_2O, and transferred to a new tube. One hundred ul of DEPC treated 3M NaAc (pH 5.2) and 2.7 ml 100% ethanol (EtOH) were added, followed by storage at -20^0 C for 15-30 minutes. The precipitated RNA was spun at 10,000 rpm for 10 minutes, the supernatant was removed, and the pellet was resuspended in 1 ml DEPC treated H_2O. The quantity of total RNA was determined by optical density (OD) absorbance reading at 260 nm using a A_{260} dilution factor of 40ug/ml. The total RNA yield was as follows; X-irradiated RTECs, 172.8 ug; control RTECs, 211.2 ug; X-irradiated C-18 cells, 238.9 ug; control C-18 cells, 247 ug.

Total RNA from each cell population was treated with DNase (Genhunter, Brookline, MA) to remove exogenous DNA, which could have served as a template for the DD reaction. 50 ug total RNA in a volume of 50 ul was added to 5.7 ul of 10X reaction buffer, and 1 ul DNase (10 unit /ul). The reactions were incubated for 30 minutes at 37^0 C. Phenol/chloroform ($CHCL_3$) (40 ul) was added and the mixture was vortexed for 30 seconds. The tubes were spun at 12,000 rpm for 10 minutes, the supernatant was collected, and the DNA free total RNA was precipitated using 5 ul 3M Na Ac and 200 ul 95% EtOH at -80^0 C for 1 hour. The tubes were then spun at high speed for 10 minutes at 4^0 C. The pellets were washed with DEPC treated 80% EtOH, centrifuged again, dried, and resuspended in 50 ul of DEPC treated H_2O. The yield was determined by OD reading at 260 nm.

DD of mRNA from x-irradiated and control RTECs and C-18 cells

The concentration of the DNA free total RNA was adjusted to 0.25 ug/ul. DD (Genhunter) was done using 2 ul (0.5ug) of total RNA. Phase 1 of DD, called reverse transcription, used the semispecific downstream primer oligodT_{12}MC. The primer consisted of the following nucleotides from 5' to 3'; 12 thymine residues connected to a degenerate nucleotide (M) consisting of a mixture of the four bases (ATGC), and a terminal cysteine residue. The reaction conditions follow.

Four reverse transcriptase reactions were done in duplicate, 2 for each cell population. Duplicate DD reactions were done for total RNA from irradiated and control cells to eliminate false positives due to nonspecific amplification by PCR. The reactions were carried out in a total volume of 20 ul using MMLV reverse transcriptase (Gibco) and the components in Table 3.

Table 3. DD reverse transcription reaction conditions (Genhunter).

Rxn. Component	Volume
distilled H_2O	9.4 ul
5X Reverse Transcription Buffer	4.0 ul
dNTP (250 uM)	1.6 ul
Total RNA (.25 ug/ul)	2.0 ul
T_{12}MC (10 uM)	2.0 ul
MMLV Reverse Transcriptase	1 ul

The reaction mix, except for the reverse transcriptase, was heated at 65^0 C for 5 minutes. The reverse transcriptase was added 10 minutes into a 60 minute incubation at 37^0 C. Following the incubation period, the entire mix was heated to 95^0 C to denature the enzyme. The 4 reaction tubes were stored at 4^0 C prior to DD amplification.

Phase 2 of DD was PCR amplification of the mRNA reverse transcribed into cDNA. A modified form of the DD reaction was used that included a Taq start antibody® (Ab) for a hot start. This Ab (Clontech, Palo Alto, CA) binds the active site of the DNA polymerase enzyme used in PCR. Binding of the Ab prevented amplification until the reaction mix was heated to 94^0 C, denaturing the Taq start Ab®, and freeing the DNA polymerase. The efficiency of PCR amplification is improved when a hot start is utilized, preventing nonspecific amplification as the PCR reaction tubes pass through the 72^0 C active range of the Taq polymerase during the first cycle (Li, 1990). The Taq start Ab/DNA polymerase mix was prepared as follows. A working solution of Ab was prepared by mixing 8.8 ul of Taq start Ab® with 35.2 ul of dilution buffer. 36.0 ul of Taq start Ab working solution was added to 7.2 ul of Taq DNA polymerase, and the mixture was stored at 4^0 C during preparation of the DD reaction mix.

The cDNA from control and irradiated RTECs and C-18 cells were amplified using the $T_{12}MC$ downstream primer and five upstream primers. The upstream primers, consisting of 10 arbitrarily chosen nucleotides (10mer), were designated as AP-6 through AP-10. Table 4 contains the sequences of the AP primers used in the experiment. Two ul from each of the four RT reactions was used in combination with each of the 5 arbitrary upstream primers, yielding 20 primer combinations. Table 5 shows the amplification

Table 4. Arbitrary upstream primer sequences (Genhunter).

Arbitrary primer	Sequence (5'-3')
AP-6	GCAATCGATG
AP-7	CCGAAGGAAT
AP-8	GGATTGTGCG
AP-9	CGTGGCAATA
AP-10	TAGCAAGTGC

Table 5. DD amplification reaction mix.

Rxn. Components	Volume
Distilled H_2O	7.7 ul
10x PCR buffer	2.0 ul
dNTPs (25 uM)	1.6 ul
AP primer (2 uM)	2.0 ul
$T_{12}MC$ (10 uM)	2.0 ul
RT reaction mix	2.0 ul
^{35}S-dATP (1,200 Ci/mmole)	1.0 ul
Taq start Ab/Taq polymerase	1.8 ul

mix, which included the following; 7.7 ul distilled H_2O, 2.0 ul 10x PCR reaction buffer, 1.6 ul dNTPs (25 uM), 2.0 ul upstream primer (2uM), 2.0 ul $T_{12}MC$ (10 uM), 2.0 ul from 1 of the 4 RT reaction tubes, 1.0 ul ^{35}S-dATP (1,200 Ci/mmole), and 1.8 ul Taq start Ab/taq polymerase mix. Table 5 lists the DD amplification mix. Each reaction mix was covered with 25 ul of sterile mineral oil and amplified using the following thermocycle: 94^0 C for 30 seconds, 40^0 C for 2 minutes, and 72^0 C for 30 seconds. This cycle was repeated 40 times followed by a 5 minute extension at 72^0 C (Liang and Pardee, 1992).

Identification of differentially expressed genes and reamplification of cDNA

The products of the DD reactions were stored at -20^0 C prior to separation on a 6% polyacrylamide denaturing gel. Loading dye (3.0 ul) was added to 3.5 ul from each DD reaction tube, and loaded onto the polyacrylamide gel. The amplified cDNAs were separated for approximately 3 hours at 60-80 watts. The gel was then dried, oriented by position marks on X ray film using needle holes, followed by exposure for 48 hours. The DD autorads for RTECs and C-18s are shown in figures 4 and 10 respectively.

Lanes containing amplified cDNA from irradiated RTECs and C-18 cells were compared to amplified cDNA from control RTECs and C-18 cells respectively. Bands present in irradiated lanes, but absent or diminished in control lanes were identified as potential X ray response genes and were cut from the gel . The X ray induced bands extracted from the gels were soaked in 100 ul dH_2O for 10 minutes. The tubes were then boiled for 15 minutes and centrifuged at 12,000 rpm for 2 minutes. The supernatant was recovered and precipitated using 10 ul 3M NaAc, 5 ul glycogen (10 mg/ml), and 450 ul 100% EtOH at -80^0 C for 1 hour.

