UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

Characterization of the Etoposide-Resistant Cell Line CHO E-126

A Thesis submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree of

Master of Science

by

James Kofi Chapman Nyaho

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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Acknowledgements

"...any scientist who cannot explain to an eight year old what he is doing is a charlatan." From "Cats Cradle" by Kurt Vonnegut

The preceding could be one of my favorite science-related quotations. Coming in a close second:

"A lab is just another place to play." Kary Mullis, Nobel Prizewinner 1993.

My dearest reader,

Fortunately for me, I have never been asked by an eight year old what my research was about. When I started my Master's it was my hope that through my research I would *exēgi monumentum aere perennius*. Time will tell. Maybe the most important things I did in the last three years will never be published in a journal. These years have been an interesting time for me during which I have learned a great many things. On occasion, I have shared some of these things with others...

The excerpt that follows is the transcript of a speech given by J. Kofi Chapman Nyaho to undergraduate science students at the University of Manitoba. (Thank you to the University of Manitoba Transcription Services Archive Division).

....but I digress, as I was saying... If ten of you were to start graduate school today, 2 would eventually leave science to pursue alternative careers; 3 would go on to do a PhD; 2 would quit their program before finishing; and 3 would finish their Master's and pursue a career in science. Regardless of what you may do, I was asked here to provide advice to those starting Graduate School in scientific research, and that I will do.

An initial consideration a student must make is where they want to do their research. For many, the answer is "wherever they will let me in the door". For those a

little luckier, location is an issue. If one could become a member of MICB, it is a membership they would be unlikely to regret. A location like MICB provides to a student both the technical and personnel resources needed to do good, relevant science. In addition, MICB offers a collegial (for the most part) atmosphere, unusual in many science institutions.

The second thing a student needs to find is a supervisor of which there are three general types. Supervisors are micro-managers, medium-managers, or macro-managers. Those who micro-manage their labs will ask the result of every gel, PCR, sequencing reaction, etc. that a student attempts. This type of supervision can be a benefit for a student who needs/wants continual feedback, advice, etc. At the other end of the spectrum are the macro-managers. These supervisors hand-over a project to a student leaving them to design and troubleshoot experiments and interpret results. With my supervisor Dr. Mowat, I was lucky enough too have had a midi-manager. I really felt like the project was my own but knew any question would result in the suggestions or help I need to answer the question. In addition, Dr. Mowat offered unwavering support and encouragement in all my decisions.

As a Master's student, who you know is much more important than what you know. Techs, R.A.s, P.D.F.s, other students, and support staff are invaluable, while journals and P.I.'s can be less so. As a young student I had an excellent introduction into the realities of research by Dr. Jennifer Brown Gladden. Joining the lab later was Shannon Neumann, a wealth of technical knowledge and friendship; Alex Hare, my right-hand man and attaché; and Yuan Gu, friend, and the man who help me end my Northern/Southern dry-spell. Other MICB members who I credit with any success I have attained include, Dr. Luke DeLange, Ludger, Kendra, Mario, Jimmy, The Gu, Lyndon, Debbie, Dr. Eisenstat, and others I have forgotten.

Now attentive audience, I will enlighten....

Unfortunately the remainder of Kofi's speech was lost in a fire due to the errant discard of a cigarette butt.

My tenure at MICB has been filled with memorable moments including: Greg Smith's discontent, Greg Smith's bachelor party, a security guard's unsettling interest in me, Justin Gawaziuk's near expulsions from his Master's program, drinking Mead in the afternoon, Christmas party hang-overs, being accused of being a dilettante, anything Dr. Lorne Brandes said, and many others.

I fear this has become entirely too long. Most acknowledgment sections comprise ½ a page however they exhibit no originality. I suppose I would be remiss here if I didn't give some word of thanks to my family. Over the last 7 years they have been willing to listen to the sermon, and contribute to the collection plate when passed around. I would be equally remiss if no mention was made of the support offered to me by Drs. Mai and Pind. Dr. Pind went out of his way to help me write the best thesis I could and both he and Dr. Mai were continually friendly, supportive, knowledgeable, and fair in their expectations of me. As I end this discourse, I would like you leave you with two quotations always at the forefront of my mind.

My life is spent in one long effort to escape from the commonplace of existence. – Sherlock Holmes

All men dream, but not equally. Those who dream by night in the dusty recesses of their minds wake in the day to find that it was vanity: but the dreamers of the day are dangerous men, for they may act their dream with open eyes, to make it possible. – T. E. Lawrence

I remain humbly yours,
J. K. Chapman Nyaho

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Abstract

The purpose of this study was the characterization of CHO E-126, an etoposide-resistant cell-line created by the transfection of CHO cells with a promoter-trap retrovirus. Experiments with the CHO E-126 cell-line suggest there has been successful promoter-trapping in these cells without the trapping construct undergoing any rearrangement. As reported in this study, sequence data has been obtained from both viral junctions and from 5' RACE experiments, with none of this sequence matching any known genes including *gadd7*, initially thought to be the viral integration site. Our results suggest that the U3NeoSV1 promoter-trap has integrated into a gene sharing homology with a putative transcribed region on mouse chromosome 11. We believe that viral integration into the unknown gene has led to resistance to the chemotherapeutic drug etoposide, decreased sensitivity to hydrogen peroxide, and a slight decrease in Cisplatin sensitivity. Interestingly the protein levels of topoisomerase II α, a target of etoposide, appear similar between CHO E-126 and parental CHO Cl-22 cells. In addition, CHO E-126 cells have a slower growth rate than parental cells and show distinct morphological changes. Similar morphological changes were seen with inducible *gadd7* cell lines, however the *gadd7* levels of induction were never quantified nor were they normalized to background levels. Other *gadd7* experiments performed prior to the correct identification of the viral flanking regions failed to show any involvement with this gene in apoptosis. At the trapping-construct's suspected site of integration the transcript produced contains a region sharing homology with the prolyl hydroxylase gene family. Apoptosis

involvement by this gene is suggested by the involvement of members of this gene family in detecting and regulating cellular oxygen radicals.

Chapter 1 – Literature Review and Background

1.1 Introduction

This thesis discusses the characterization of CHO E-126, an etoposide-resistant cell-line created by the transfection of CHO cells with a promoter-trap retrovirus. Cells containing a single viral integration in a transcriptional unit were screened for etoposide-resistance. These experiments were carried out in an attempt to identify novel components of the cellular-death process known as apoptosis. It was hoped that the etoposide-resistant cell lines generated with the U3NeoSV1 promoter-trapping construct and directed screening would also be apoptosis-resistant due to the disruption of a gene important in the execution or regulation of apoptosis. What follows is an outline of the origin, uses, and various types of gene-trapping techniques, an examination of the promoter-trap retrovirus U3NeoSV1 used in this project, a look at some of the important elements of apoptosis, a description of the apoptosis-inducing drug etoposide including it's mechanisms of action, and finally the progress made on this project prior to and since my involvement.

1.2 Genomics: Structural and Functional Approaches:

Advances in DNA sequencing technologies and computational analysis have made possible the study of an organism at the whole-genome level. Genomics, the term given to this process involves the examination of genome structure and the function of these genes in the context of an organism's entire genetic content (Keon *et al.,* 2003). Analysis of a genome requires interplay between various scientific fields in order to collect and transform raw biomolecular data into biologically significant

information. Two important elements in genomic research are structural and functional genomics.

Structural genomics provides a link between genomic sequences and the three-dimensional structure of the proteins these sequences encode. While the number of different genes within a mouse or a human may run in the tens of thousands, it is speculated that there are only 1000-5000 stable polypeptide chain folds in nature (Chothia, 1992) (Brenner *et al.,* 1997). The large globular proteins found in eukaryotes are really chains of these smaller folds performing a specialized function characteristic of their three-dimensional folding pattern. Once the biological function of polypeptide domains is determined and related to the function of the entire protein, reasonable hypotheses can occur respecting the function of gene sequences producing a similar predictive structure.

Functional genomics (as the name suggests) attempts to determine the function of the genes making up a genome. A myriad of large and small-scale techniques have been applied in accomplishing this end including: the use of "gene chip" techniques to examine the transcription of genes, Representational Differential Analysis (RDA) to analyze the varying expression of genes dependent on either cellular conditions or the type of cell (Liang and Pardee, 1992), and different techniques to knock-out or silence genes followed by the phenotypic analysis of gene-specific mutants.

Mutagenesis strategies initially focused on X-ray and chemical mutational techniques as a means to generate gene mutants. Multiple rather than single specific gene mutations are common with these techniques, making identification of a specific

gene's function problematic. Insertional mutagenesis techniques (gene targeting and gene-trapping) helped researchers overcome these limitations and now serve as an indispensable approach in functional genomic discovery and the characterization of novel and known genes (Stanford *et al.,* 2001).

Insertional mutagenesis gained prominence and underwent much development during the early 1990s. Gene-targeting experiments lead to mutagenesis in a specific chromosomal location through homologous recombination between the targeting construct and target DNA. This approach is primarily used to generate "knock out" mice with a defined mutation from embryonic stem (ES) cells targeted for that mutation (Thomas and Capecchi, 1987). Conditional recombinase systems were developed following the realization that mutations made in ES cells had the potential to be developmentally lethal, thereby preventing the study of the targeted gene after development. These systems allowed researchers to specifically control when and where a mutational event was to occur (Orban *et al.,* 1992). While gene-targeting has been an effective tool in functional genomics for experiments, including the creation of mouse models for human diseases such as DiGeorge syndrome (Lindsay *et al.,* 1999), leukemia (Collins *et al.,* 2000), and models for chromosomal translocations in human tumors (Smith *et al.,* 1995), there exists the need for a more phenotypic-driven approach in functional mutational analysis. Gene-trapping is one means in accomplishing this end.

1.3 Gene-Trapping Basics

Gene-trapping techniques allow the analysis of a gene's function following random mutagenesis, providing a useful tool in the discovery of novel genes in which a loss of function mutation produces a selectable phenotype. In general, gene-trapping is an insertional mutagenesis technique consisting of the random disruption of genes throughout a genome of interest, with gene-trap constructs acting both as a mutagen and genetic tag. The inserted DNA element contains a reporter gene whose activation signals a mutation of the gene at the locus of interest, and whose expression presumably mimics the expression of that gene (Lako and Hole, 2000). Through selection for the reporter gene's activity, up to 100% of recovered clones may contain a gene disruption. The detection of novel genes with important roles in a particular biological system is possible after observing the phenotypic effects caused by mutated or impaired genes after directed screening.

Like gene-targeting mutagenesis, gene-trapping may develop mutated cell lines as the subject of study. Additionally, ES cells containing a single characterized mutation can be used to generate transgenic organisms. There are presently many large-scale gene-trapping projects underway including: The Centre for Modeling Human Disease – Toronto, The BayGenomics Gene Trap Project – California, and The University of Manitoba, Manitoba Institute of Cell Biology – Winnipeg. The collective aim of this research is the creation of mouse cell lines with defined mutations from which new models of development, disease, and other functionally related processes may be constructed.

Efficient gene-trapping experiments require mutation of the gene by the trapping construct along with expression of the reporter gene. In addition, both the sequence of the genomic DNA at the integration site and the trapped cDNA must be straightforward and free from significant repetitive elements (Lako and Hole, 2000). Enhancer, promoter, "gene-trap", or poly(A)-trap constructs are some of the specific DNA construct types that may be used in gene-trapping experiments and each carries with it particular advantages and disadvantages.

Enhancer-trap constructs contain a reporter gene fused to a minimal promoter. Significant reporter-gene activity requires that the trapping construct integrates into or near a gene under the control of an endogenous enhancer of gene expression. Gene-trapping techniques with enhancer constructs were first developed using a bacteriophage transposable element to insert a reporter gene into the *E. coli* genome (Casadaban and Cohen, 1979). The approach was later adapted for systems other than *E. coli,* including plants and *Drosophila.* Problematic with enhancer-trapping is the ability of enhancers to act over large distances, making identification of the specific gene involved difficult and limiting the number of loss-of-function mutations. Despite this, enhancer-trapping experiments continue with the Berkely *Drosophilia* Genomics Project currently using these enhancer-traps to disrupt genes required for adult viability.

Promoter-trapping serves as one alternative to enhancer-trapping and requires integration of the trapping-construct within an actual transcriptional unit for reporter gene activity to be detected (Friedrich and Soriano, 1991). This eliminates some of the limitations of enhancer-trapping (including distance problems leading to low

generation of loss-of-function mutations), however, due to orientation and location requirements, promoter-trap constructs are less likely (up to 200-fold lower) to lead to reporter gene activity than enhancer-traps (Stanford *et al.,* 2001). After these constructs integrate into an exon a fusion transcript is produced containing the upstream exonic sequence along with the reporter gene. Successful identification of the fusion transcript is possible through use of the rapid amplification of cDNA ends (RACE) (Frohman *et al.,* 1988).

The "gene-trap" constructs contain a promoterless reporter gene with a splice acceptor (SA) site at the 5' end. Following integration into the intron of an actively transcribing gene a 5' fusion transcript is produced with the upstream coding sequence and the reporter gene. Again, 5' RACE experiments can lead to identification of the particular fusion transcript. Some gene-trapping constructs contain an element directing for the production of a polyadenylic sequence (poly(A)), whereas others rely on the integrated gene to provide the poly(A) signal. When screened for the selectable marker, the presence of a poly(A) site on the trapping construct may preferentially select for cells in which the virus has integrated into an exon as the presence of a poly(A) site in an intron alone may not be enough to terminate transcription (Levitt *et al.,* 1989). Constructs without the poly-A signal have the added advantage of allowing identification of not only 5' but 3' coding regions or untranslated regions, increasing the chance for successful gene identification. Because introns are generally larger than exons, gene-trap vectors are at least 50 times more likely than promoter-trap vectors to lead to reporter gene activity (Stanford *et al.,* 2001), however, intronic insertion carries the risk of

alternative splicing around the virus leading to the production of wild-type transcripts (McClive *et al.,* 1998).

A gene-trapping construct being developed and used to a larger extent recently is the poly(A)-trap. In these type of vectors, the poly(A) signal has been removed from the *neo* gene leading to the requirement that the virus integrates adjacent to an endogenous poly(A) site for G418 resistance (Niwa et. al.1993). In theory, these vectors would integrate exclusively in exons with a possible bias towards the 3' end of genes. These constructs have been used successfully in large-scale projects of disruption and identification of genes in mouse embryos (Zambrowicz, 1998).