The tubes were then centrifuged at 12,000 rpm for 1 minute, washed with 80% EtOH, centrifuged again, and the pellets were dried. The cDNA pellets were resuspended in 10 ul of dH_2O and stored at -20^0 C prior to reamplification. Reamplification utilized the same reagents used in DD. The 40 ul reaction mix included the following; 20.4 ul dH_2O, 4 ul 10x PCR buffer, 3.2 ul dNTPs (250 uM), 4 ul arbitrary upstream primer (2 uM), 4 ul $T_{12}MC$ (10uM), 4 ul cDNA template (from resuspended cDNA bands), and 0.4 ul Taq DNA polymerase. The thermocycle program used in DD was repeated; 94^0 C for 30 seconds, 40^0 C for 2 minutes, 72^0 C for 30 seconds, and a 5 minute extension period at 72^0 C. Ten ul of the reamplified cDNA product was analyzed on a 1.2% agarose minigel to confirm that the reamplified band matched the size of the band extracted from the DD gel.

TA cloning of reamplified cDNA bands

The PCR amplified cDNA fragments were cloned using a TA cloning kit (Invitrogen, San Diego, CA) that exploits the addition of an extra adenine nucleotide at the end of PCR amplified fragments. The lyophilized pCRII vector was resuspended by adding 8.8 ul TE buffer (25 ng/ul). Each cDNA fragment was ligated to the vector using the following reaction: 5ul sterile H_2O, 1 ul 10x ligation buffer, 2 ul resuspended pCRII vector, 1 ul PCR amplified cDNA fragment, and 1 ul T4 DNA ligase. The reaction was incubated overnight at 12^0 C, centrifuged at 12,000 rpm, and stored at -20^0 C prior to transformation.

The pCRII vectors containing cDNA fragments were transfected into One Shot competent cells®. Each ligation reaction tube was treated as follows. Two ul of β-

mercaptoethanol and 1 ul of the ligation reaction were added to each vial of compentent cells, and the mixture was incubated at 4^0 C for 30 minutes. The tubes were then heat shocked at 42^0 C for 30 seconds and returned to 4^0 C for 2 minutes. SOC (450 ul) (BRL, Gaithersburg, MD) medium was added and tubes were incubated at 37^0 C for 1 hour, shaking at 225 rpm. The tubes then were stored at 4^0 C. LB agar plates were prepared that contained ampicillin (50 ug/ml). 25 ul X-Gal (40 mg/ml stock) was spread on the surface of the ampicillin containing LB agar plates 1 hour prior to use. The presence of the ampicillin antibiotic and X-Gal served for double selection criteria so that only cells that incorporated the pCRII vector with a ligated cDNA fragment would form white colonies. Cells that contained pCRII vector without insert form blue colonies.

Plates were streaked with 25 ul of transformed competent cells from each ligation reaction and incubated at 37^0 C for 24 hours. White colonies were chosen for plasmid purification, restriction digest, and further analysis of cDNA clones.

Mini prep and midi prep procedures

White colonies were picked from the LB agar plates using sterile toothpicks to inoculate cultures for mini preps of plasmid DNA. Toothpicks were placed in sterile 5 ml plastic tubes (Falcon) containing 3 ml terrific broth (TB) (BRL) with ampicillin (50 ug/ml) and incubated overnight in a 37^0 C shaker water bath. The tubes containing cultures were split, 1.5 ml frozen with 0.25 ml sterile glycerol and 1.5 ml centrifuged at 8,000 rpm for 1 minute. After removing the supernatant, the pellets in each tube were washed with 1.2 ml sterile dH_2O, and centrifuged again. The sterile dH_2O was removed and 200 ul of lysis buffer was added. The freshly prepared lysis buffer consisted of the

following: 50 mM glucose, 20 mM Tris-HCL (pH 8.0), 10 mM EDTA, and 4 mg/ml lysozyme. The tubes were vortexed and set at 20^0 C for 5 minutes, then 400 ul of alkaline solution was added to each tube. The alkaline solution included 0.2 N NaOH, and 1% SDS. The tubes were inverted 3-6 times and placed on ice for 5 minutes. Following the 5 minutes at 4^0 C, 300 ul of 7.5M ammonium acetate (NH_4Ac) was added and each tube was mixed well. The tubes were placed on ice for 10 minutes and centrifuged for 5 minutes at 12,000 rpm. The supernatant from each tube was transferred to a new tube and centrifuged again at 12,000 rpm for 5 minutes. The supernatant was transferred to a new tube and 500 ul of isopropanol was added. Tubes were left at -20^0 C for 15 minutes, centrifuged at 12,000 rpm, washed with 80% EtOH, centrifuged again, dried, and resuspended in 100 ul TE buffer.

The resuspended plasmid DNA from each mini prep was used to do a restriction enzyme digests to check the size of the TA clone cDNA fragment insert. An *EcoR*1 restriction digest was done for each mini prep as follows. Five ul DNA from the mini prep was added to 12 ul of distilled H_2O, 2 ul of React 3 buffer®, and 1 ul of *EcoR*1 enzyme (10 unit/ul). The reactions were incubated at 37^0 C for 1 hour, and 10 ul of cut and uncut plasmid mini prep DNA were analyzed in adjacent lanes on a 1.2% agarose gel to confirm the presence and size of inserts.

Larger batches of plasmid DNA (midi preps) were made from the mini prep cultures that contained inserts of appropriate size. Frozen cells from mini prep cultures were thawed and spread on ampicillin treated (50 ug/ml) LB plates with 25 ul X-Gal (40ug/ul). A white colony from each plate was picked with a sterile toothpick and grown in 20 ml of

TB broth with 50 ug/ml ampicillin. The cultures were then transferred to new sterile 30 ml tubes and centrifuged for 2 minutes at 8,000 rpm in a Sorvall SS34. The supernatant was removed and pellets were washed with 10 ml sterile H_2O. The tubes were centrifuged again, the supernatant was removed, and the pellets were resuspended in 3 ml of lysis buffer. The lysis buffer was the same used in the mini prep protocol. The tubes were incubated at room temperature for 5 minutes. Six mls of 0.2N NaOH with 1% SDS was added, each tube was inverted 3-6 times, and tubes were stored at 4^0 C for 5 minutes. Four and one half mls of 7.5 M NH_4Ac were added to each tube and stored at 4^0 C for 10 minutes. The tubes were spun at 10,000 rpm for 3 minutes, the supernatant was transferred to new tubes, and centrifuged again. The supernatant was transferred to a third tube and 7 mls isopropanol was added. The tubes were stored at room temperature for 15 minutes, centrifuged, washed with 80% EtOH, centrifuged again, and the pellet was resuspended in 500 ul of TE buffer.

RNA gel and Northern blot protocol

Total RNA from irradiated and control RTECs and C-18 cells respectively were separated on a denaturing RNA gel. The gel was prepared by mixing 0.6 g agarose, 48 mls DEPC treated H_2O, and 1.2 mls 0.5 M Na_2HPO_4 (pH 6.5), heating the solution to a boil then cooling to 55^0 C. Heated (55^0 C) 37% formaldehyde (10.8 mls) was added along with 10 ul ethidium bromide (EtBr). The gel was cast in a gel apparatus and allowed to cool for 1 hour.

The loading mix for RNA samples consisted of the following components; 300 ul deionized formamide, 108 ul warmed 37% formaldehyde, and 12 ul .5 M Na_2HPO_4 (pH

6.5). The samples were prepared by mixing 3 ul of total RNA (3.3 ug/ul), 9 ul loading mix, and 6.3 ul 5x RNA loading buffer. The RNA loading buffer contained 1mM EDTA (pH 8.0), 0.25% bromphenol blue, 0.25% xylene cyanol, and 50% glycerol. These samples were loaded and separated using 10 mM Na_2HPO_4 (pH 6.5) circulating buffer.

The RNA gel was blotted to Genscreen® (NEN Research Products, Boston, MA) membrane by capillary action overnight. The membrane was then washed for 10 minutes in DEPC treated 2x SSC, pictures were taken with a ruler to position RNA, and the blot was dried for 2 hour at 80^0 C in a vacuum oven. Figure 3 shows the Northern blot with the 18s and 28s ribosomal RNA bands.