1.4 The U3NeoSV1 Promoter-Trap

A promoter-trap retroviral system was utilized in order to identify novel components in apoptosis through the creation of apoptosis-resistant cell lines. Specifically, the creators of this project chose and used the U3NeoSV1 promoter trap retrovirus (obtained from Dr. Earl Ruley – Vanderbilt University) [Figure. 1] and the Chinese Hamster ovary (CHO) K1 cell line with a transfected ecotropic retrovirus receptor gene. The CHO K1 cell line was chosen due to its constant diploid chromosome number and because it is functionally hemizygous at approximately 20% of loci (Siminovitch, 1976). This means that only one mutational event will be necessary in some cases to result in a loss of autosomal gene function. The U3 enhancer region of the long terminal repeat (LTR) has been removed in the trapping vector used and the bacterial neomycin phophotranferase gene (*neo*) inserted, providing resistance to the aminoglycoside analogue G418. In addition, the provirus contains the ampicillin (amp) resistance gene, the pBR322 plasmid origin of

replication (Ori) and both LTR regions contain sequences directing polyadenylation and processing (Hicks *et al.,* 1995)

The U3NeoSV1 promoter trap vector was designed to integrate widely in a genome, however G418 drug resistance should only occur when the virus integrates within or closely located to a promoter of an actively transcribed gene (Hicks *et al.,* 1995). G418 resistance from the trapping construct requires only minimal expression of the trapped gene (Jeannotte *et al.,* 1991). It was believed that the two poly(A) sites on the virus would not only prevent transcription downstream of the virus but would also lead to preferential integration at exonic locations. In fact, half of all viral integrations are intronic (Hicks *et al.,* 1997). Because of a cryptic 3' splice site found 28 nucleotides downstream of the *neo* gene's start codon, intronic viral integration may result in G418 resistance without a disruption of cellular gene expression. Intronic viral integration may lead to the production of a fusion transcript with the target gene or the gene's wild-type transcript may be produced (Roshon *et al.,* 2003). Specifically, when U3NeoSV1 intronic integration occurs the 5' proviral poly(A) becomes located at the end of an alternative exon, which may be excluded and wild-type transcripts produced. Alternatively, the alternative exon may be included, resulting in fusion transcripts and G418 resistance (Roshon *et al.,* 2003).

1.5 Basics of Apoptosis

In our experiment gene-trapping and selection of gene-disrupted clones was followed by directed screening. The screening approach Dr. Jeannick Cizeau adopted attempts to identify novel genes whose products play an important role in the cellular death process known as apoptosis.

The term apoptosis was used in 1972 in the description of morphological changes occurring in cells during the death process. These changes included cellular shrinkage, DNA and nuclear fragmentation, cytoplasmic vacuolization, and chromatin condensation in the nuclear periphery (Kerr *et al.,* 1972). These morphological changes have since become hallmarks of the apoptotic process. This process is an energy-dependent one which may switch to necrosis (another type of cellular death) if the ATP levels required are not present (Leist *et al.,* 1997). Dysregulation of apoptosis is associated with diseases such as sepsis, HIV, multiple organ dysfunction syndrome, neurodegenerative diseases, Parkinson's disease, and the cell death triggered in healthy tissue following heart-attacks or strokes (reviewed in Kam and Ferch, 2000). An inability to induce, or a deficiency in the process of apoptosis can result in lymphoproliferative diseases or cancer. Despite the extensive research into apoptosis, including the definition of various pathways of apoptosis, many intricacies of this process have yet to be full discovered and described.

During development of the nematode *Caenorhabditis elegans* 1090 cells are produced and 131 cells undergo programmed cell death making this a useful system for studying programmed cell death [(Sulston and Horvitz, 1977) (Sulston *et al.,* 1983)]. Research with *C. elegans* indicated that the apoptosis mechanism of cell death relied on chromosomally encoded factors (Ellis and Horovitz, 1986). Early research in *C. elegans* identified two genes (*ced*-3 and *ced*-4) that were needed to initiate cell death in this organism. If these genes were reduced or lost, cells that normally died would continue to proliferate. Later research identified the *C. elegans* gene *ced*-9, which prevented cell death by negatively regulating the activities of other

apoptotic molecules (Hengartner *et al.,* 1992). Mammalian homologues to these nematode cell death genes were soon identified. Cysteine proteases, now known as caspases were found to be homologous to c*ed*-3 (Yuan *et al.,* 1993) and *ced-9* shared homology with the mammalian proto-oncogene *Bcl*-2 (Korsmeyer, 1992). Following years of research with *C. elegans* it became clear that apoptosis is a genetically conserved and controlled process in which specific genes act in a defined molecular genetic pathway (Horvitz, 1999). It is the genetic control of apoptosis, the determination of genes necessary and sufficient to drive or inhibit the pathway, which is of interest in this study.

There are many triggers of apoptosis. Some of the various initiators include: hypoxia (Sheridan *et al.,* 1984), viral infection (Meyaard *et al.,* 1992), treatment with cytotoxic agents including anti-cancer drugs (Burger *et al.,* 1979), activation of death receptors (Laster *et al.,* 1988), DNA damage (Allan and Harmon, 1986), physiological triggers such as glucocorticoid hormones (Wyllie, 1980), and many others. These triggers have been divided into two general categories. The first consists of death receptor-mediated apoptosis (see Schmitz *et al.,* 2000), the second being mitochondria-mediated apoptosis (see Debatin *et al.,* 2002). Despite the fact that the causes of apoptosis may vary, it was suggested that there exists a shared mechanism guiding apoptosis (Wyllie *et al.,* 1980). The mechanism of apoptosis control following apoptotic stimuli is now known as the genetically controlled "decision phase" followed by the "execution phase" in which proteases and nucleases act against the cell causing the morphological changes of apoptosis and death (Kam and Ferch, 2000).

Receptor-mediated apoptosis relies on the binding of a ligand to a member of the TNF-receptor superfamily, which results in receptor trimerization and recruitment of other proteins (such as TRADD or FADD) that in turn recruit pro-caspases 8 and 10. When this receptor/adaptor-protein/pro-caspase complex is formed auto-proteolytic cleavage by the pro-caspases results in active caspases (for a review see Rossi and Gaidano, 2003.). Following caspase activation two different pathways may be activated depending, on the cell-type (Scaffidi *et al.,* 1998). The initiator caspases (8 or 10) can directly activate executioner caspases 3 and 7 (Type I cells) (Scaffidi *et al.,* 1998). Alternatively, caspase 8 may cleave the pro-apoptotic protein Bid, which when truncated, translocates to the mitochondrial leading to mitochondrial permeabilization and the activation of executioner caspases (Li *et al.,* 1998).

The involvement of the mitochondria and mitochondrial proteins in apoptosis is extensive. The mitochondria may be involved in amplifying the apoptotic signal from the receptor-mediated apoptosis pathway through caspase 8 activation (Kuwana *et al.,* 1998) or may act through it's own pathway of apoptosis induction. Pro-apoptotic members of the Bcl-2 family such as Bax (Rosse *et al.,* 1998), and Bid (Luo *et al.,* 1998) can translocate to the mitochondria upon an apoptotic stimuli, resulting in the release of proteins from the intermembrane space in the mitochondria. One of the proteins released, cytochrome c, is encoded by the nucleus but when imported to the mitochondria becomes coupled with a heme group (Yang *et al.,* 1997) and in this form can induce caspase activation (Kluck *et al.,* 1997). Other gene products including Bcl-2 can block cytochrome c release and prevent apoptosis (Yang *et al.,* 1997). The mitochondrial protein, Smac/DIABLO, is released along with cytochrome

c and promotes apoptosis by binding inhibitors of apoptosis proteins (IAPs) and preventing their inhibition of caspase activity (Du *et al.,* 2000). IAPs are also inhibited by the mitochondrial protein Omi/HtrA2 which (like Smac/DIABLO) contains IAP-binding motifs (Hegde *et al.,* 2002). What triggers the release of the Omi/HtrA2 and Smac/DIABLO proteins from mitochondria remains unknown. Two other important factors found in the mitochondrial intermembrane space are AIF and Hsp60/10. The chaperone proteins Hsp10 and 60 are found in a complex with pro-caspase 3 and promote the activation of this protein (Samali *et al.,* 1999). AIF is a flavoprotein, synthesized in the cytosol and translocated to the mitochondria (Daugas *et al.,* 2000), which when released leads to chromatin condensation, DNA fragmentation, and apoptosis (Susin *et al.,* 1999).

The two genes of most importance in the decision phase of apoptosis are *Bcl-2* and *p53*. The *Bcl-2* gene was found as the translocated locus in a B-cell leukemia. Specifically, the region of chromosome 18 containing the *Bcl-2* locus underwent a reciprocal translocation with a region on chromosome 14 containing an immunoglobulin heavy chain locus, placing the *Bcl-2* gene near the immunoglobulin gene's enhancer (Tsujimoto *et al.,* 1984). This leads to overexpression of the *Bcl-2* gene, whose product was found to prevent apoptosis (Henderson *et al.,* 1991). Later research determined that *Bcl-2* was actually a member of a gene family whose members may also prevent apoptosis such as Bcl-x_L (Boise *et al.,* 1993), or may promote apoptosis such as Bax (Oltvai *et al.,* 1993), Bad (Yang *et al.,* 1995), Bak (Kiefer *et al.,* 1995), and Bcl-x_S (Boise *et al.,* 1993). After the discovery that the members of the Bcl-2 family could form heterodimers with each other (Oltvai *et al.,*

1993) it became evident that the expression and subsequent ratio of pro-apoptotic to anti-apoptotic proteins was a determining factor in whether apoptosis would be triggered or not (Oltvai and Korsmeyer, 1994).

The *p53* tumor suppressor gene determines the cellular response to a wide variety of stresses, including: oxidative and reducing stresses; depletion of ribonucleotides; oncogenic stimuli; DNA damage; and many others [For a review see: (Giaccia and Kastan, 1998)]. P53 has a well established role in cell cycle progression and also plays a significant role in the control of apoptosis (Yonish-Rouach *et al.,* 1991). A few of the many ways the *p53* gene exerts control over apoptosis include: inhibiting expression of the anti-apoptotic protein Bcl-2 directly (Miyashita *et al.,* 1994) or activating proteins such as Cdc42 which inactivate Bcl-2 (Thomas *et al.,* 2002); activating transcription of pro-apoptotic genes such as *bax* (Miyashita and Reed, 1995), PAC1 (Yin *et al.,* 2003); APAF1 (Robles *et al.,* 2001) and others (for a review see Vogelstein *et al.,* 2000); activating genes involved in mitochondrial apoptotic signaling (Polyak *et al.,* 1997); and/or inducing death receptors such as Fas (Muller *et al.,* 1998) and the Fas related death receptor DR5 (Sheikh *et al.,* 2000).

Once apoptosis is activated, many proteins comprise the machinery which executes the cell death program. Some of the more important components of this pathway are members of the caspase family of proteins. Overexpression of cDNAs encoding these cysteine proteases can induce apoptosis in cultured cells (Kumar *et al.,* 1994) (Wang *et al.,* 1994) and either viral genes (Turner and Moyer, 1998), or specific peptide inhibitors (Thornberry *et al.,* 1994) preventing the activities of caspases can suppress successful apoptosis execution. Studies (reviewed in Stennicke

and Salvesen, 2000) have revealed a pathway of caspase activity in which initiator caspases activate executioner caspases responsible for the cleavage of cellular components. In the cell, caspase activity is regulated by conversion of the proteins from their pro-enzyme state in response to apoptotic or inflammatory stimuli and by the specific inhibition of active caspases by natural caspase inhibitors including members of the inhibitors of apoptosis protein family (IAP) (reviewed in Denault and Salvesen, 2002). Members of the IAP protein family contain between one to three domains of the zinc-binding baculovirus inhibitor of apoptosis repeats (BIR) necessary for these proteins to bind to active caspases (Deveraux and Reed, 1999). This binding prevents caspases from interacting with their substrates and executing apoptosis (Shiozaki *et al.,* 2003). To date, 8 members of the mammalian IAP family have been identified (Verhagen *et al.,* 2001) with XIAP, cIAP-1, and cIAP-2 being the most effective (Roy *et al.,* 1997). The execution of receptor-mediated apoptosis may also be inhibited by FLIP (FLICE-inhibitory protein) (Irmler *et al.,* 1997), a caspase-8-like protein that competes with initiator caspases for the death-receptor binding sites (Hu *et al.,* 1997). FLIP may also interact with TNF-receptor associated factors (TRAF) 1 and 2 and the kinases RIP and Raf-1, leading to the activation of the Nf-kappaB and extracellular signal regulated kinase (Erk) involved in pro-survival signaling (Kataoka *et al.,* 2000).

1.6 Etoposide and Topoisomerases

Initial screening for apoptosis resistant clones following promoter-trapping was accomplished with the chemotherapeutic drug etoposide (VP-16) by Dr. Jeannick Cizeau. Etoposide is an antineoplastic agent derived from the plant *Podophyllum*

peltatum also known as the May apple or mandrake. This plant produces podophyllotoxin, an aryltetralin-type lignan which was discovered to be an effective treatment for penile condylomata acuminata (von Krogh, 1978) and had potential as a treatment for different cancers including lung, testicular, and leukemia (Jardine, 1980) (Arnold, 1979). Etoposide went on to become the most widely prescribed chemotherapy drug until the development and use of taxane-based drugs (paclitaxel and docetaxel).

While developing the model of the DNA double helix it was hypothesized that it was essential for the two DNA chains to untwist to allow them to separate (Watson and Crick, 1953). The topoisomerase family of enzymes plays a crucial role in gene transcription and chromosomal replication. The first type of topoisomerase (topoisomerase I) generates single-stand nicks in DNA to relieve supercoiling stresses and allow gene transcription to proceed. The second class of topoisomerases (topoisomerase II) binds to and cleaves double-stranded DNA, retaining hold of the two cleaved ends (Liu *et al.,* 1983). Following cleavage, a double-stranded helix is passed through the break, effectively untangling and resolving any knots generated during the replication process (Wang, 1985). Either an absence or excess of the topoisomerase II-DNA complex (cleavage complex) can have detrimental effects on a cell. When deprived of topoisomerase II and lacking the cleavage complex cells die of mitotic failure (Wang, 1996). Under normal conditions the cleavage complex is short-lived and tolerated by a cell, however an excess of the cleavage complex has toxic effects on cells (Wang, 1990).

Because of the essential role topoisomerase II has in biological functions it has been a target for antineoplastic agents. An interaction with topoisomerase II was first suggested as the mechanism by which etoposide acts against a cell (Long and Minocha, 1984). When treated with etoposide, cells underwent protein-associated DNA breaks; however the mechanism by which this occurred was unknown. It was later determined that etoposide treatments led to increased levels of topoisomerase II-induced cleavage complexes in cells along with an inhibition of the enzyme's ability to religate cleaved DNA (Osheroff, 1989). The name "topoisomerase II poison" was given to etoposide and other drugs that convert the enzyme into a cellular toxin creating DNA lesions which when detected result in a pathway of apoptosis (Sordet *et al.,* 2003). Specifically, DNA lesions are detected by the protein kinases DNA-PK, ATM, and ATR, which bind to DNA breaks and phosphorylate substrates including kinases such as c-Abl and Chk2 (Kharbanda *et al.,* 1997) (Shafman *et al.,* 1997) (Xu *et al.,* 2002). c-Abl then functions as an activator of cell death pathways such as SAPK, p53, and p73 and an inhibitor of cell survival pathways such as PI(3) kinase (Sordet *et al.,* 2003). Chk2 also functions as an apoptosis inducer by the phosphorylation and activation of the promyelocytic leukemia (PML) protein (Yang *et al.,* 2002) and p53 (Chehab *et al.,* 2000). In addition, etoposide treatment can lead to the activation of serine palmitoyltransferase, the initial and rate-limiting enzyme in the pathway for the *de novo* synthesis of ceramide (Perry *et al.,* 2000), a molecule important in the cellular response to stress and induction of apoptosis (For a review see Hannun and Luberto, 2000). Other drugs such as merbarone (Drage *et al.,* 1989) and dioxopiperazine derivatives (Tanabe *et al.,* 1991) do not stabilize cleavage

complexes causing fatal double-stranded DNA breaks but do inhibit topoisomerase II activity. Drugs acting in this manner are termed "topoisomerase II catalytic inhibitors". The mechanisms by which catalytic inhibitors and topoisomerase II poisons work appear to be distinct with cells resistant to catalytic inhibitors showing no cross-reactivity to topoisomerase II poisons (Sehested *et al.,* 1998).

1.7 Prior Progress and Results in the creation of Etoposide-resistant cell-lines

All cell-lines used in this project were created by Dr. Jeannick Cizeau. In the experimental approach CHO K1 cells transfected with an ecotropic retrovirus receptor gene were infected with the pU3NeoSV1 retrovirus at a ratio of one virus per 20 cells to prevent more than one virus integrating into a single cell. These cells were selected for viral integration with G418 and approximately 5 x 10^4 clones on 200 plates were collected and separated into 50 pools. It has been estimated that a library containing 10^4 - 10^5 gene-trapped cells could represent a disruption of potentially all single-copy number cellular genes (Chang *et al.,* 1993). Our recovery of null mutations (with relation to apoptosis-related genes), should have been enhanced by the selective pressure the cells were subjected to. Cells from the 50 pools were re-plated in the presence of the apoptosis inducing drug etoposide at a high concentration (greater than three logs of kill or 99.9%) to give little or low background survival during selection. One thousand colonies per plate were treated for 48 hours then washed with PBS and allowed to recover. Drug resistant colonies were picked and re-selected at a higher etoposide concentration. Parallel experiments were carried out using the same population of uninfected parental CHO K1 cells and

the same platting conditions. These control experiments did not result in the production of any etoposide-resistant colonies.

Etoposide-resistant clones were examined by Dr. Cizeau for resistance to other apoptosis inducers including granzyme B or granzyme K, gamma irradiation, ceramide, staurosporine, TNF-α, and anti-Fas antibodies, in order to eliminate clones resistant to apoptosis through any mechanism other than the disruption of an apoptosis-related gene [Table 1]. Cell lines showing cross-resistance to other apoptosis inducing drugs were identified as starting points for future study because these cells have a high probability of carrying a disruption in an apoptosis-related gene. The detection of cross-resistance may help to determine the function of the gene inactivated by the virus, however it is important to realize that different xenobiotic chemicals inducing apoptosis may operate through different pathways (Dini *et al.,* 1996).

Preliminary experiments to determine the site of viral integration in the etoposide resistant cell line 126 (CHO E-126) suggested that the promoter-trap construct was integrated into the *gadd7* gene, also known as *adapt15*. This gene was identified in 1996 as a UV (Hollander *et al.,* 1996) and hydrogen peroxide (Crawford *et al.,* 1996) inducible gene. The suggestion that our promoter-trap had integrated into *gadd7* was interesting as *gadd7* is a single-copy number gene induced by alkylating agents such as methyl methanesulfonate (MMS) and mechlorethamine HCL (HN2), growth arrest signals such as medium depletion, and DNA damaging agents. While *gadd7* is polyadenylated, no large open reading frame is apparent in this gene, however, *gadd7* is associated with polysomes (Crawford *et al.,* 1996). This led to the

suggestion that *gadd7* acts as a functional RNA protecting cells from stressful conditions.

1.8 Rationale and Approach of the current study

The goal of this study was to characterize the etoposide-resistant cell line CHO E-126. It is our hypothesis that promoter-trapping and directed-screening with etoposide has created multiple cell-lines (CHO E-126 being one) being apoptosis deficient due to a gene interruption. Of immediate interest was the correct identification of the trapped-gene along with examination of any virally-induced fusion transcript. Inverse PCR and 5' RACE experiments yielded these results. Work on this cell-line prior to this study had suggested an alternative site of viral integration. Northern blots, Southern blots, a Reverse-transcriptase PCR experiment, and analysis of the sequence data obtained in this study did not support the previously suspected site of viral integration.

An assessment of CHO E-126 cells at the phenotypic level was of interest in order to relate the viral mutation with the gene's function. This included measuring the cross-resistance of E-126 cells to other apoptosis inducers. E-126 cells were also examined for other changes found in cells resistant to etoposide including altered growth rates, topoisomerase IIa protein levels, and cellular morphology.

Chapter 2 – Experimental Procedures

2.1 Cell Culture Conditions

Unless otherwise stated, all cells were grown in a humidified atmosphere of 5 % CO_2 at 37°C in Minimum Essential Medium-alpha (MEM-α) medium (Invitrogen, Burlington, Ontario) containing 3.7 g of sodium bicarbonate per litre, 100 μg/ml streptomycin, 100 U/ml penicillin, and supplemented with 10 % fetal bovine serum.

2.2 Viral re-arrangement analysis by genomic PCR

2.2.1 Primers (See Figure 2):

Primer Name	Primer Basis	Primer Sequence 5'→ 3'
Neo1	U3NeoSV1 Neomycin Resistance Gene	CCA TCT TGT TCA ATC ATG CGA AAC GAT CC
Amp1	U3NeoSV1 Ampicillin Resistance Gene	GCC GAG CGC AGA AGT GGT CCT G
NeoF1	U3NeoSV1 Neomycin Resistance Gene	ATG ATT GAA CAA GAT GGA TTG CAC G
NeoR2	U3NeoSV1 Neomycin Resistance Gene	GGA ACT CGG ACC GCT TGT CAA G

2.2.2 Genomic DNA extraction

Genomic DNA from CHO E-126 and CHO Cl-22 cells grown under standard conditions was isolated using the GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis). DNA concentrations were determined spectrophotometrically.

2.2.3 PCR, Electrophoresis, and Visualization

0.2 μg of genomic DNA was placed in 100 mM Tris-HCl (pH8.3), 10mM dNTP Mixture (TaKaRa Biomedicals), 5 units/μl *TaKaRa Taq* (TaKaRa Biomedicals), with primers of Neo1 (20 pmols) and Amp1 (20 pmols), or NeoF1 (20 pmols) and NeoR2 (20 pmols), and brought to a final volume of 50 μl. The samples were heated to 94 °C for 2 minutes and then subjected to 35 cycles of 94 °C for 20 seconds, 65 °C for 30 seconds, and 72 °C for 2 minutes. A final extension took place for 15 minutes at 72 °C. After amplification the samples were separated by electrophoresis and visualized on a 1 % agarose gel containing 0.5 μg/ml ethidium bromide.

2.3 MTT examination of cross-resistance to apoptosis inducers

Two thousand cells in a volume of 100 μl of medium per well were seeded in 96 well plates. The cells were incubated at 37 °C (5 %) CO_2 for 24-48 hours (until wells were ~50 % confluent). Cells were treated with various concentrations of Etoposide, Doxorubicin, Cisplatin, and Taxol and returned to the incubator for 48

hours. For Hydrogen Peroxide analysis cells were treated with either 200 or 400 μM Hydrogen Peroxide in media for 1 hour, washed with PBS, and normal medium added to wells. The plates were returned to the incubator for 24 hours.

After treatment for the appropriate time 10 μl of MTT Formazan [1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan (Sigma-Aldrich, St. Louis)] (5 mg/ml in PBS) was added to each well and the plates returned to the incubator for 3 hours to allow formazan crystal formation. Plates were placed in the centrifuge at 2000 rpm for 10 minutes to sediment the formazan product. The medium was then removed, 100 μl of DMSO was added per well, and plates were mixed thoroughly with a mini-shaker. The optical density (OD) was determined at 640 nm using a Titertek Multiskan MCC/340.

2.4 Western Immunoblot analysis of topoisomerase IIa protein levels

2.4.1 Protein Sample Preparation

CHO Cl-22 and E-126 cells were harvested, washed twice with PBS and resuspended in NP-40 Cell lysis buffer (1 % Nonidet P-40, 10 mM HEPES, 10 mM NaCl, 1 mM EDTA, 0.1 mM Sodium Orthovanadate, 80 mM B-glycerophosphate, 1 mM PMSF, 50 mM NaF, 10 μg/ml aprotinin, and 5 μg/ml leupeptin). Cells were placed on ice for 10 minutes with intermittent shaking. Cellular debris was removed by microfuge centrifugation at maximum speed at 4 °C and the supernatant was stored at -80 °C. Protein concentration was determined using the Bio-Rad *DC* Protein assay.

2.4.2 Electrophoresis and Protein Transfer

For western immunoblot analysis, 5, 10 or 20 μg of protein were loaded onto a 12 % polyacrylamide gel in loading buffer (50 mM Tris pH 6.8, 100 mM DTT, 2 % SDS, 0.1 % BPB dye, and 10 % glycerol) and separated by electrophoresis at 40 mA in running buffer (25 mM Trizma base, 192 mM glycine, 0.1 % w/v SDS). Separated protein from the gel was transferred to an Immobilon-P Polyvinylidene fluoride (PVDF) hydrophobic membrane (Millipore) for 1 hour at 100V in transfer buffer (25 mM Tris, 192 mM glycine, and 20 % methanol).

2.4.3 Immunodetection

For immunodetection, the membrane was blocked for 1 hour at room temperature with 5 % skim milk powder in TTBS buffer [TBS (10 mM TRIS, 150 mM NaCl pH 7.4) with 0.1 % Tween 20]. Following three 15 minute washes in TTBS the primary antibody was added as indicated in 1 % skim milk powder in TTBS and incubated overnight at 4 °C with shaking. Following two 20 minute washes the secondary antibody (Horseradish Peroxidase conjugate) was added and incubated for 1 hour at room temperature with shaking. Two 15 minute, and two 30 minute room temperature washes in TTBS preceded detection using the ECL Plus Western blotting reagents (Amersham Pharmacia Biotech).

2.4.4 Stripping and Re-probing membrane

To strip and re-probe following ECL detection, the membrane underwent two 15 minute washes in TTBS at room temperature and was incubated for 30 minutes at

50° C in Stripping buffer (100 mM β-mercapto-ethanol, 2 % SDS, 62.5 mM Tris-Cl pH 6.8) with occasional shaking. After two 15 minute washes in TTBS the membrane was ready to be re-blocked, and re-probed.

2.4.5 Antibodies

The following antibodies were used in this study: anti-topoisomerase II (Dr. Jack Yalowich – Associate Professor of Pharmacology, Pittsburgh School of Medicine) 1:5000 dilution; anti-actin (Sigma, product no. A20666) 1:150 dilution; and anti-rabbit IgG (Whole Molecule) Peroxidase Conjugate (Sigma, product no. A0545) 1:15 000 dilution.

2.5 Growth Rate Assessment of CHO Cl-22 and E-126

One thousand (10^3) CHO E-126 and Cl-22 cells were plated in triplicate on 10 cm dishes for 4 time periods. Cell number was determined using a Reichert Bright-Line Hemacytometer. Triple aliquots of both cell types were counted at times: 0, 36 hours, 84 hours, 136 hours, and 165 hours. To count total cells on a plate, the cells were washed with PBS and harvested with 0.05 % trypsin. Trypsinzation was stopped using complete media and cells were pelleted at 2000 rpm for 5 minutes at 4 °C. The cells were resuspended in media and three counts were taken for each sample.

2.6 Immunocytochemistry analysis of cellular morphology

2.6.1 Cell preparation

Cells were grown on coverslips until they were 60-70 % confluent. The coverslips were washed once with PBS, and cells were fixed with 4 % paraformaldehyde in PBS for half an hour at room temperature. The cells were washed once with PBS and three times for 10 minutes in PBS with 0.05 % Triton X-100 with shaking.

2.6.2 Antibody staining

Following the washes, coverslips were blocked for 2 hours at room temperature in blocking buffer (1 X PBS, 5 % Fetal Bovine Serum, 0.2 % Triton X-100, 0.02 % Sodium Azide, and 0.1 % Bovine Serum Albumin) with shaking. The appropriate primary antibody was diluted (as indicated below) in blocking buffer, placed on the coverslips, and incubated in a humidity chamber overnight at 4 °C. The next day the coverslips were washed 3 times for 10 minutes in PBS with 0.05 % Triton X-100 with shaking and then the appropriate secondary antibody was diluted (as indicated below) in blocking buffer, placed on the coverslips, and incubated at room temperature for 2 hours. After incubation the coverslips were washed 3 times for 10 minutes in PBS with 0.05 % Triton X-100 with shaking, rinsed briefly with PBS alone, and mounted on slides with mounting medium containing DAPI (Vectashield Mounting Medium for Fluorescence with DAPI – Vector Laboratories Inc).