The blot contained three sets of four lanes with X ray and control lanes for C-18 and RTEC total RNA respectively. The lanes were as follows; (1) C-18 X ray RNA; (2) C-18 control RNA; (3) RTEC X ray RNA; (4) RTEC control RNA; (5) C-18 X ray RNA; (6) C-18 control RNA; (7) RTEC X ray RNA; (8) RTEC control RNA; (9) C-18 X ray RNA; (10) C-18 control RNA; (11) RTEC X ray RNA; (12) RTEC control RNA. The blot, shown in Figure 3, was then split into three independent blots for probing.

Preparation of radioactive probes and secondary screen of gene expression

Probes were prepared by purifying inserts from midi preps. An *EcoR*1 enzyme digest was done using 10 ug of purified plasmid in a total volume of 50 ul. Reaction conditions were previously described. The enzyme digests were run on a 6% polyacrylamide gel, and bands containing inserts were cut out and purified as follows: the gel slices were crushed using a micropipette tip and mixed with 200 ul DNA extraction buffer. The buffer consisted of .5 M NH_4OAc, 2.5 mM magnesium acetate (MgAc), 1mM EDTA,

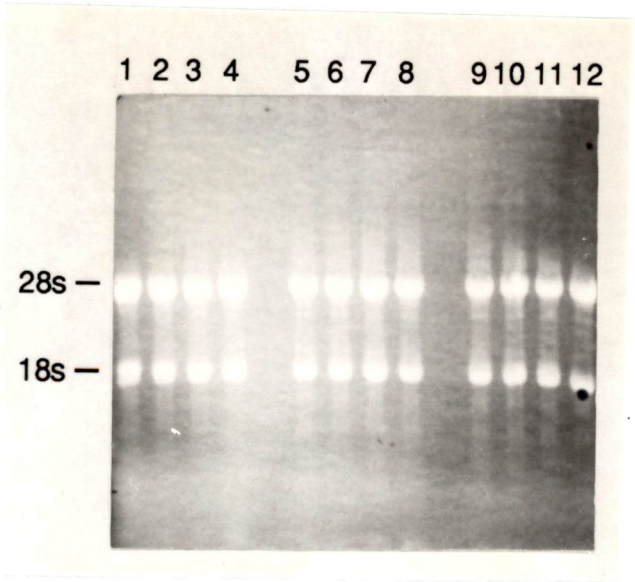


Figure 3. Northern blot with total RNA from irradiated and control C-18 cells and RTECs respectively.

and 0.1% SDS. The tubes were incubated overnight at 37^0 C, centrifuged, and the supernatant was transferred to a new tube. The DNA was precipitated using 1/10 volume of 3M NaAc, and 2.5x volume 100% EtOH at 20^0 C for 1 hour. The tubes were then centrifuged, pellets washed with 80% EtOH, centrifuged again, and the dried pellets were resuspended in 50 ul TE buffer.

The purified cDNA inserts were labeled with a ^{32}P-dCTP DNA labeling kit (Pharmacia) for hybridization of northern blots. Purified cDNA (30-50 ug) in a volume of 34 ul was used in the labeling reaction. The cDNA template was boiled for 3 minutes and cooled to 4^0 C before adding 10 ul of reaction buffer, 5 ul radioactive isotope, and 1 ul of

Klenow fragment. The reaction was incubated for 1 hour at 20^0 C and precipitated to remove unincorporated ^{32}P-dCTP label. H_2O (150 ul), 25 ul 3M NaAc, 2 ul yeast tRNA (50 ug/ul), and 650 ul 100% EtOH were added and the tubes were stored at -20^0 C for 1 hour. The tubes were centrifuged, washed with 80% EtOH, centrifuged again, and the pellets were resuspended in 52 ul of TE buffer. Liquid scintillation counts were done on 1 ul and $1x10^6$ to $10x10^6$ counts were used to hybridize northern blots.

The Northern blots were washed in prewash solution for 30 minutes at 41^0 C. The prewash consisted of the following components; 2M NaCl, 10 mM Tris-Hcl(pH 8.0), 1 mM EDTA, and 0.1% SDS. The membranes were blotted dry with 3MM paper, and placed in hybridization solution overnight at 41^0 C. The hybridization solution consisted of the following; 20 g dextran sulphate, 80 ml deionized formamide, 40 ml 20x SSC, 1.4 ml 1M Tris-HCl (pH 7.5), 3.2 ml 50x Denhardt solution, 2.0 ml herring sperm DNA (2 mg/ml), and 72 mls of H_2O.

Radioactively labeled probes were boiled for 3 minutes and immediately put on ice. The volume of each probe needed was determined by scintillation count. Ten mls of hybridization solution was heated to 41^0 C and the labeled probe was added. This mixture was immediately added to the prewarmed membrane in hybridization solution. The membranes and radioactively labeled cDNA were incubated overnight at 41^0 C in a roller oven.

The membranes were washed stringently to remove probe that was bound nonspecifically. The washing condition were as follows; 2 washes at 41^0 C in DEPC treated 2x SSC with 0.1% SDS, 3 washes in DEPC treated 0.2x SSC with 0.1% SDS at

55^0 C, and 1 wash in DEPC treated 2x SSC without SDS at 55^0 C. The membranes were then dried and exposed on X ray film. The time of exposure for each Northern blot is listed in the figure legend.

DNA sequencing and analysis by database searches

TA clones were that were confirmed by northern blot analysis were sequenced using a dideoxy sequencing kit (Pharmacia). The ligation sites were flanked by sites for the SP6 and T7 primers (Sigma). Two sequencing reactions were done for each sample, 1 from the T7 primer direction and 1 from the SP6 primer direction. Each sequencing reaction was prepared as follows. Five ul of 2N NaOH was added to 5 ug of plasmid DNA in 20 ul TE buffer and incubated at 20^0 C for 5 minutes. To this mix 5 ul H_2O, 7.5 mls 3M NaAc, and 150 ul 100% EtOH were added and stored at -20^0 C for 30 minutes. The tubes were then centrifuged, washed with 80% EtOH, centrifuged again, and the pellets dried.

Each annealing reaction was prepared by adding each of the following to the purified, dried plasmid: 2 ul of reaction buffer, 7 ul of H_2O, and 1 ul of T7 or SP6 primer. The annealing reactions were incubated at 37^0 C for 30 minutes, then centrifuged, and stored at 4^0 C prior to the sequencing reaction.

The following solutions were added to the annealing reaction; 1 ul 0.1M DTT, 2 ul diluted labeling mix, 1 ul magnesium chloride ($MgCl_2$) buffer, 0.5 ul ^{35}S-dATP, and 2 ul of diluted Sequenase®. The enzyme dilution included 9.75 ul enzyme dilution buffer, 1.5 ul Sequenase, and 0.75 ul Pyrophosphatase®. The sequenase reaction was incubated at 20^0 C for five minutes, then 3.5 ul of the reaction was added to each of 4 termination mixes; ddATP, ddGTP, ddCTP, and ddTTP. The termination mixes were prewarmed to

37^0 C. The tubes were incubated at 37^0 C for 5 minutes, before 4 ul of stop solution was added. The reaction tubes were stored at -20^0 C prior to loading on a sequencing gel.

The 4 sequencing reactions for each clone from the T7 and SP6 primer directions were analyzed on a 6% polyacrylamide denaturing gel for 2 hours at 90 watts. Sequences of each clone were determined and inserted into a computer file. These files were used to search databases (Genbank) to compare the sequences of the DD clones to known sequences.

CHAPTER 3

RESULTS

Six cDNA fragments were identified by DD that appeared to be differentially expressed in irradiated cell populations. Each band, and the secondary screening results from each are discussed in the following sections.

Potential X ray responsive RTEC genes

The amplifed cDNA from RTEC DD reactions using the semispecific downstream primer $T_{12}MC$ and arbitrary upstream primers AP-6 through AP-10 revealed 2 potential X ray responsive genes. These results are shown in Figure 4. There were 2 bands 255 bp in length that were amplified in the lanes containing DD reactions from irradiated RTECs, but were absent in the lanes with the DD reactions from control RTECs. These bands were identified as X-7 and X-10 for the arbitrary upstream primers AP-7 and AP-10 respectively. The bands were cut from the gel, reamplified, and TA cloned as described in the Methods and Materials. The cDNA insert from the X-7 clone was labeled and used to probe the Northern blot displayed in Figure 5. The Northern blot was exposed for 7 days at -80 C, revealing 2 sizes of hybridizing bands. The large bands were about 3,000 bases in length, the smaller bands were about 1,600 bases, and neither appeared to be enhanced in irradiated cell populations. The cDNA insert from the X-10 TA clone was used to probe the Northern blot in Figure 6. The Northern blot was exposed for 7 days at -80^0, revealing a 1.9 Kb transcript that was equally intense in X-irradiated and control lanes.

Figure 4. Differential display of mRNA isolated from X-irradiated and control RTEC total RNA. All reactions used oligodT_{12}MC and one of the arbitrary upstream primers AP-6 through AP-10. Two cDNA bands, X-7 and X-10, were identified as potential X ray response genes.

AP-6
Control 1
Control 2
X-ray 1
X-ray 2
AP-7
Control 1
Control 2
X-ray 1
X-ray 2
—X-7
Marker
255
216
198
185
172
AP-8
Control 1
Control 2
X-ray 1
X-ray 2
AP-9
Control 1
Control 2
X-ray 1
X-ray 2
AP-10
Control 1
Control 2
X-ray 1
X-ray 2
—X-10

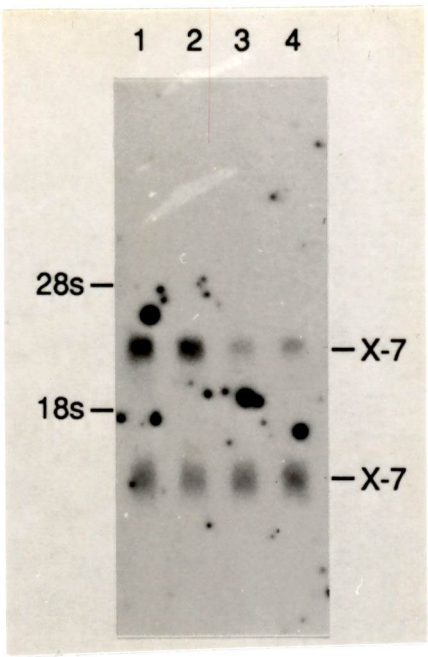


Figure 5.Northern blot analysis of cDNA fragment X-7.

(1) C-18 X ray; (2) C-18 control; (3) RTEC X ray; (4) RTEC control.

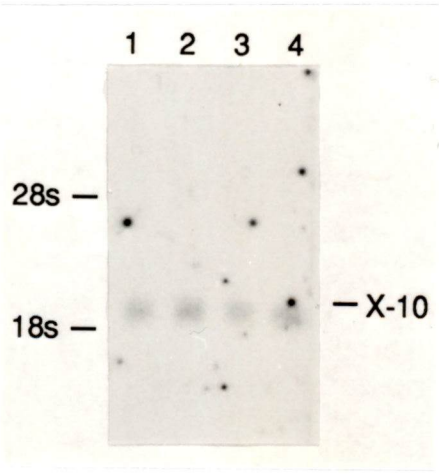


Figure 6. Northern blot analysis of cDNA fragment X-10

(1) C-18 X ray; (2) C-18 control; (3) RTEC X ray; (4) RTEC control

The X-7 and X-10 clones were then sequenced (Fig. 7) and compared to known sequences in Genbank. Both clones matched known genes with sequence identity above 98%. The X-7 clone displayed 99% identity (251/255 bases) to mouse embryogenic preimplantation gene 2C4d (Temeles *et al.*, 1994) shown in Figure 8. The X-10 clone had 98% identity (145/148 bases) to the human interferon related gene (Tirone *et al.*, 1989). The comparison of the X-10 sequence and the interferon related gene sequence is shown in Figure 9.

ggcttaaacc atgagtgcca caccagtgaa tatacagctc atgaataact
taaaatgtat ttcccattta aaaaggcaac acatagcata caaaaaccta
tactaaacaa ataagctata atatggatac atgattaatg tgtctaaaat
agatatatat cagtct

X-7

CTTCTAATGT ACATAAGCTT TTAGAGACTT TTTTATCTTG GTCAACTTAG
ATAATTTTTG ATGTAGGGAT GGGTTATATT TTAATTTAAT GTACAGTGTT
ACAAATTAAT GAGTTCTTTA TTCTGTAAAA ATAACTGATA ACCACAAATA
AAAGTGTTTG TGATGCTTGG TCAAAAAAAA AAAAAA

X-10

Figure 7. DNA sequences of the X-7 and X-10 cDNA fragments

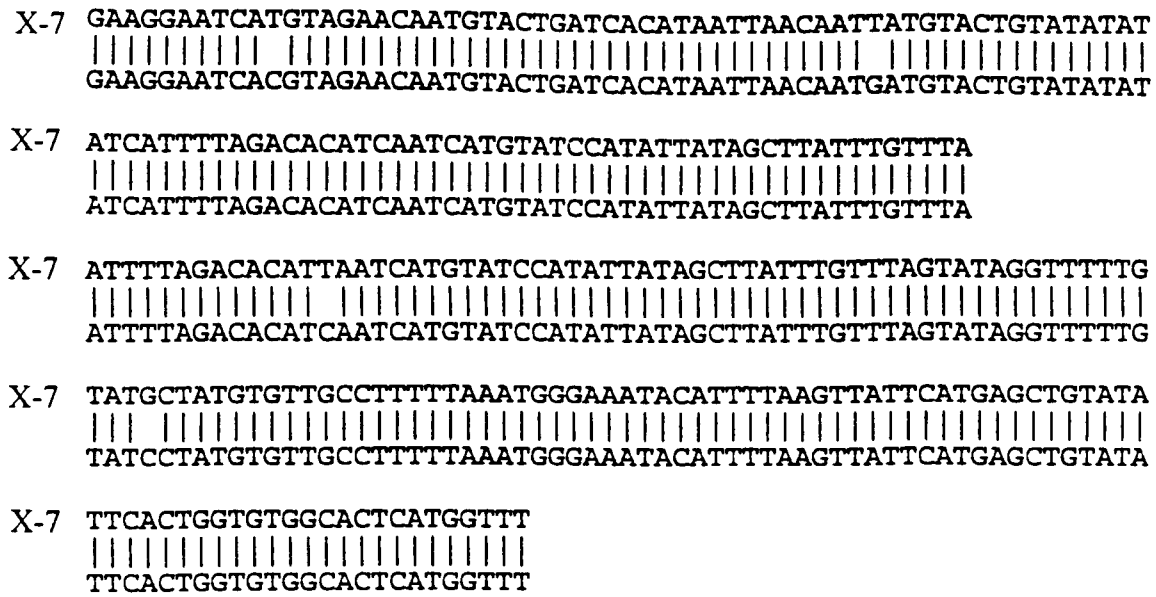


Figure 8. Genbank comparison of the X-7 sequence to the mouse embryogenic preimplantation gene.