2.6.3 Antibodies

γ-Tubulin Staining:

Primary Antibody: γ-Tubulin (Santa Cruz Biotechnology sc-7396) 1:50

Secondary Antibody: Fluorescein (FITC)-conjugated Mouse Anti-Goat IgG (H+L) (*Jackson ImmunoReearch* Laboratories Inc. [205-095-108]) 1:100

Lamin A Staining:

Primary Antibody: Lamin A (Cell Signaling Technology #2032) 1:100

Secondary Antibody: Anti-Rabbit IgG (whole molecule)-FITC (Sigma F0382) 1:100

2.7 Fusion and wild-type transcript analysis by 5' and 3' RACE

2.7.1 Primers (See Figure 2):

Primer Name	Primer Basis	Primer Sequence 5'→ 3'
Universal Primer Mix (1 Part Long: 5 Parts Short)	SMART™ RACE cDNA Amplification Kit (Clontech, Palo Alto, California)	Long CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA ACA ACG CAG AGT Short CTA ATA CGA CTC ACT ATA GGG C
NeoES	U3NeoSV1 neomycin resistance gene	AATCCATCTTGTTCAATCATGC
NeoIN10	U3NeoSV1 neomycin	ACA GGT CGG TCT TGA CAA AAA

	resistance gene	GAA C
3RaceA	5' RACE Unique Sequence	TCA GTT CTC TTG GGT ATG TG
3RaceB	5' RACE Unique Sequence	TGT GCA CTG ATG AAT G

2.7.2 Total-RNA extraction

Total RNA was isolated from CHO E-126 and Cl-22 cells grown in using the RNeasy® Mini Kit (Qiagen, Mississauga Ont.) and resuspended in 30 µl of RNase-free water.

2.7.3 cDNA synthesis

5'RACE-Ready and 3'RACE-Ready cDNA was synthesized with 2 µl of Total RNA following the Smart™ RACE cDNA Amplification Kit protocol (Clontech).

2.7.4 5' RACE

5' RACE-Ready cDNA (2.5 µl) was placed in 100 mM Tris-HCl (pH 8.3), with 10 mM dNTP Mixture (TaKaRa Biomedicals), 5 units/µl *TaKaRa Taq* (TaKaRa Biomedicals), 10 pmols Universal Primer Mix (Clontech), and 10 pmols of the primer Neo IN10. The mixture was brought to a final volume of 50 µl and sample heated to 94 °C for 2 minutes, then subjected to 35 cycles of 94 °C for 20 seconds, 67 °C for 30

seconds, and 72 °C for 2 minutes. A final extension took place for 10 minutes at 72 °C.

2.7.5 3' RACE

Three µl of 3' RACE-Ready cDNA was placed in 100 mM Tris-HCl (pH8.3), with 10mM dNTP Mixture (TaKaRa Biomedicals), 5 units/µl *TaKaRa Taq* (TaKaRa Biomedicals), 10 pmols Universal Primer Mix (Clontech), and 10 pmols of the primer 3RaceA or 3RaceB. The mixture was brought to a final volume of 50 µl and sample heated to 94 °C for 2 minutes, then subjected to 35 cycles of 94 °C for 20 seconds, 67 °C for 30 seconds, and 72 °C for 4 minutes. A final extension took place for 15 minutes at 72 °C.

2.7.6 PCR product visualization and sequencing

After 5' and 3' PCR amplification, the samples were separated by electrophoresis and visualized on a 1 % agarose gel containing 0.5 µg/ml ethidium bromide. The 5' Race PCR product was purified using the QIAquick PCR Purification Kit (Qiagen, Mississauga Ont.) and 1-2 µl subjected to sequencing with 1.67 µl BigDye Dilution buffer (Applied Biosystems, Foster City, California), 0.33 µl BigDye Reaction Mix (Applied Biosystems, Foster City, California), 20 pmols NeoES primer, and ddH_20 to a final volume of 5 µl. Cycle-sequencing was carried out under the following conditions: 25 cycles of 96 °C for 10 seconds, 50 °C for 5 seconds, and 60 °C for 4 minutes. To the sequencing reaction 30 µl of ddH_20 and 65 µl of 100 % ethanol was added and sample allowed to precipitate for 2 hours at room

temperature in the dark. The DNA was pelleted in a micro-centrifuge for 15 minutes at top speed, all traces of ethanol removed, and sequence analyzed on the ABI Prism® 310 Genetic Analyzer (Applied Biosystems).

2.8 Inverse PCR to determine the viral integration junction

2.8.1 Primers (See Figures 2 and 3)

Primer Name	Primer Basis	Primer Sequence 5'→ 3'
NeoIN1	U3NeoSV1 neomycin resistance gene	ACC AAG CGA AAC ATC GCA TC
NeoIN10	U3NeoSV1 neomycin resistance gene	ACA GGT CGG TCT TGA CAA AAA GAA C
NeoES	U3NeoSV1 neomycin resistance gene	AATCCATCTTGTTCAATCATGC
NeoIN21	U3NeoSV1 neomycin resistance gene	CAA GGA CCT GAA ATG ACC CTG TG
NeoIN22	U3NeoSV1 neomycin resistance gene	ATG ATA TTC GGC AAG CAG GCA TC
NeoLTR3	U3NeoSV1 neomycin resistance gene	AGT TGC ATC CGA CTT GTG

2.8.2 Restriction enzyme digestion of genomic DNA

Genomic DNA (2.5 µl) was combined with 0.25 µl *Nsp*I (New England Biolabs), 1 µl of restriction enzyme buffer (New England Buffer 2 + 1mg/ml BSA), 6.25 µl ddH_2O, mixed and incubated at 37 °C for 2 hours. *Nsp*I was inactivated by heating the mixture for 20 minutes at 65 °C.

2.8.3 Ligation of digested DNA

Digested DNA (10 µl) was added to 90 µl of DNA ligase mix (0.5 µl T4 DNA Ligase [New England Biolabs – 400U/µl], 10 µl 10x ligase buffer, and 79.5 µl ddH_2O) and mixed by pipetting up and down once. This mixture was incubated overnight at 14-15 °C.

2.8.4 Purification of viral/flanking DNA

Ligated viral/flanking DNA was purified using a QIAquick PCR Purification Kit (Qiagen, Mississauga Ont.) as follows: 500 µl of buffer PB was piptetted into the spin column followed by 100 µl of ligated DNA. The sample was mixed by pipetting up and down twice. The column was centrifuged at 1000 rpm for 30 seconds and the flow-through was discarded. 750 µl of buffer PE was then added to the spin column, the column was centrifuged for 30 seconds at 1000 rpm and flow-through discarded. The column was centrifuged for 1 minute at 1000 rpm to remove any remaining ethanol. The DNA was eluted by the addition of 15 µl of buffer EB to the column and centrifuging the column for 1 minute at 1200 rpm.

2.8.5 PCR for the left viral junction (5' end)

Qiagen purified DNA (2.5–5 µl) was heated at 94 °C for 20 minutes and placed on ice. The DNA was added to PCR mix (100 mM Tris-HCl (pH 8.3), 10 mM dNTP Mixture (TaKaRa Biomedicals), 5 units/µl *TaKaRa Taq* (TaKaRa Biomedicals), with 20 pmols of the primer NeoIN10 and 20 pmols of the primer NeoIN1, and brought to a final volume of 50 µl.

The sample was heated to 94 °C for 2 minutes and then subjected to 35 cycles of 94 °C for 20 seconds, 65 °C for 30 seconds, and 72 °C for 2 minutes. A final extension took place for 15 minutes at 72 °C. 1 µl of the resulting product was added to 24 µl of the same PCR mixture as before and PCR was carried out under the following conditions: 94 °C for 1 minute, and 20 cycles of 94 °C for 20 seconds, 65 °C for 30 seconds, and 72 °C for 3.5 minutes. A final extension took place for 8 minutes at 72 °C. After amplification the sample was separated by electrophoresis and visualized on a 1 % agarose gel containing 0.5 µg/ml ethidium bromide. Both the 1.7 kb band and 0.5 kb band were cut from the agarose gel and purified using the QIAquick® Gel Extraction Kit (Qiagen, Mississauga Ont.).

1-2 µl of gel-extracted PCR product was sequenced with 1.67 µl BigDye Dilution buffer, 0.33 µl BigDye Reaction Mix, 20 pmols NeoES primer, and ddH_20 to a final volume of 5µl. Cycle-sequencing was carried out under the following conditions: 25 cycles of 96 °C for 10 seconds, 50 °C for 5 seconds, and 60 °C for 4 minutes. To the sequencing reaction 30µl of ddH_20 and 65 µl of 100 % ethanol was added and sample allowed to precipitate for 2 hours at room temperature in the dark. The DNA was pelleted in a micro-centrifuge for 15 minutes at top speed, all traces of

ethanol removed, and sequence analyzed on the ABI Prism® 310 Genetic Analyzer (Applied Biosystems).

2.8.6 PCR for the right viral junction (3' end)

Qiagen purified DNA (2.5–5 µl) was heated at 94 °C for 20 minutes and placed on ice. The DNA was added to PCR mix ((100 mM Tris-HCl (pH 8.3), 10 mM dNTP Mixture (TaKaRa Biomedicals), 5 units/µl *TaKaRa Taq* (TaKaRa Biomedicals), with 20 pmols of the primer NeoIN21 and 20 pmols of the primer NeoIN22, and brought to a final volume of 50 µl. The sample was heated to 94 °C for 2 minutes and then subjected to 35 cycles of 94 °C for 20 seconds, 65 °C for 30 seconds, and 72 °C for 2 minutes. A final extension took place for 15 minutes at 72 °C. After amplification the sample was separated by electrophoresis and visualized on a 1 % agarose gel containing 0.5 µg/ml ethidium bromide. The PCR product was purified using the QIAquick PCR Purification Kit (Qiagen, Mississauga Ont.) cycle-sequenced in the same manner as above, however the primer NeoLTR3 was used in place of the primer NeoES.

2.9 Analysis of the *gadd*7 message in CHO Cl-22 and E-126 cells

2.9.1 Primers

Primer Name	Primer Basis	Primer Sequence 5'→ 3'
ORF1F	The immediate 5' end	TCC AAG CTT GGG AAG CTG

	of *gadd7*	AGG TTT TTC CTA
G7-Reverse Rest	The immediate 3' end of *gadd7*	TCC TCT AGA CAA ATG AAC ATT TAT TTC AGC AGT CAG

2.9.2 Total-RNA extraction and quantification

Total RNA was isolated from CHO E-126 and Cl-22 cells treated with 400μM hydrogen peroxide for 1 hour using the RNeasy® Mini Kit (Qiagen, Mississauga Ont.) and resuspended in 30μl of RNase-free water. The concentration of Total RNA was calculated spectrophotometrically.

2.9.3 One-Step RT-PCR

The SuperScript™ One-Step RT-PCR with Platinum® *Taq* System (Invitrogen, Burlington, Ontario) was used for cDNA synthesis and PCR amplification. 1 μg of Total RNA was combined with 25 μl of 2X Reaction Mix, the sense and anti-sense primers ORF1F and G7-Reverse each at a concentration of 10 μM, 1 μl RT/ Platinum® *Taq* Mix, and ddH_2O to a final volume of 50 μl.

2.9.4 cDNA synthesis and pre-denaturation

The cDNA was synthesized by incubating the samples at 45 °C for 30 minutes. The samples were then heated to 94 °C for 2 minutes prior to PCR amplification.

2.9.5 PCR amplification of *gadd7* cDNA

Following pre-denaturation the samples were subject to 40 cycles of: 94 °C for 15 seconds, 55 °C for 30 seconds, and 72 °C for 2 minutes. A final extension for 10 minutes at 72 °C ended the program. Following amplification, the samples were separated by electrophoresis and visualized on a 1 % agarose gel containing 0.5 μg/ml ethidium bromide.

2. 10 Northern Blots for the *gadd7*, viral-fusion, and wild-type messages

2.10.1 Primers

Primer Name	Primer Basis	Primer Sequence 5'→ 3'
ORF1F	The immediate 5' end of *gadd7*	TCC AAG CTT GGG AAG CTG AGG TTT TTC CTA
G7-Reverse Rest	The immediate 3' end of *gadd7*	TCC TCT AGA CAA ATG AAC ATT TAT TTC AGC AGT CAG

2.10.2 Gel preparation

One hundred ml of Agarose gel solution was prepared by heating 1 g of agarose in 10 mls of 10x MOPS (0.4 M morpholinopropanesulfonic acid, 0.1 M Na-acetate, 10 mM EDTA) and 85 ml of ddH_20 until the agarose completely dissolved.

After the solution cooled to ~65°C, 5 ml of 37 % Formaldehyde was added, the gel was immediately cast and allowed to harden for 30 minutes.

2.10.3 RNA sample preparation

Ten µg of CHO E-126 and Cl-22 total RNA (isolated as described earlier) was brought to 10 µl using Loading buffer (10 % 10 x MOPS pH 7.2 (see above), 18.5 % Formaldehyde, 50 % Formamide, 4 % Ficoll 400, 10 % bromophenol blue and ddH_20 to 1 ml) and 0.5 µl of Ethidium Bromide (0.5 µg/ul) was added to each sample. The samples were heated for 5 minutes at 90°C and cooled on ice.

2.10.4 RNA electrophoresis

Before running the samples, the gel box was cleaned with 5 N NaOH. Electrophoresis was carried out in a fume hood at 40 volts overnight in Northern Running buffer [1 x MOPS (see above), and 0.2 M Formaldehyde in ddH_20]. Electrophoresis was carried on until the bromophenol blue marker had moved to the end of the gel.

2.10.5 Transfer of separated RNA from gel to membrane

After electrophoresis, the gel was washed 4 times for 5 minutes in ddH_20, stained with ethidium bromide, and photographed. A nylon transfer membrane (Maximum Strength Nytran - Schleicher & Schuell, Keene, New Hampshire) was cut to the appropriate size, placed briefly in ddH_20, and a standard capillary blot system was set-up using 10 x SSC as the transfer buffer (1.5 M NaCl and 0.15 M Sodium

Citrate). RNA transfer proceeded for 24 hours at room temperature with occasional changes of the blotting paper. Following transfer the membrane was washed briefly in 2 x SSC (0.3 M NaCl and 0.03 M Sodium Citrate), exposed to a UV cross-linker while damp for 30 seconds, then baked at 80° C for 1-2 hours.

2.10.6 Probe Preparation

The probe for *gadd7* was generated using the primers ORF1F and G7-Reverse on *gadd7* cDNA cloned into the mammalian expression vector pcDNA3.1+ (Invitrogen, Burlington, Ontario) [vector obtained from Dr. Dana Crawford, Center for Immunology & Microbial Disease – Albany Medical College]. The probe for the 5' sequence was the fragment generated during the 5' Race experiment or the 5' Inverse PCR fragment. All probes were purified with the QIAquick PCR Purification Kit (Qiagen, Mississauga Ont.) and concentrations determined spectrophotometrically. 25 ng of probe DNA was labeled with 50 μCi of [α-^{32}P]dCTP using the Random Primers DNA Labeling System (Invitrogen, Burlington, Ontario).

2.10.7 Hybridization of probe to membrane-bound RNA

The membrane was prehybridized at 42° C for 1-4 hours in Prehybridization buffer (5 x SSC [0.75 M NaCl and 0.075 M Sodium Citrate], 50 % Formamide, 5 x Denhardt's solution [50x Denhardt's solution: 1 % (w/v) Ficoll 400, 1 % (w/v) polyvinylpyrrolidone, 1 % (w/v) bovine serum albumin in dH_2O], 1 % SDS, and 100 μg/ml heat-denatured Salmon sperm) with rotation. The probe was heated to 95 °C for 3 minutes, chilled on ice, added to Hybridization buffer (Prehybridization buffer with

5 % Dextransulfate (MW 500,000) and without Salmon sperm), and probe hybridization to the membrane-bound RNA carried out at 42° C overnight with rotation. The next day the membrane was washed once for 15 minutes with 2 x SSC (0.3 M NaCl and 0.03 M Sodium Citrate) at room temperature and once at 65° C with 2 x SSC containing 0.1 % SDS for 40 minutes or until the background radiation level became low. The wet membrane was wrapped in Saran wrap and exposed overnight at room temperature to a phosphor screen and for 4 days at -80 °C to x-ray film.

2.10.8 Membrane stripping for re-hybridization

To remove the radioactive probe membranes were washed for 30-90 minutes in Stripping solution (5mM Tris pH 8, 0.2 mM EDTA, 0.05 % Sodium pyrophosphate, and 0.1 x Denhardt's solution) until no radioactivity could be detected on the membrane. Following a wash with 5 x SSC (0.75 M NaCl and 0.075 M Sodium Citrate) the membranes were ready for pre-hybridization.

Note: Northern Blot Protocol Adapted from Sambrook and Russell, 2001.

2.11 Plasmid Rescue for determination of the viral flanking regions

Note: Unpublished protocol from Dr. Luke DeLange – Manitoba Institute of Cell Biology

2.11.1 Plasmid preparation from genomic DNA

Twenty μg of Genomic DNA (obtained as described earlier) was digested with 50 Units of *Eco*RI (Invitrogen, Burlington, Ontario) overnight at 37 °C. The digest was heat inactivated at 68 °C for 15 minutes and cooled to room temperature.

40 µl of 10x T4 DNA Ligase Buffer was added to the cooled sample along with 2 µl of High Concentration T4 DNA Ligase (400 U/µl -New England Biolabs) and ddH_2O to a final volume of 400 µl. Ligation occurred overnight at 14 °C.

2.11.2 Plasmid purification

The resulting plasmids from the overnight ligation were purified using the QIAquick PCR Purification Kit (Qiagen, Mississauga Ont.) and eluted in a 50 µl volume. Added to this were 5 µl of chilled 3M Sodium Acetate and 100 µl of chilled absolute ethanol. Plasmids were precipitated for 15 minutes at -80 °C in the micro-centrifuge at maximum speed for 15 minutes at 4 °C. The ethanol was carefully removed and the plasmids washed with cold 70 % ethanol, dried, and resuspended in 5 µl ddH_2O.

2.11.3 Plasmid amplification

Three µl of the resuspended plasmids was incubated on ice with one tube of TOP 10F' chemically competent *E. coli*. cells (Invitrogen, Burlington, Ontario) for 30 minutes, heat-shocked for 30 seconds at 42 °C, and incubated in 2 ml LB broth for 30 minutes at 37 °C. 1 ml of this was spread on LB plates with 50 µg/ml ampicillin and incubated overnight at 37 °C. The resulting individual colonies were picked and grown in 2 ml LB broth with 50 µg/ml ampicillin overnight.

2.11.4 Plasmid isolation from bacterial cultures

Note: Protocol Adapted from Sambrook and Russell, 2001.

The overnight culture (1.5 ml) was centrifuged at maximum speed for 30 seconds at 4 °C. The medium was removed and bacterial pellet resuspended in 100 µl of cold Alkaline lysis solution I (50 mM glucose, 25 mM Tric-Cl pH 8.0, and 10 mM EDTA pH 8.0). 200 µl of freshly prepared Alkaline lysis solution II (0.2 N NaOH, 1 % SDS) was added to the bacterial suspension and contents mixed by inverting the tube several times. 150 µl of cold Alkaline lysis solution III (60 % 5M potassium acetate, 11.5 % glacial acetic acid and 28.5 % ddH_2O) was immediately added, tube contents mixed by inverting several times, and the mixture was placed on ice. After 5 minutes, the bacterial lysate was placed in a micro-centrifuge running at maximum speed for 5 minutes and the supernatant transferred to a new tube. An equal volume of pehol:chloroform was added to the supernatant, samples mixed, then placed in a micro-centrifuge running at maximum speed at 4 °C for 2 minutes. The aqueous layer was transferred to a new tube, and plasmids precipitated with the addition of 2 volumes of absolute ethanol and centrifugation at 4 °C at maximum speed for 5 minutes. The supernatant was carefully removed, plasmids washed with 70 % ethanol followed by centrifugation at 4 °C at maximum speed for 5 minutes. The supernatant was again removed, and the plasmids dissolved in 40 µl of ddH_2O.

2.12 Southern Blot analysis of viral integration site

2.12.1 Preparation of Genomic DNA

Ten μg of the genomic DNA was digested in a total volume of 400 μl with 6 units of *Eco*RI, *Bam*H1, or *Xba*I per μg of DNA at 37 °C overnight. One volume (10 μl) of 3M sodium acetate was added along with 2.5 volumes of 100 % ethanol and samples precipitated for 1 hour at -80 °C. Following precipitation, the DNA was spun in a microfuge at room temperature at maximum speed for 20 minutes. The supernatant was removed and the samples were air-dried for 5 minutes. The digested DNA was resuspended in 15 μl of 10 mM Tris pH 7.5.

2.12.2 Agarose gel electrophoresis of restriction digested genomic DNA

A 0.8 % agarose gel was prepared with 1 x TAE (40 mM Tris-acetate and 1 mM EDTA) and contained 2 μl of 1 % ethidium bromide. The gel was submerged in 1 x TAE, 3 μl of DNA-loading buffer was added to samples, and markers and digested DNA loaded. Electrophoresis was carried out for 6 hours at 40 Volts and the gel photographed alongside a fluorescent ruler.

2.12.3 Transfer of separated DNA from gel to nylon membrane

The gel was soaked in Denaturation Solution (1.5 M NaCl and 0.5 N NaOH) twice for 15 minutes with shaking and washed for 15 minutes in Neutralization Buffer (0.5 M Tris pH 7.5, 1.5 M NaCl). Transfer of DNA from the gel to the membrane was carried out as described in the Northern Blot experiment.

2.12.4 Probe Preparation

The probe for the 5' sequence was the fragment generated during the 5' Race experiment or the 5' Inverse PCR fragment. The *gadd7* probe was the same probe used in the Northern Blot experiment. All probes were purified and labeled as described as in the Northern Blot protocol.

2.12.5 Hybridization, Membrane Stripping, and Re-hybridization

See Northern Blot protocol.

Note: Southern Blot Protocol Adapted from Sambrook and Russell, 2001.

2.13 The effect of gadd7 transfection on CHO cells

2.13.1 Transient transfection

Two thousand CHO Cl-22 cells in a volume of 100 µl of medium per well were seeded in 96 well plates and incubated at 37 °C (5 % CO_2) for 24 hours. Cells were washed once with Opti-Mem® serum-reduced medium and transfected with 0, 0.5, 1, 1.5, or 2 µg of the pcDNA3.1 mammalian expression vector containing *gadd7* cDNA cloned into the mammalian expression vector pcDNA3.1 using Lipofectin® Reagent (Invitrogen, Burlington, Ontario) and the enclosed protocol. Cells were incubated for 20 hours at 37 °C after which the transfection solution was removed and cells incubated in normal serum for a further 48 hours.

2.13.2 Cell survival determination following *gadd7* transfection

Cell survival was determined using the MTT assay as previously described.

2.14 gadd7 Induction System

2.14.1 Cloning of gadd7 cDNA into pIND(SP1)/Hygro

One μg of *gadd7* cDNA in the mammalian expression vector pcDNA3.1+ [obtained from Dr. Dana Crawford, Center for Immunology & Microbial Disease – Albany Medical College] was digested with 10 Units of *Hind*III (Invitrogen, Burlington, Ontario) and 10 Units of *Xba*I at 37 °C. The resulting products were separated by electrophoresis and visualized on a 1 % agarose gel containing 0.5 μg/ml ethidium bromide. The full-length *gadd7* cDNA product was cut from the agarose gel and purified using the QIAquick® Gel Extraction Kit (Qiagen, Mississauga Ont.). This cDNA was cloned into the *Hind*III and *Xba*I site of pIND(SP1)/Hygro (Invitrogen, Burlington, Ontario). The ligation mixture was transformed into chemically competent *E. coli* TOP 10F' cells and selected on LB plates containing 75 μg/ml ampicillin. Clones were obtained, plasmids isolated (as per Plasmid Rescue), and analyzed by restriction digestion and sequencing for the presence of the *gadd7* insert.

2.14.2 Stable-transfection of pVgRXR into HCT 116 and CHO cells

HCT 116 and CHO cells were transfected with 1 μg of the pVgRXR vector following the Lipofectin® Reagent protocol (Invitrogen, Burlington, Ontario). 48

hours post transfection, selection for pVgRXR clones was begun with 150-250 μg/ml Zeocin™ (Invitrogen, Burlington, Ontario) and continued until the resulting colonies could be picked and expanded. The resulting clones were examined for ponasterone-inducible gene expression by x-gal screening following the Ecdysone-Inducible Mammalian Expression System published protocol (Invitrogen, Burlington,, Ontario).

2.14.3 Transfection of pIND(SP1)/Hygro/gadd7

pVgRXR clones showing high levels of inducible expression after x-gal screening were transfected with the pIND(SP1)/Hygro/gadd7 construct following the Lipofectin® Reagent protocol (Invitrogen, Burlington, Ontario). Selection was carried out with 500 μg/ml (CHO), and 300 μg/ml (HCT 116) Hygromycin (Sigma-Aldrich, St. Louis) until single clones could be isolated.

2.14.4 gadd7 Induction

For gadd7 induction, HCT 116 or CHO clones containing both the pVgRXR vector and the pIND(SP1)/Hygro/gadd7 construct were grown under standard conditions on plates or coverslips and induction was carried our with the addition of 5-10 μM Ponasterone (Invitrogen, Burlington, Ontario) to the media for 24-72 hours.

Chapter 3 – Results

3.1 Overview

The following results demonstrate my analysis of one etoposide-resistant clone, CHO E-126, the subject of my thesis project. The aim of my project was to determine the site of viral integration, identify any fusion transcript produced in CHO E-126, and characterize the cell-line. Also included in this section is a brief discussion of the results leading up to my involvement in the project.

3.1 Previous results in the creation of etoposide-resistant cell-lines

During the initial screening of U3NeoSV1 virally infected cells by Dr. Jeannick Cizeau, 45 etoposide-resistant clones were isolated, indicating a ratio of nine resistant colonies per 10^4 cells plated. Parallel experiments with uninfected CHO cells led to a much lower number of etoposide-resistant colonies (approximately $1/10^6$).

From the etoposide-resistant clones treated with other apoptosis inducers several clones were isolated that displayed a cross-resistance suggesting a disruption of apoptosis-related genes rather than a specific drug resistance mechanism. It is unlikely that drug resistance could be attributable to the use of a multidrug resistance pathway or other drug resistance mechanisms as our screening selects for "loss of" rather than "gain of" function mutations.

The viral infectivity used (approximately 1 virus per 20 cells) should lead to a single virus integration per cell and this was confirmed with a Southern blot. When genomic DNA from etoposide resistant clones was probed with a region of the *neo* sequence, a single band was detected for each clone as shown in Figure 4. While it is

possible that other mutational events may have occurred in our clones independent of the viral integration, direct viral mutational events are limited to one.

3.2 Viral configuration analysis

Besides ensuring that there is only one virus integrated in a cell line of interest it is also important to determine whether the inserted construct has remained intact or has undergone some form of re-arrangement. Because E-126 cells are resistant to G418 *neo* gene rearrangement is unlikely. In order to confirm there has been no viral re-arrangement, genomic DNA was isolated from the E-126 cell line and two PCR reactions were done using primers based on U3NeoSV1 sequences. One PCR utilized primers based in the *neo* gene and the expected amplification product was 520 base pairs. Based on this product there doesn't appear to be any re-arrangement in the *neo* gene of the retrovirus [Figure 5:A]. The second PCR reaction amplifies a region between the ampicillin resistance gene and the *neo* gene. The expected and observed amplification product for this PCR was 1.6 kilobase pairs [Figure 5:B]. Because these PCR reactions cover greater than half of the promoter-trap sequence, the finding that neither reaction provided evidence of a viral re-arrangement, and because E-126 shows G418 resistance, viral re-arrangement is unlikely. This would suggest that the promoter-trap is acting as expected with respect to its integration in the genome of the CHO E-126 clone. Furthermore, provided there has been exonic and not intronic integration, it is expected that viral integration has completely disrupted the activity of a gene.

3.4 Cross-resistance to apoptosis inducers

Preliminary experiments indicated that the E-126 cell line was resistant to apoptosis inducers other than etoposide [Table 1]. The MTT assay was used to determine the sensitivity of CHO E-126, CHO Cl-22 (CHO cells containing the retrovirus receptor) and CHO Neo610 (Un-cloned U3NeoSV1 infected cells) clones to Doxorubicin, Cisplatin, Taxol and Hydrogen Peroxide. These agents all stimulate apoptosis through methods different than etoposide. Doxorubicin, also known as Adriamycin, primarily kills cells through the intercalation of double-stranded GC base-pair DNA regions (Zeman *et al.,* 1998), the inhibition of topoisomerase II, (Bodley *et al.,* 1990), and the production of ceramide (Delpy *et al.,* 1999), Cisplatin causes interstrand and intrastrand cross-links in DNA (Comess and Lippard, 1993) both of which are cytotoxic to a cell. Cisplatin may cause protein-DNA crosslinks (Banjar *et al.,* 1983), and interfere in DNA repair (Turchi *et al.,* 2000). Taxol binds to β-tubulin, stabilizing microtubules leading to mitotic arrest and apoptosis (Jordan *et al.,* 1996). Taxol can act independently of p53 in triggering apoptosis (Woods *et al.,* 1995), and at higher levels can lead to the induction of genes including tumor necrosis factor α (Bogdan and Ding, 1992) and interleukin-8 (Collins *et al.,* 2000), which will result in cellular death. When Taxol acts independently of p53 in triggering apoptosis there is increased sensitivity to the drug through avoidance of the p21-dependent G1/S checkpoint and endoreduplication leading to apoptosis (Stewart *et al.,* 1999). Finally, hydrogen peroxide generates oxygen free radicals inside a cell damaging DNA (Bradley and Erickson, 1981), lipids, the mitochondria (Richter *et al.,* 1995),

and other macromolecules (Bjork *et al.,* 1980), leading to apoptosis (for a review see Chandra *et al.,* 2000).