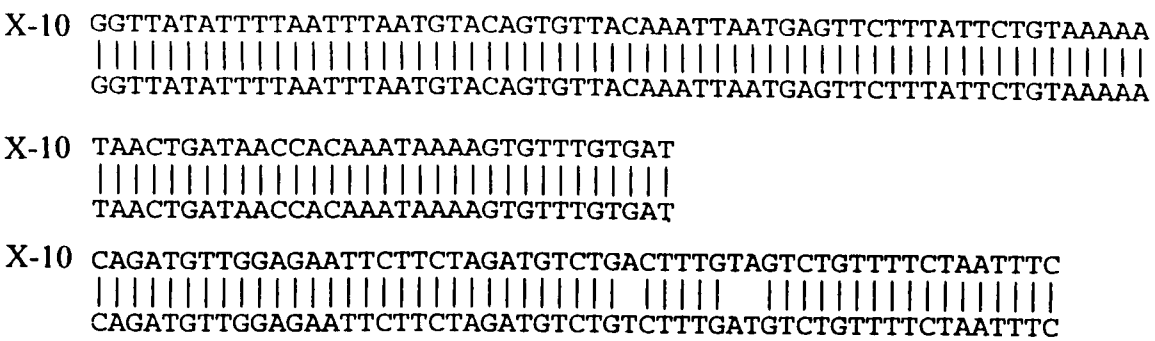


Figure 9. Genbank comparison of the X-10 sequence and the sequence of the interferon related gene.

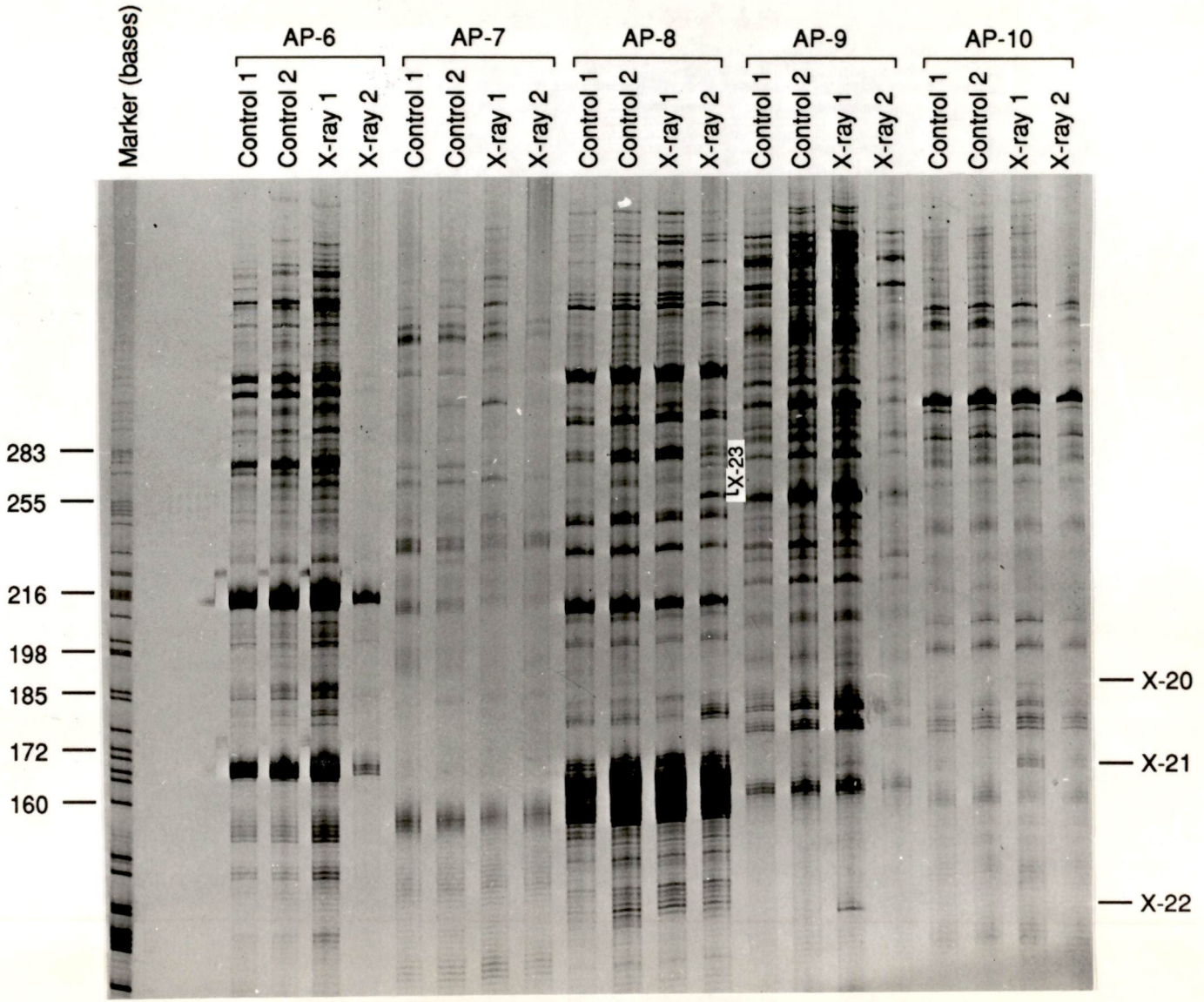
Potential X ray responsive C-18 genes

Four cDNA gene fragments were identified that were differenetially amplified from X-irradiated C-18 total RNA. They are referred to as X-20, X-21, X-22, and X-23. The C-18 DD gel and differentially amplified cDNA bands are displayed in Figure 10. Each of these bands was cut from the DD gel, reamplified, TA cloned, and sequenced. Each cDNA insert was used to do Northern blot analysis. Figure 11 shows the Northern blot analysis of cDNA fragment X-20. The 1,870 base transcript's hybridization intensity appeared equal in all lanes except lane 4. The decreased intensity in lane 4 is due to inefficient transfer of RNA in lane 12 of the northern blot (Fig. 3) shown in Material and Methods. The clone was subsequently sequenced and no matches were identified in Genbank. The sequence of clone X-20 is shown in Figure 12.

Northern blot expression of clone X-21 is shown in Figure 13, revealing a 1,800 base band that appeared equally intense in X-irradiated and control RNA. Expression levels of the transcript does appear to be dependent on rat strain and cell line, with different levels for rat lung endothelial (RLE) cells, RTECs, and C-18s. The sequence of clone X-21, when entered into Genbank, revealed that the clone had 78% identity (87/112) over 112 bases to the human elongation factor 1 δ (EF-1δ) shown in Figure 14 (Sanders, 1993).

X-22 was used as a probe, displaying the Northern blot pattern in Figure 15. The 4,500 base transcript was seen following a 10 day exposure period. It showed identical intensity in X-irradiated and control lanes. The DNA sequence of X-22, when entered into Genbank showed 67% identity (36/53 bases) to a mouse zinc finger (Zfx) mRNA (Mardon *et al.*, 1990). The comparison of X-22 and mouse Zfx mRNA is in Fig. 16.

Differential display of mRNA from X-irradiated and control C-18 cells. All reactions used the oligodT_{12}MC and one of the arbitrary upstream primers, AP-6 through AP-10. Four cDNA bands, X-20, X-21, X-22, and X-23, were identified as potential X ray response genes.