As expected, the E-126 cell line shows a significant resistance to etoposide in comparison to Cl-22 and Neo610 cells [Figure 6:C]. Interestingly, the Neo610 cells with random viral integrations appear more sensitive to etoposide than uninfected Cl-22 cells. E-126 cells did not display increased resistance to Doxorubicin in spite of this being another drug acting on topoisomerases [Figure 6:B] When treated with Doxorubicin, E-126 and Cl-22 cells showed a similar survival profile, whereas Neo610 cells appear more sensitive [Figure 6:B]. The E-126 cell line did not show an increased resistance to Taxol [Figure 6:D] and the slope of the death curve was similar for all clones treated with Cisplatin [Figure 6:A]. Besides etoposide, the only cytotoxic agent the E-126 cells show a noticeable resistance to is hydrogen peroxide [Figure 7]. At both 200 and 400 μM concentrations of hydrogen peroxide, E-126 cells show upwards of 15 % greater survival over Cl-22 and Neo610 cells.

3.5 Growth Rate and Topoisomerase II-α levels

Experiments on an etoposide-resistant human glioma cell line (Matsumoto *et al.,* 1999) revealed reduced levels of topoisomerase II mRNA and protein, as well as decreased activity of this protein and a reduced cellular growth rate. In addition, these cells had a 47-fold increase in resistance to topoisomerase II poisons. This same observation has occurred in several different cell lines (Matsumoto *et al.,* 1997). Etoposide resistance does not always mean there is a decrease in topoisomerase II levels as human leukemia cells resistant to etoposide have displayed lower etoposide-

induced DNA cleavage without a decrease in topoisomerase II levels or activity (Fukushima *et al.,* 1999).

To detect and compare topoisomerase II levels in CHO E-126 with levels in CHO Cl-22 cells, a polyclonal topoisomerase II antibody derived from the 70 Kilodalton c-terminal domain of topoisomerase II were obtained from Dr. Jack Yalowich (University of Pittsburgh School of Medicine). This antibody detects both topo II alpha (170 kDa) and beta (180 kDa), however it is not as effective in detecting the beta form as the alpha form. Topoisomerase II α levels fluctuate during the cell cycle and it is found primarily in proliferating cells (Woessner *et al.,* 1991), whereas topoisomerase II β levels remain constant throughout the cell cycle and may not be required for cellular mitosis as many of the essential mitotic functions of the alpha isoform are not adopted by the beta isoform (Grue *et al.,* 1998).

We were unable to detect any significant changes in topoisomerase II α levels in the E-126 cell line in comparison to parental Cl-22 cells [Figure 8] with either 5, 10, or 20 μg of protein loaded. While the protein levels of topoisomerase II α appear similar, this analysis does not measure the activity of this protein in E-126 in comparison to Cl-22 cells. Topoisomerase II activity in cells is regulated by a variety of ways including phosphorylation dependent mechanisms (Chikamori *et al.,* 2003) (Wells and Hickson, 1995) and phosphorylation-independent mechanisms (Kimura *et al.,* 1996).

The cellular growth rate of the E-126 cell line was compared with the rate of Cl-22 cells. From the four time-points taken over the 170 hour experiment period, E-126 cells displayed a lower growth rate than Cl-22 cells [Figure 9]. A lower activity

of topoisomerase II α has been related to a slower growth rate (Shin *et al.,* 1999) and correspondingly, an increase in topoisomerase II expression correlates with an increased tissue growth rate (Nelson *et al.,* 1987). Because the protein levels of topoisomerase II α are similar in CHO E-126 and CHO Cl-22, if an analysis of the topoisomerase II activity revealed a decrease in CHO E-126 cells, it may account for the decreased growth rate in this cell line. Future studies will be needed to test this hypothesis.

3.6 Morphological Changes in CHO E-126

Etoposide resistance has been linked with morphological and karyotypic changes in many cell lines. Human chronic promyeloctic leukemia cell lines resistant to etoposide are susceptible to spontaneous polyploidization, and have karyotypic changes (Meliksetian *et al.* 1999). Human chronic myelogenous leukemia cells resistant to etoposide have an increased rate of spontaneous polyploidization coupled with a down regulation of topoisomerase II α (Melixetian *et al.,* 2000). Polyploidy is also observed in cells following treatment with topoisomerase II inhibitors (Zucker *et al.,* 1991).

In order to view cellular morphology we carried out immunocytochemistry using γ-tubulin antibodies. Specifically, we were interested in examining the number of centrosomes in E-126 to determine whether polyploidy occurred. γ-tubulin is a member of the tubulin family (Oakley and Oakely, 1989) associated with the centrioles and microtubule organizing centres (Kornarova *et al.,* 1997), however the majority of γ-tubulin is found within the cellular cytoplasm (Patel and Stearns, 2002).

In contrast, DAPI stains the DNA with little or no cytoplasmic staining. The morphology of CHO Cl-22 cells stained with γ-tubulin [Figure 10:A] and DAPI [Figure 10:B] provides an indication of the normal appearance of CHO cells. When stained with γ-tubulin the appearance CHO E-126 cells was different than CHO Cl-22 cells [Figure 10:C]. CHO E-126 cells appear larger and there is a less-defined cellular shape. DAPI staining on these cells shows the appearance of larger, differently shaped and in some cases multiple nuclei in many of the cells [Figure 10:D]. This may indicated a deficiency in cellular control over mitosis. Cellular morphology was also examined using Polyclonal antibodies against Lamin A, a component of the nuclear membrane along with DAPI staining. Enlarged nuclear membranes as well as differences in the appearance, number, and size of the nuclei are again evident in the CHO E-126 cell line [Figure 11]. It should be noted that in neither case, were the different morphologies seen in E-126 quantified with respect to the Cl-22 cell line. At this point the question remains as to whether it is the viral mutation itself, the selection of E-126 cells in etoposide, (as seen above), or a combination of both factors leading to the altered cellular morphologies seen in CHO E-126 cells. Interestingly, the *gadd*7 induction system produced similar results to the E-126 cell line in both CHO and HCT cells, however the levels of *gadd*7 induction were never quantified, nor were the results normalized to background levels; therefore, whether or not *gadd*7 induction leads to polyploidy and altered cellular morphologies remains unanswered.

3.7 5' and 3' RACE

When U3NeoSV1 promoter-trap integration occurs proximate to a cellular promoter a 5' fusion transcript will be produced with the upstream coding sequence and a region of the virus including the G418 resistance gene (*neo*). Identification of the fusion transcript is possible through the use of the 5'-rapid amplification of cDNA ends (RACE) procedure. Our 5' RACE experiments generated an amplification product of approximately 400 base pairs [Figure 12:A]. Sequence analysis of this product had the expected homology to both *neo* and the U3NeoSV1 Long Terminal Repeat sequences, as well as providing some sequence representing genomic flanking DNA [Figure 13:A]. When examined for low complexity and repetitive elements, (Repeat Masker www.genome.washington.edu/UWGC/analysistools/RepeatMasker.cfm), neither were detected.

Because the complete genome sequence does not exist for the Chinese hamster, comparisons were made with the mouse genome. The 103 base pairs of unique 5' RACE flanking sequence produced 76% identity with a region on chromosome 11 of the mouse genome (25535-25634) [Figure 13:B]. Within this region the Ensembl Mouse Genome Browser predicts a transcript (supported by similarity to protein or cDNA sequences) and predicts a transcript based on EST evidence. The 103 base pairs of unique 5' RACE sequence had no significant identity with the human genome.

Two primers were designed for 3' RACE PCR based on the unique 5' RACE sequence. It was hoped that a 3' RACE on CHO Cl-22 cells using these primers would identify the specific gene product in which the virus is integrated.

Unfortunately, even with a high annealing temperature too many amplification products were produced for identification of the wild-type gene product corresponding to the 5' RACE fusion [Figure 12:B].

3.8 Inverse PCR to clone viral junctions

To determine the location of viral insertion in the CHO E-126 cells we used the process known as Inverse PCR. This technique allows the genomic regions flanking the virus to be determined using viral specific PCR primers following circularization and ligation of restriction-enzyme digested genomic DNA. The restriction enzyme *Nsp*I cuts once in the U3 region of the virus (at both 5' and 3' U3 sequences) and in the flanking DNA surrounding the virus. This allows the generation of plasmids containing either the 5' or 3' end of the promoter-trap along with the flanking genomic DNA at either end. We were able to determine the genomic DNA sequence at both the 5' and 3'end of the U3NeoSV1 promoter-trap construct using this method.

Sequence analysis of the 5' integration amplification product showed homology with both the U3NeoSV1 virus and 44 nucleotides of the sequence obtained from the 5' RACE (the absence of overlap with the other 59 nucleotides may be due to splicing). In addition, sequence was obtained representing 205 base pairs of unique sequence [Figure 14:A]. Examining this region for repetitive elements and low complexity regions revealed 153 bases of this type [Figure 14:B]. Again, the only significant homology was found on the mouse chromosome 11.

Sequence analysis of the 3' integration amplification product from the virus had a short region homologous to the U3NeoSV1 virus and 431 unique base pairs [Figure 15:A]. Screening for repetitive and low complexity regions found 109 nucleotides of this type [Figure 15:B]. When the masked sequence (repetitive elements hidden) was compared to the mouse genome, the highest homology was again found on chromosome 11 (24933-25146) with 76% identity found (164/215) having very low "e value" (9.4 e-18) [Figure 16]. When compared to the human the highest homology was found with a region on chromosome 19, having 66% identity (109/165).

Based on the 5' RACE, 5' Inverse PCR, and 3' Inverse PCR experiments, our site of viral integration appears to be homologous to the region including (24933-25634) on mouse chromosome 11. At both the 5' and 3' end of viral integration there appears to be some repetitive elements, and the region of integration is predicted (based on similarities to protein and cDNA sequences) to produce a transcript and within the chromosome region is a cloned mouse cDNA. According to the database, our viral integration is within a large intron found in this gene.

3.9 Viral integration and *gadd7*

Initial work by Dr. Jeannick Cizeau on the CHO E-126 cell line attempted to determine viral integration by the technique known as Plasmid Rescue. In this technique genomic DNA is digested with either *Eco*RI or *Bam*HI. These enzymes cut once in the integrated virus and in the flanking genomic DNA regions allowing cloning of the 5' or 3' flanking sequences respectively. The DNA is ligated,

transformed into *E. coli* and ampicillin/kanamycin resistant colonies produced with plasmids containing DNA corresponding to either 5' or 3' flanking sequences. Sequencing of an *Eco*RI generated 5' flanking DNA plasmid showed homology to the gene *gadd7*. No *gadd7* homology was found with Inverse PCR from either junction nor with the 5' RACE. It was reported that *gadd7* is a single copy number gene (Hollander *et al.,* 1996), so if our promoter-trap was integrated into *gadd7* there should be no detectable message produced for that gene.

A Reverse-Transcriptase PCR performed on Total-RNA isolated from CHO Cl-22 and CHO E-126 cells produced the expected full-length product for *gadd7* in both cell lines [Figure 17]. A Northern blot using total RNA extracted from CHO Cl-22 and CHO E-126 cells and a probe based on 95% of the *gadd7* gene product revealed a single band of the same intensity and at the same location from CHO Cl-22 and CHO E-126 cells [Figure 18]. A Southern blot digestion of Cl-22 and E-126 genomic DNA with either *Bam*H1, *Eco*R1, or *Xba*I and probed with the full-length *gadd7* message produced identical banding patterns in both cell lines [Figure 19]. If the promoter-trap retrovirus had integrated into *gadd7*, one would expect to see some difference in the banding pattern, especially if *gadd7* is a single-copy number gene as reported. Because *gadd7* integration was not confirmed by sequence data, 5' RACE data, RT-PCR, or Northern analysis, we believe that our promoter-trap construct is not integrated into *gadd7*, but into a novel gene. The Plasmid Rescue experiment that indicated the virus had integrated into *gadd7* is currently being repeated to completely remove any doubt about the viral integration site.

3.10 Apoptosis Induction by *gadd7*

Because we initially believed the U3NeoSV1 promoter-trap had integrated into *gadd7*, we were interested in apoptosis induction by this gene. CHO Cl-22 cells were transiently transfected with *gadd7* cDNA in the mammalian expression vector pcDNA3.1 and cell survival determined 48 hours post transfection [Figure 20]. No appreciable cell death was seen following *gadd7* transfection. CHO cells transfected and selected for the *gadd7* expression vector in later experiments did show a decrease in colony formation as previously reported (Hollander *et al.,* 1996) [data not shown].

3.11 Virally-trapped gene's copy number and expression

Northern Blots with the DNA fragments generated during the 5' RACE or 5' Inverse PCR experiments were attempted with the hope that they would reveal the full-length message produced in Cl-22 cells and possibly, a truncated version from E-126 cells. Alternatively, the message from E-126 cells might also remain full-length if the promoter-trap has integrated into a diploid gene or intronically with alternative splicing. Surprisingly, neither probe produced any specific bands. When probed with the 5' RACE fragment, a smear was seen for both samples. The 5' Inverse PCR based probe also failed to produce any clear results.

To learn whether the virally-integrated gene is a single-copy number gene Southern Blots were attempted using the 5' Inverse PCR product and the DNA fragment generated during the 5' RACE. The Inverse PCR fragment yielded the clearest results and both Cl-22 and E-126 show 3 possible bands along with smearing at higher molecular weights [Figure 21]. Because the probe used contains a small

viral region it may have picked up Long Terminal Repeat sequences common in the Chinese hamster genome. In addition, the 5' Inverse PCR product contains a low complexity/repetitive region [Figure 14], which may also be common to the Chinese hamster genome.

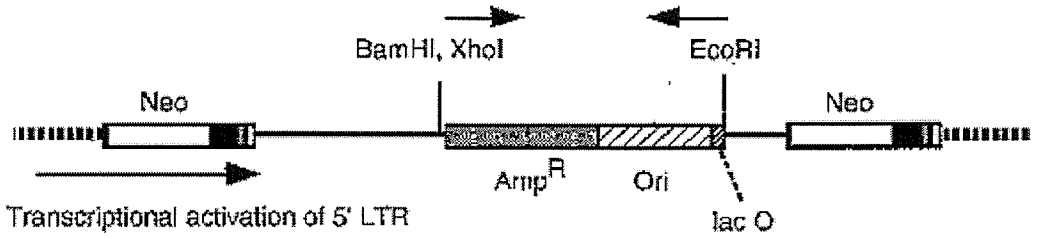

Figure 1: The U3NeoSV1 promoter-trap retrovirus.

From: Hicks, G.G., E-G. Shi, J. Chen, M. Roshon, D. Williamson, C. Scherer, and H.E. Ruley. 1995. Retrovirus Gene Traps. *Methods Enzymol.* **254**:263-75.

↓Viral-Host Junction ←NeoES

tgaaagaccccacctgtaggtttggcaagctagctgaggatcgtttcgcatgaattg

NeoF1→ ↓Cryptic Splice Site

aacaagatggattgcacgcaggttctccggccgcttgggtggagaggctattcggct

NeoF2→ ←NeoR2

atgactgggcacaacagacaatcggctgctctgatgccgccgtgttccggctgtcag

←NeoIN10

cgcaggggcgcccggttctttttgtcaagaccgacctgtccggtgccctgaatgaac

tgcaggacgaggcagcgcggctatcgtggctggccacgacgggcgttccttgcgcag

ctgtgctcgacgttgtcactgaagcgggaagggactggctgctattgggcgaagtgc

cggggcaggatctcctgtcatctcaccttgctcctgccgagaaagtatccatcatgg

ctgatgcaatgcggcggctgcatacgcttgatccggctacctgccattcgaccacca

agcgaaacatcgcatcgagcgagcacgtactcggatggaagccggtcttgtcgatca

←NeoR2

ggatgatctggacgaagagcatcaggggctcgcgccagccgaactgttcgccaggct

caaggcgcgcatgcccgacggcgaggatctcgtcgtgacccatggcgatgcctgctt

gccgaatatcatggtggaaaatggccgcttttctggattcatcgactgtggccggct

gggtgtggcggaccgctatcaggacatagcgttggctacccgtgatattgctgaaga

gcttggcggcgaatgggctgaccgcttcctcgtgctttacggtatcgccgctcccga

ttcgcagcgcatcgccttctatcgccttctttgacgagttcttctgagcgggactct

ggggttcgaaatgagctagcttaag

Figure 2: Orientation of primers in the 5' Long-Terminal Repeat of U3NeoSV1. Grey-highlighting indicates primer sequence overlap for NeoES and NeoF1. Blue-highlighting indicates primer sequence overlap for NeoF2 and NeoIN12.

gagcatcaggggctcgcgccagccgaactgttcgccaggctcaaggcgcgcatgccc

←**NeoIN22**

gacggcgaggatctcgtcgtgacccatggcgatgcctgcttgccgaagatcatggtg

gaaaatggccgcttttctggattcatcgactgtggccggctgggtgtggcggaccgc

tatcaggacatagcgttggctacccgtgatattgctgaagagcttggcggcgaatgg

gctgaccgcttcctcgtgctttacggtatcgccgctcccgattcgcagcgcatcgcc

ttctatcgccttcttgacgagttcttctgagcgggactctggggttcgaaatgagct

agcttaagtaacgccattttggaaggcatggaaaaatacataactgagaatagagaa

gttcagatcaaggtcaggaacagatggaacagtctagagaaccatcagatgtttcca

NeoIN21→

gggtgccccaaggacctgaaatgaccctgtgccttatttgaactaaccaatcagttc

gcttctcgcttctgttcgcgcgcttctgctccccgagctcaataaaagagcccacaa

cccctcactcggggcgccagtcctccgattgactgagtcgcccgggtacccgtgtat

NeoLTR3→

ccaataaaccctcttgcagttgcatccgacttgtggtctcgctgttccttgggaggg

tctcctctgagtgattgactacccgtcagcgggggtctttcatt

Figure 3: Orientation of primers in the 3' Long-Terminal Repeat of U3NeoSV1.

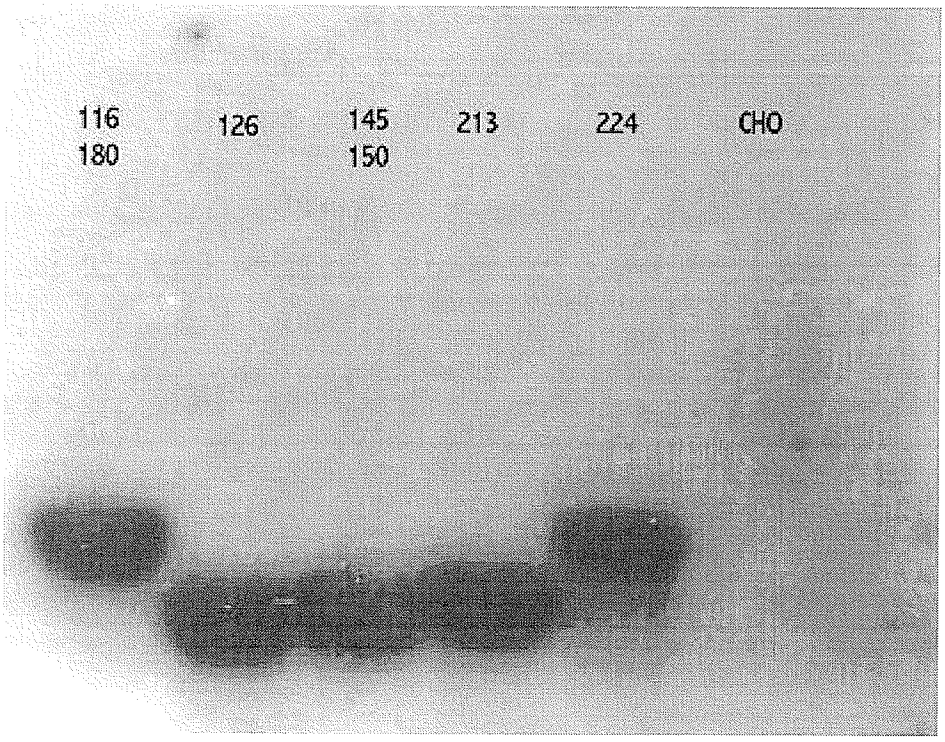

Figure 4: Southern blot analysis of *Hind*III digested genomic DNA from etoposide-resistant clones probed with the proviral Neo sequence. Data from Dr. Jeannick Cizeau.

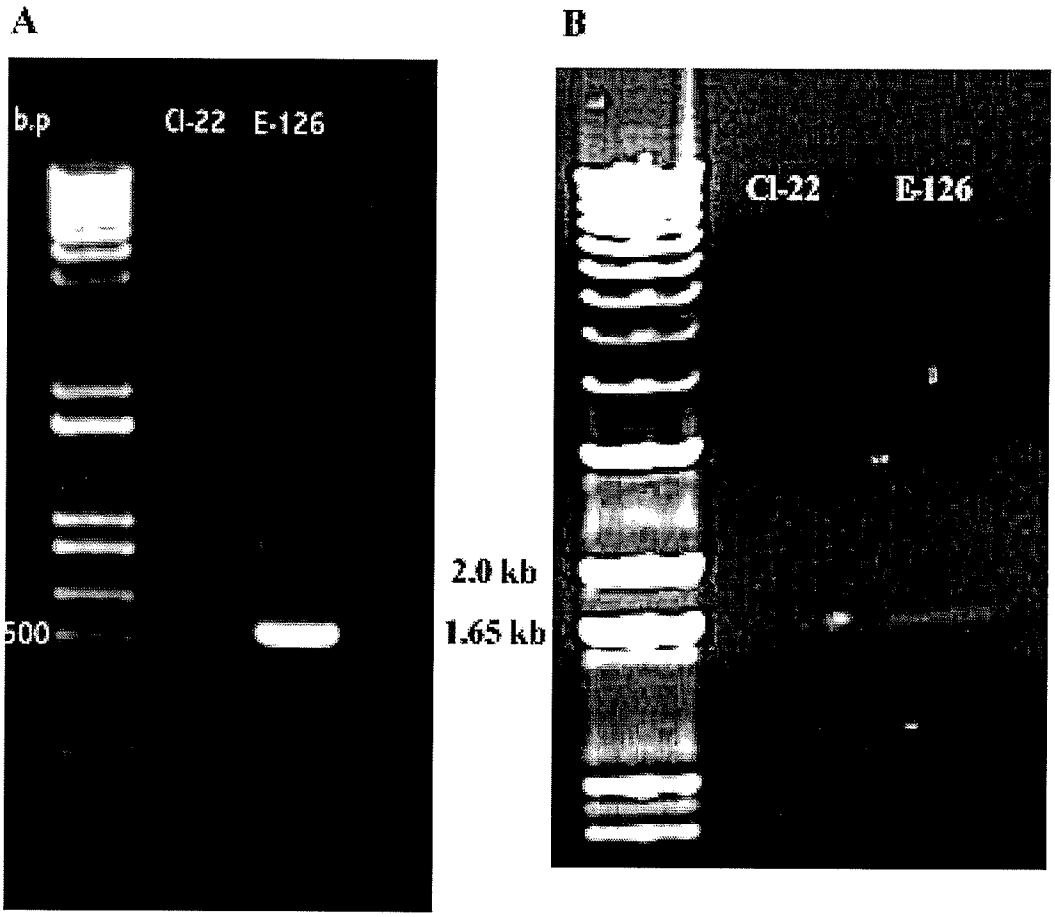

Figure 5: Viral configuration determination by Genomic PCR. **(A)** Genomic DNA of Cl-22 and E-126 clones amplified by PCR with the primers NeoR2 and NeoF1. Lane 1: 1 Kb Plus DNA Ladder (Invitrogen), Lane 2: Cl-22 PCR product (negative control), Lane 3: E-126 PCR product. **(B)** Genomic DNA of Cl-22 and E-126 clones amplified by PCR with the primers with the primers Amp1 and Neo1. Lane 1: 1 Kb Plus DNA Ladder (Invitrogen), Lane 2: Cl-22 PCR product (negative control), Lane 3: E-126 PCR product.

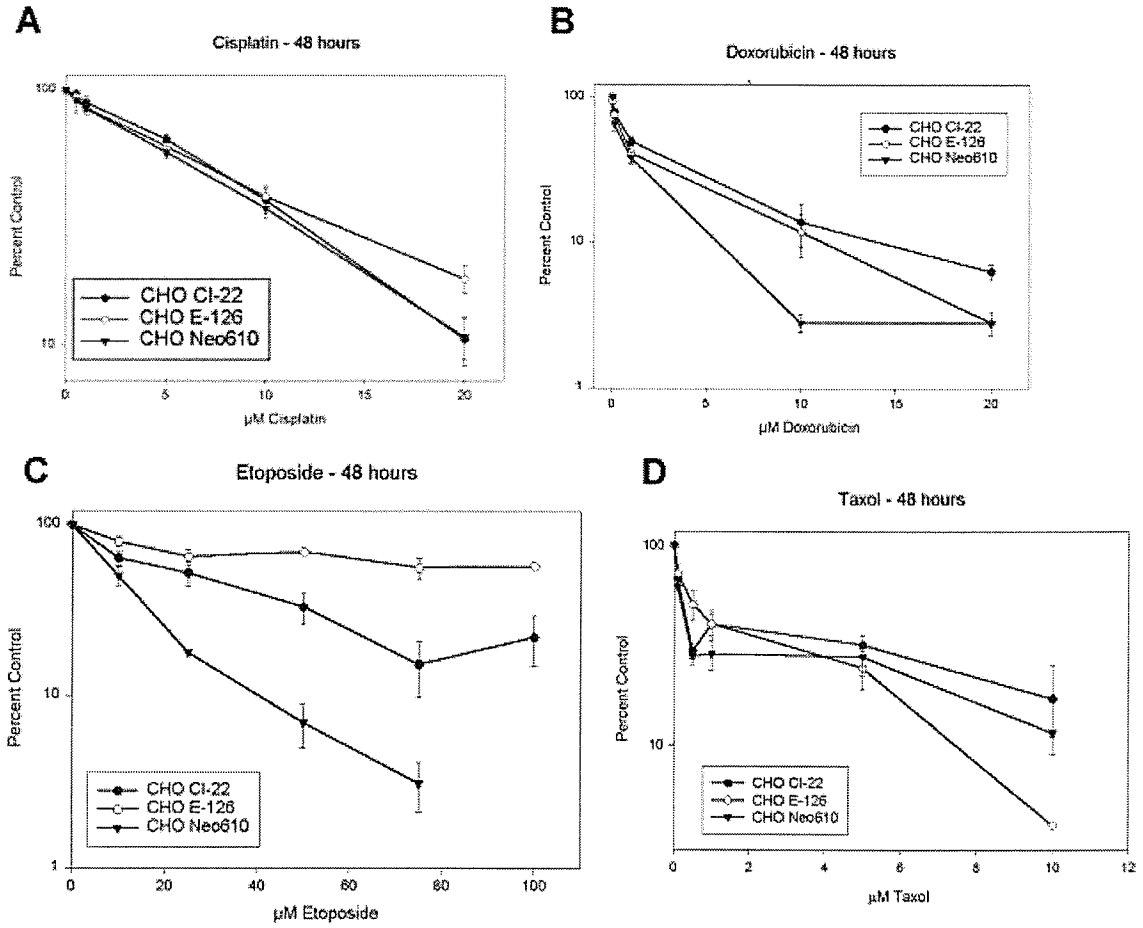

Figure 6: Cytotoxic activity of Cisplatin, Doxorubicin, Etoposide, and Taxol on CHO Cl-22, E-126, and Neo 610 cells. Cells were incubated for 48 hour with **(A)** 0, 0.5, 1, 5, 10 or 20 μM Cisplatin, **(B)** 0, 0.01, 0.1, 1, 10, or 20 μM Doxorubicin, **(C)** 0, 10, 25, 50, 75, or 100 μM Etoposide, or **(D)** 0, 0.1, 0.5, 1, 5, or 10 μM Taxol. Cytotoxicity was determined by the MTT assay and is presented as the surviving cell fraction (optical density in treated cells as a fraction of the optical density in untreated cells).

Note: Error bars represent standard error. Results indicate the average of at least five independent experiments consisting of no less than 8 samples for each point.