Marker (bases)
AP-6
AP-7
AP-8
AP-9
AP-10
Control 1
Control 2
X-ray 1
X-ray 2
283
255
216
198
185
172
160
LX-23
X-20
X-21
X-22

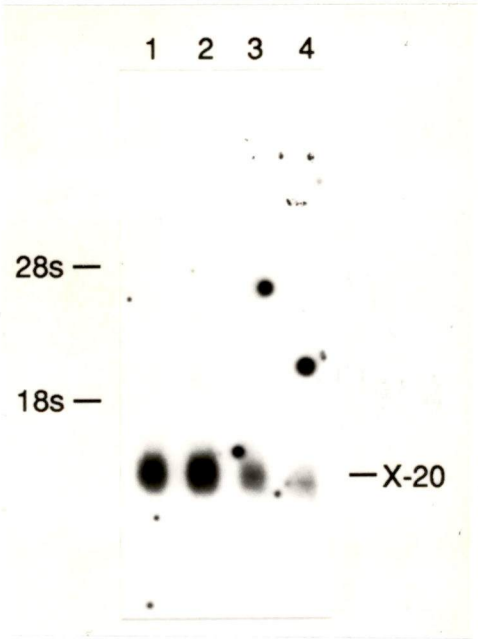


Figure 11. Northern blot pattern of clone X-20

(1) C-18 X ray; (2) C-18 control; (3) RTEC X ray; (4) RTEC control

tagcaagtgc ccagatggaa caataaaaga cgttgcctgt aacagagtaa

agttcattgc caaggaaata gctttacctt aagcaaatgc agtgggcctg

tttggactca tatgtaggag tcaaatatcc ctccatacag tgtacacttt

aatgtctaac caagaatttt aatttattat cttgcaaaaa aaaaaaa

Figure 12. DNA sequence of clone X-20

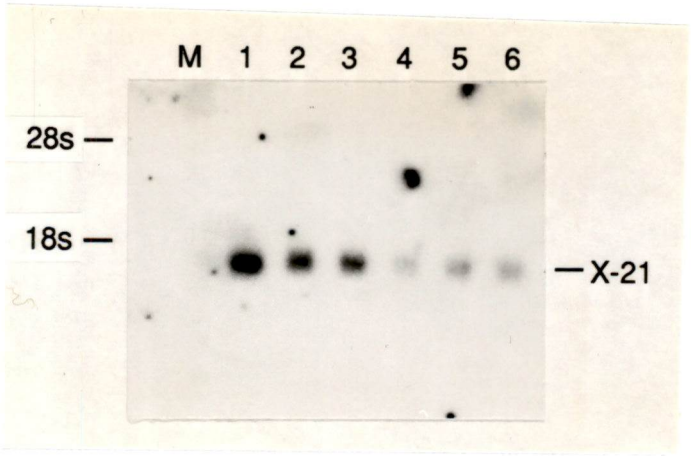


Figure 13. Northern blot pattern of X-21

(1) RLE tot. RNA; (2) C-18 control RNA; (3) C-18 X ray

(4) RTEC X ray RNA; (5) RTEC control RNA;

(6)RTEC X ray RNA

```
X-21 AGCAAGTGCAGAGTGTCGACATTGCAGCCTTCAACAAGATCTGAA
     |||| ||||||||||||||| || ||||| ||||||||||||||||
     AGCACGTGCAGAGTGTCGATATCGCAGCTTTCAACAAGATCTGAA

X-21 GAGAGTGTGTGGTTTCATGTGTGTGAGCACTGCTGAGATTAAAGACTGAGACTCCGCAAA
     | | ||  |||     | || ||| | || ||||   ||||||||||||||||     |||
     GTGTGTACGTGCGCGCGTGCGTGAGGGCCCTGCCACGATTAAAGACTGAGACCGGCAAAA

X-21 AAAAAAA
     |||||||
     AAAAAAA
```

Figure 14. Genbank sequence comparison of X-21

with human elongation factor-1 δ (EF-1δ)

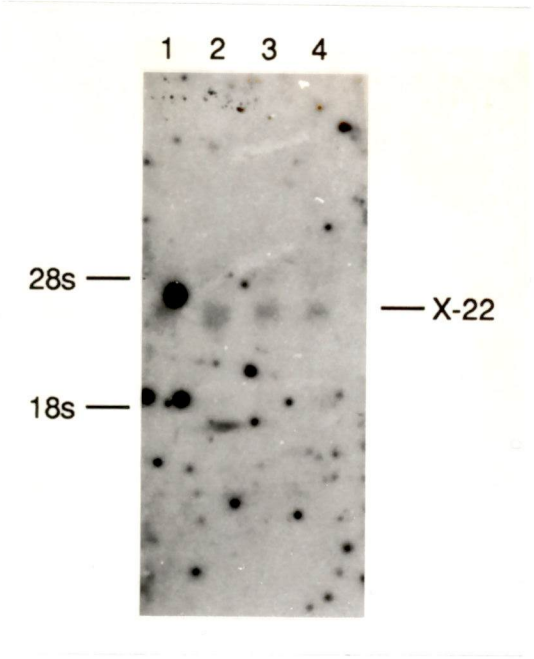


Figure 15. Northern blot analysis of X-22

(1) C-18 X ray; (2) C-18 control; (3) RTEC X ray; (4) RTEC control

```
X-22  CTCCAGATTTGTTTTGTTTTGGGTTCCCCATTTGTTTCATCTGTGTCATTGCC
      ||  |||||||||||| || |  |||| | |    |||||||| | |  | ||
      CTTAAGATTTGTTTTATTATCAGTTCACAACACATTTCATCTGAGGCCCTACC

X-22  CTCCAGATTTGTTTTGTTTTGGGTTCCCCATTTGTTTCATCTGTGTCATTGCC
      ||  |||||||||||| || |  |||| | |    |||||||| | |  | ||
      CTTAAGATTTGTTTTATTATCAGTTCACAACACATTTCATCTGAGGCCCTACC
```

Figure 16. Genbank sequence comparison of X-22

to mouse zinc finger mRNA

The final C-18 band, X-23, was used as previous cDNA fragments for Northern blot analysis. The faint transcripts, seen after a 12 day exposure, were approximately 6,000 bases in length. The band pattern of the northern probed with X-23, shown in figure 17, was not influenced by exposure to ionizing radiation. Furthermore, DNA sequence analysis did not reveal significant identity to known sequences in Genbank. The sequence of clone X-23 is presented in Figure 18.

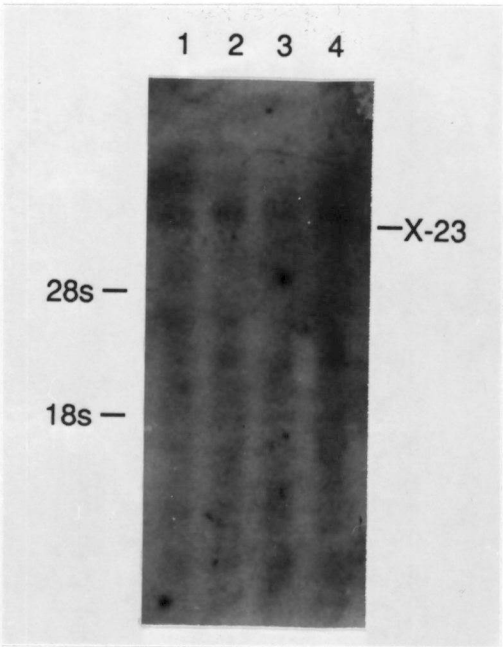


Figure 17. Northern blot analysis of clone X-23

(1) C-18 X ray; (2) C-18 control; (3) RTEC X ray; (4) RTEC control

```
ttttttttt ttttgcggtt gttaaacaaa ttctattcag tagctggaaa
agtcaatttc ctgtggaatg acaggaatta catagaagca agcaatttta
tttcctcaaa gtagc
tggattactg cttaaggacc tagaaaagaa cagcagtaag gaaagttgga
gatgctaatg tcctccccca tccccactgc accttaaagt atgcatgtca
ccttcaaggt tttataattg cactgtttt
```

Figure 18. DNA sequence of X-23 cDNA fragment

Additional clones isolated from DD bands X-10 and X-20

The efficiency of reamplification and TA cloning was checked by doing an independent reamplification and cloning of TE suspended DD bands X-10 and X-20. Each cDNA band was independently reamplifed and cloned revealing distinct gene fragments. These secondary clones were called X-10B and X-20B. When used as probes, these cDNA fragments displayed Northern blot expression patterns that were different from the original clones isolated from bands X-10 and X-20.

RTEC clone X-10B

Clone X-10B was independently produced by reamplification and cloning of DD band X-10. X-10B was used as a probe for Northern blot analysis exposing a 4,500 base

transcript in Figure 19 that is expressed at slightly higher levels in control populations. This contradicts the RTEC DD results in figure 4 where the X-10 cDNA fragment was differentially expressed in X-irradiated populations. DNA sequence analysis in Genbank, shown in Figure 20, revealed a 95% identity over a span of 50 bases to mouse cyclin A mRNA (Ravin, Genbank).

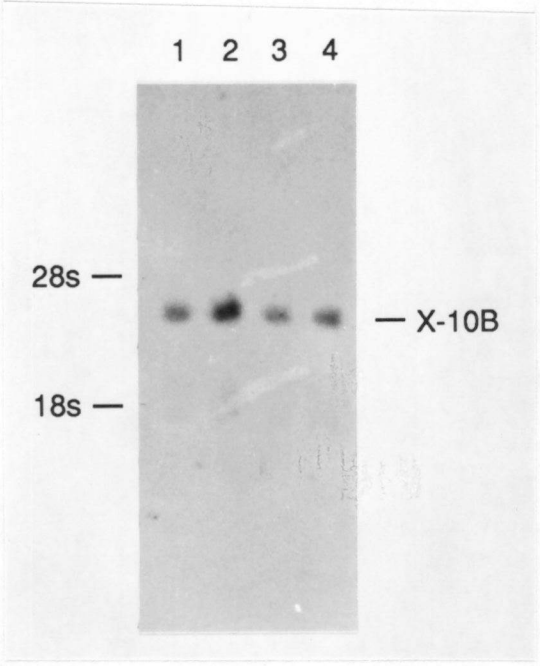


Figure 19. Northern blot analysis of X-10B

(1) C-18 X ray; (2) C-18 control; (3) RTEC X ray; (4) C-18 control

```
X-10B  CAACAGAAAAGTTTTCCAGAAAGTTTATTCGAAGCATAATGTTAAAGACAAAACAGTTTA
       ||||||||||||||||||||||||||||||| ||||||||||||||||||||||||| ||
       CAACAGAAAAGTTTTCCAGAAAGTTTATTCAAAGCATAATGTTAAAGACAAAACAGTGTA

X-10B  TTAAG
       | |||
       TCAAG
```

Figure 20. DNA sequence comparison of

X-10B to mouse cyclin A mRNA

C-18 clone X-20B

A C-18 clone, designated X-20B, was independently made from C-18 DD band X-20 shown in Figure 10. When used as a probe in Northern blot analysis, X-20B revealed a increase in intensity and number of bands in RTEC X-irradiated RNA. There were no specific transcripts observed in the C-18 lanes. This Northern blot, shown in Figure 21, also revealed 2 large transcripts that are intensified in the RTEC X ray lane relative to the RTEC control lane. In addition, several faint bands of smaller sizes appear increased in intensity in the RTEC X ray lane relative to the RTEC control lane. DNA sequence analysis, shown in Figure 22, shows over 75% identity to a wide variety of rat genes. The homology appears limited to a rat mRNA repetitive sequence (Ivanov, 1987).

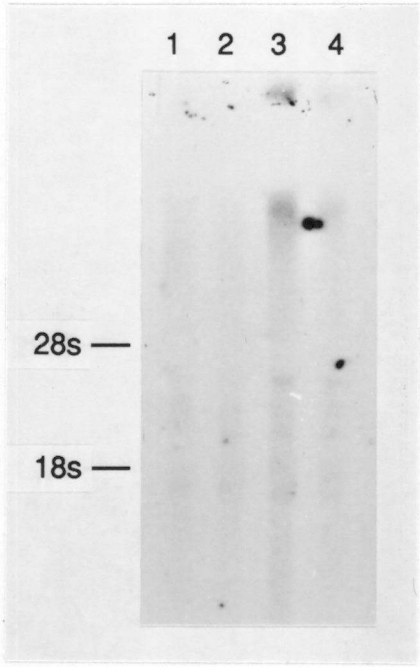


Figure 21. Northern blot analysis of X-20B

(1) C-18 X ray; (2) C-18 control; (3) RTEC X ray; (4) RTEC control

Figure 22. DNA sequence comparison of X-20B

to rat line 1 mRNA repetitive sequence

CHAPTER 4

DISCUSSION

General Conclusions

The purpose of this project was to conduct a search for X ray response genes using differential display of mRNA from rat tracheal epithelial cells. Six cDNA fragments were identified as differentially amplified in X-irradiated RTECs (Figs. 4 and 10). All of these cDNA bands were successfully reamplified, TA cloned, and sequence analyzed. Northern blot analysis using each cDNA fragment as a probe revealed a variety of band patterns and intensities, but no significant overexpression was observed in X-irradiated RNA. The results implicated a variety of growth related genes as potential X ray response genes, although Northern blot analysis suggested further analysis of each gene fragment was necessary to demonstrate specific responses to ionizing radiation.

Identification of growth related gene fragments

The original DD experiment revealed six potential X ray responsive gene fragments. Four of the six cDNA fragments, shown in Table 6, had significant identity to growth regulatory genes.

Table 6. Growth related gene fragments identified by DD.

cDNA gene fragment	Homology to known genes (% identity)
X-7	embryogenic preimplantation gene, 2C4D (98% / 255 bases)
X-10	rat interferon related gene, PC4 (98% / 148 bases)
X-21	human elongation factor 1 δ (78% / 112 bases)
X-22	mouse zinc finger mRNA (67% / 106 bases)

RTEC fragment X-7; embryogenic preimplantation gene, 2C4D

The cDNA fragment X-7, identified in DD reactions from X-irradiated RTECs, was sequence analyzed revealing high identity to the mouse embryogenic preimplantation gene, 2C4D (Temeles *et al.*, 1994). The 255 base X-7 had 98% identity (251/255 bases) and matched 90 bases upstream of the polyA tail of the 2C4D transcript. Interestingly, the X-7 cDNA appeared to be primed at both ends by the arbitrary upstream primer AP-7. The northern blots using X-7 as a probe showed two sets of bands (Fig. 5). The band intensities appeared strain specific, but were not enhanced in X-irradiated lanes.

Temeles et. al. identified the 2C4D by subtractive hybridization of large cDNA libraries derived from different stages of early mouse development. The 2C4D gene showed increased expression during oocyte and blastocyst maturation, suggesting a role in growth and development. The loss of growth control is one of the hallmarks of carcinogenesis, and the abnormal activation of a gene in adult tissues that is normally involved in embryogenic development could contribute to transformation and cancer development.

RTEC fragment X-10; rat interferon related gene, PC4

The RTEC cDNA band X-10 was reamplified, cloned, and sequence analyzed as previously described. Sequence analysis revealed high identity to the rat interferon (*INF*) related gene, PC4 (Tirone *et al.*, 1989). X-10 had 98% identity (145/148 bases) to the rat *INF* related gene and over 80% identity to the mouse *INF* β gene.

There are several classes of INFs based on the primary structure of the gene; INFα, INFβ, and INFγ. INFs are common immune system proteins that perform a variety of

regulatory functions (Coffman *et al.*, 1990). Among these functions is the ability to regulate cellular growth and differentiation. INFs have been shown to have an inhibitory effect on the growth of RT4 human bladder cancer cells (Hawkyard *et al.*, 1992). INFs have also been described as post transcriptional regulators of c-*myc* expression (Knight *et al.*, 1985). The effects of EGF and PDGF, alluded to in the introduction, were shown to be counteracted by the effects of INFs (Clemens *et al.*, 1985). The identification of an *INF* related gene fragment in irradiated cells and the previous literature suggest a possible role for INFs in the X ray response. Northern blot analysis did not reveal enhanced bands in X-irradiated lanes relative to control lanes (Fig. 6).

C-18 fragment X-21; human elongation factor 1 δ

The cDNA fragment X-21 was reamplified, cloned and sequence analyzed, revealing 78% identity (87/112 bases) to human *EF*-1δ. X-21 matched the 3' end of the EF-1δ transcript. Furthermore, sequence identity was 91% for the 45 bases at the 5' end of the match. This upstream region of the mRNA, which is 5' of the polyA tail, is more conserved than the 3' region adjacent to the polyA tail. Therefore the increased identity in the 5' region was not surprising. There were also lower identity Genbank matches to *EF*-1δ from the species; *X. laevis* and *A. thaliana*. Northern blot analysis, using X-21 as a probe, showed a set of 1,800 base transcripts that did not appear enhanced in X-irradiated lanes (Fig. 13).

EF-1δ is involved in cellular growth and differentiation. Specifically, EF-1δ is involved in the elongation step of protein synthesis (Sanders *et al.*, 1993). EF-1δ has been shown to be overexpressed 2-3 fold in the early stages of colorectal cancer development

(Ender, 1993). In addition, *EF*-1δ was previously implicated as a X ray response gene (Jung, 1994). That study differed from this project in two important respects; Jung et. al. used squamous sarcoma cells and a very high dose of 1,000 rads.

C-18 fragment X-22; potential zinc finger mRNA

Sequence analysis of the X-22 cDNA clone showed a region of significant identity to mouse zinc finger (Zfx) mRNA. There were two independent regions of the Zfx transcript that had homology to the same 53 base segment of X-22 (Mardon *et al.*, 1990). Both regions had 67% identity (37/57 bases) to the segment of X-22.The regions of high identity were 200-300 bases upstream of the polyA tail of the Zfx transcript. Northern blot analysis showed a set of 4,500 base transcripts equally intense in irradiated and control lanes (Fig. 15).

Zinc finger binding motifs are often incorporated into proteins that play a role in transcriptional regulation. For example, the egr-1 transcription factor cited in the introduction as a X ray response gene, contains a zinc finger motif. The *egr*-1 gene was shown to increase expression 18 fold in HL-525 cells following exposure to 2,000 rads ionizing radiation (Hallahan *et al.*, 1992). The type of cells and exposure used were considerably different than those used in this project.

Another growth related protein with a zinc finger motif is the Xeroderma Pigmentosum gene, *XRAC*. The XP disorder is genetically inherited and patients are hypersensitive to UV radiation. Mutations in the *XRAC* gene are largely responsible for the susceptibility of XP patients to UV radiation (Tanaka *et al.*, 1990).

Unidentified DD cDNA fragments; X-20 and X-23

Two cDNA fragments identified by C-18 DD did not show significant homology to known genes in Genbank. Several factors made identification of gene fragments difficult in this project. First, DD amplifies the 3' end of mRNA, typically producing cDNA less than 300 bases in length. The 3' region of mRNA just upstream of the polyA tail is not in the coding region of genes and is not highly conserved (Alberts, 1989). Furthermore, the rat genome is not as well characterized as the heavily studied genomes in mice and humans. Specifically, this makes significant matches in the poorly conserved 3' end of mRNA more difficult. These two factors account for the difficulty of identifying known genes in rat DD experiments. Identifying four of six gene fragments is notable given the conditions. X-20 and X-23 may represent significant X ray response genes that are not yet characterized. Future experiments could involve searching large cDNA libraries to determine the identity of the two DD fragments.

Potential reamplification and cloning of multiple DD bands

The results of this project suggest potential problems with the reamplification and cloning of DD products. Specifically, the DD results portrayed bands that were differentially expressed in irradiated cells. However, Northern blot results showed bands that were not enhanced in RNA from irradiated cells. The possibility was considered that multiple bands of the same length and same primer combination were amplified by DD. If this occurred multiple bands would be cut from the DD gel and reamplified, providing a variety of cDNA for TA cloning. The efficiency of reamplification and cloning was tested by doing a secondary reamplification and cloning of previously resuspended DD

cDNA bands. The independent reamplification and cloning of bands X-10 and X-20 produced clones X-10B and X-20B. Northern blot analysis and sequencing of these clones showed distinct results from original clones, X-10 and X-20. These results further suggest that multiple bands of the same length may be found using the DD technique. Care must be taken so the correct DD cDNA fragment is cloned and studied.

X-10B; Potential cyclin mRNA

X-10B, produced from RTEC DD band X-10, was reamplified, cloned, and sequence analyzed. Genbank analysis showed 95% identity (62/65 bases) to mouse cyclin A mRNA (Ravnik, Genbank). In addition X-10B showed 87% identity in the same region to hamster cyclin A mRNA. Northern blot analysis revealed a set of bands 4,000 bases in length that appeared slightly enhanced in control lanes. Table 7 shows the clear distinction between the clones X-10 and X-10B.

Table 7. Comparison of clones X-10 and X-10B

cDNA fragment	Transcript size	Potential Identity
X-10	1.9 Kb	INF related gene
X-10B	4.0 Kb	Cyclin A mRNA

Analysis of cyclin A expression is difficult because levels of the transcript vary widely at different stages of the cell cycle. Cyclin A levels increase sharply at the S/G_2 boundary of the cell cycle. Ionizing radiation was shown to increase cyclin A levels at the S/G_2 boundary relative to control cells (Muschel *et al.*, 1993). In addition, cyclin A was shown

to be overexpressed in synchronized transformed alveolar epithelial cells (AEC) relative to normal rat AEC's (Bui *et al.*, 1993).

X-20B; potential mRNA repetitive sequence

The cloned cDNA fragment X-20B, produced from C-18 DD band X-20, was reamplified, cloned, and sequence analyzed. The X-20B fragment showed greater than 70% identity to over 15 rat genes. Further examination showed the segment of high identity was a rat mRNA repetitive sequence (Ivanov *et al.*, 1987). Interestingly, the X-20B fragment appeared to be primed at both ends by the semispecific downstream primer oligodT_{12}MC. Northern blot analysis showed two large bands that were enhanced in the irradiated RTEC lane (Fig. 21). In addition, there were several faint bands that were more intense in the irradiated RTEC lane. The identity of the fragment supports significant background when using X-20B as a probe. There were no bands in the C-18 lanes. Table 8 shows the comparison of X-20 and X-20B. The results of clones X-10B and X-20B suggest bands isolated from DD gel may require more in depth secondary screening than originally proposed by Liang and Pardee (1992).

Table 8. Comparison of clones X-20 and X-20B

cDNA fragment	Transcript size	Potential Identity
X-20	1.87 Kb	Unknown
X-20B	6.0 / 8.0 Kb	Rat mRNA repet. seq.

Comparison of C-18 DD gel with RTEC DD gel

A thorough comparison of how cell lines and primary cells respond to ionizing radiation is not possible from the results of this project. The project included the cell line, C-18 DD, as a qualitative comparison of how cell lines and primary cells respond to ionizing radiation. However, general observations can be made. Figures 4 and 10 show that the extremely intense bands in RTEC and C-18 lanes that use the same primer pairs appear to be present in both cell types. The less intense bands, appear quite variable in the two cell types. This observation is not surprising given what is known about cellular metabolism. Genes that play a crucial housekeeping role in the structure and metabolism of cells are expressed at high levels. These genes would be expressed constitutively in most cell types, despite the environmental changes the cells may be experiencing. The low copy transcripts appear more variable between the RTECs and C-18s. This is expected due to the subtle differences between the two cell types.

Final conclusions and future directions

This project has demonstrated several of the strengths and weaknesses of differential display. Gene fragments were differentially amplified by DD in a reproducible manner, reamplified, TA cloned, and sequence analyzed. Of the six original clones, four had significant sequence identity to known genes involved in growth and differentiation. The cDNA inserts were used to produce northern blots for all six clones, although northern blot results did not support differential expression in irradiated cells.

Several inconsistencies of DD were also portrayed. The mRNA and cDNA targeted by DD can be internally primed by the semispecific downstream primer or arbitrary

upstream primer. In addition, results of this project suggest that multiple cDNA clones are produced from 1 DD gel band. This complicates secondary screening of potentially differentially expressed genes. Despite the inconsistencies, DD remains one of the most promising means for conducting rapid searches differentially expressed genes in X ray response research.

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