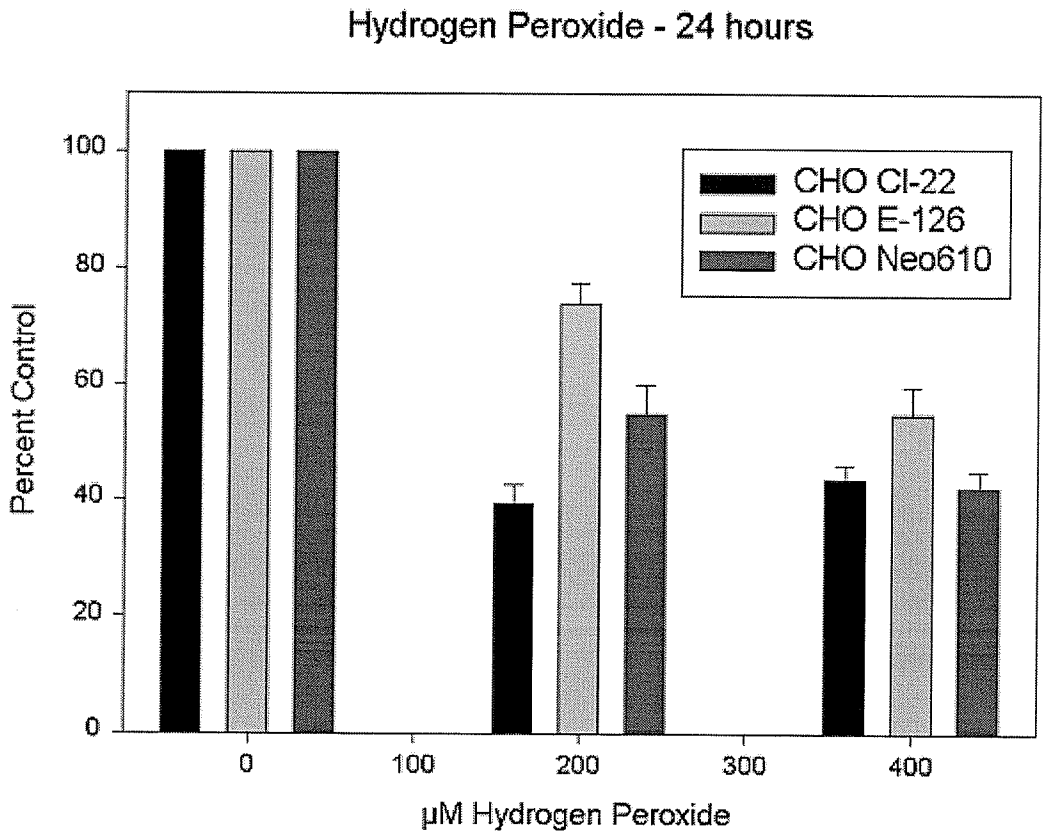

Figure 7: Cytotoxic activity of Hydrogen Peroxide on CHO Cl-22, E-126, and Neo 610 cells. Cells were incubated for 1 hour with 0, 200, or 400 µM hydrogen peroxide, washed with PBS and incubated overnight in normal medium. Cytotoxicity was determined by the MTT assay and is presented as the surviving cell fraction (optical density in treated cells as a fraction of the optical density in untreated cells).

Error Bars represent Standard Error.
Results indicate the average of at least five independent experiments consisting of no less than sixteen samples for each concentration.

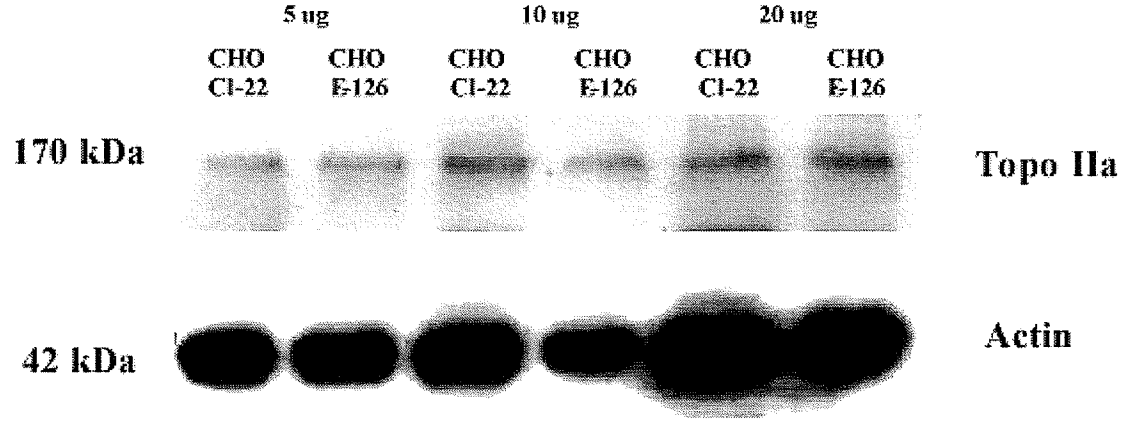

Figure 8: Comparison of Topoisomerase IIa protein levels between CHO Cl-22 and CHO E-126 clones. Protein extracts were prepared from CHO Cl-22 and CHO E-126 cells growing under normal conditions and immunoblotted for the 70 Kilodalton c-terminal domain of topoisomerase IIa and for actin.

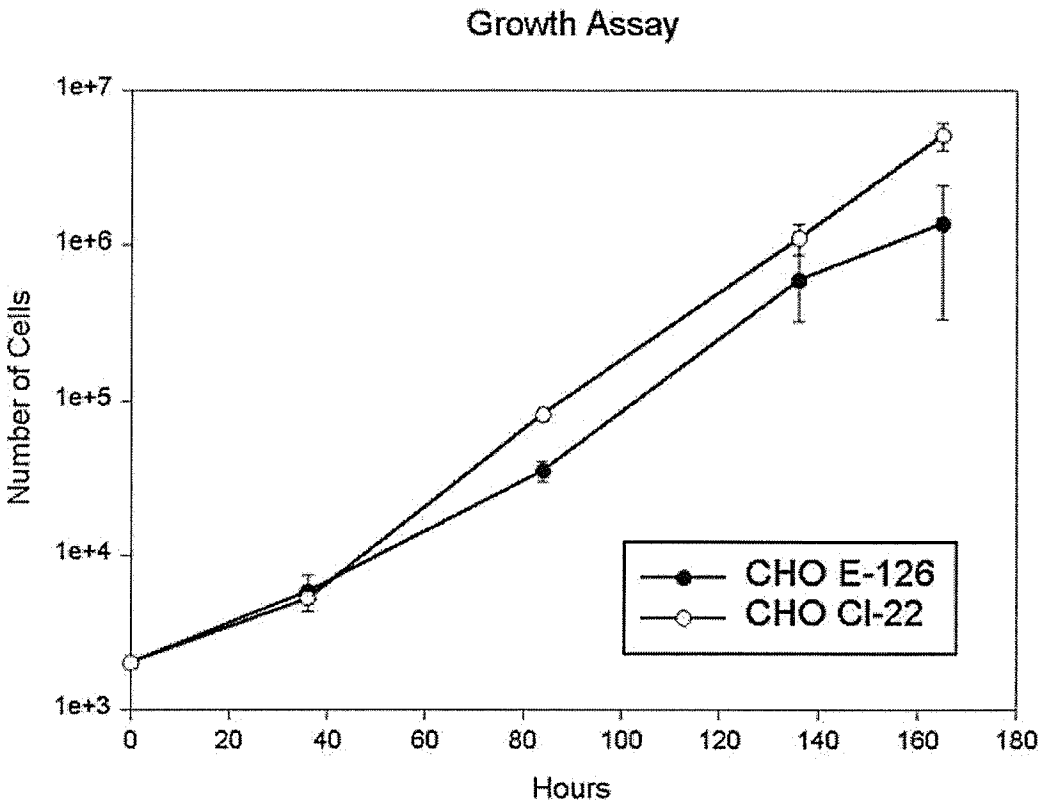

Figure 9: Cellular growth rate for CHO E-126 and CHO Cl-22 clones. One thousand cells were plated at time 0 and triple aliquots for both cell types were counted in triplicate at the indicated times.

Note: Error bars represent Standard Error.

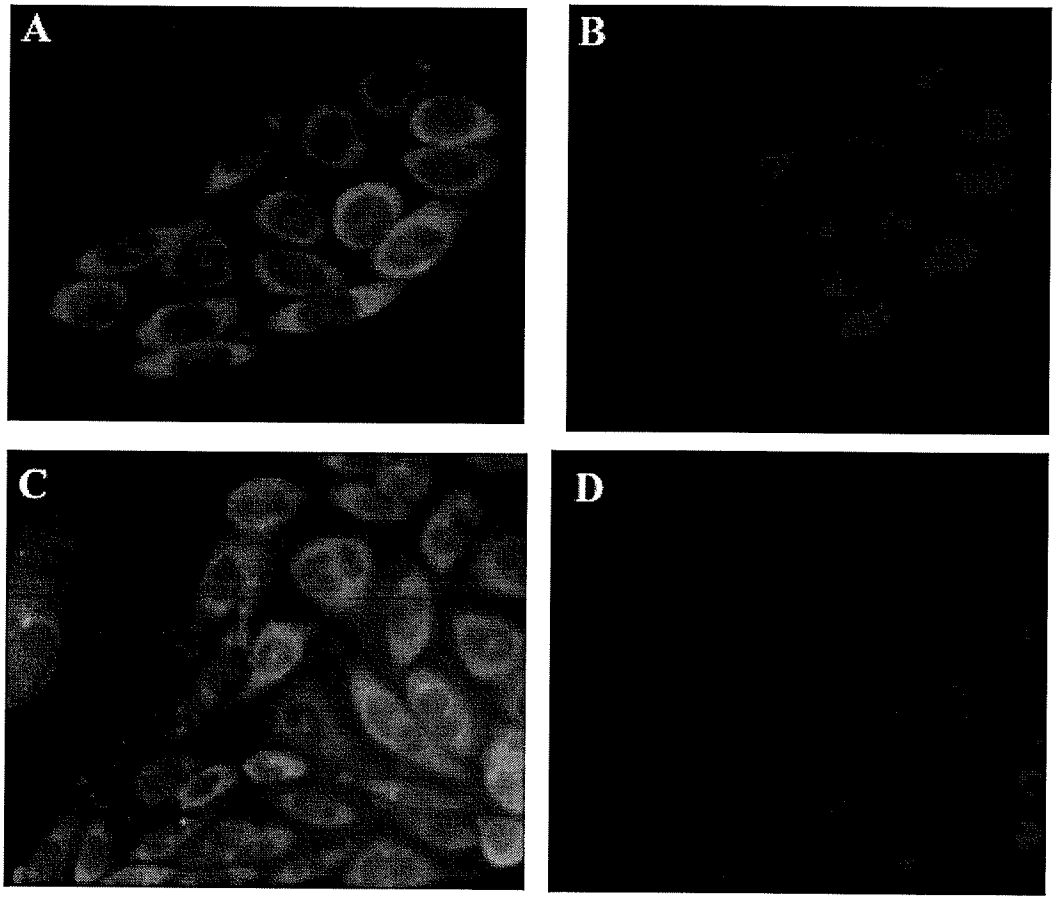

Figure 10: Morphologies of CHO E-126 and CHO Cl-22 cells grown under normal conditions and stained with γ-tubulin antibodies and DAPI. **(A)** CHO Cl-22 cells stained with γ-tubulin antibodies. **(B)** CHO Cl-22 cells stained with DAPI. **(C)** CHO E-126 cells stained with γ-tubulin antibodies. **(D)** CHO E-126 cells stained with DAPI.

Magnification:60x

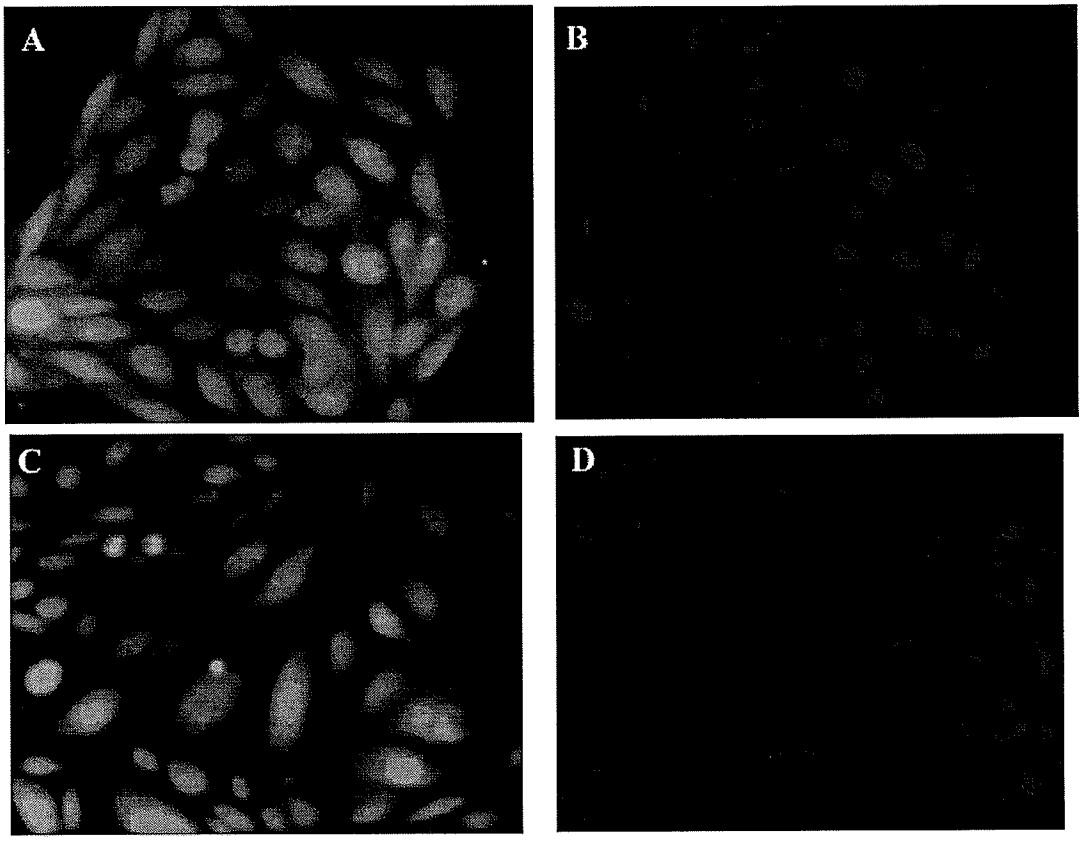

Figure 11: Morphologies of CHO E-126 and CHO Cl-22 cells grown under normal conditions and stained with Lamin A antibodies and DAPI. **(A)** CHO Cl-22 cells stained with DAPI. **(B)** CHO Cl-22 cells stained with Lamin A antibodies. **(C)** CHO E-126 cells stained with DAPI. **(D)** CHO E-126 cells stained with Lamin A antibodies.

Magnification: 60x

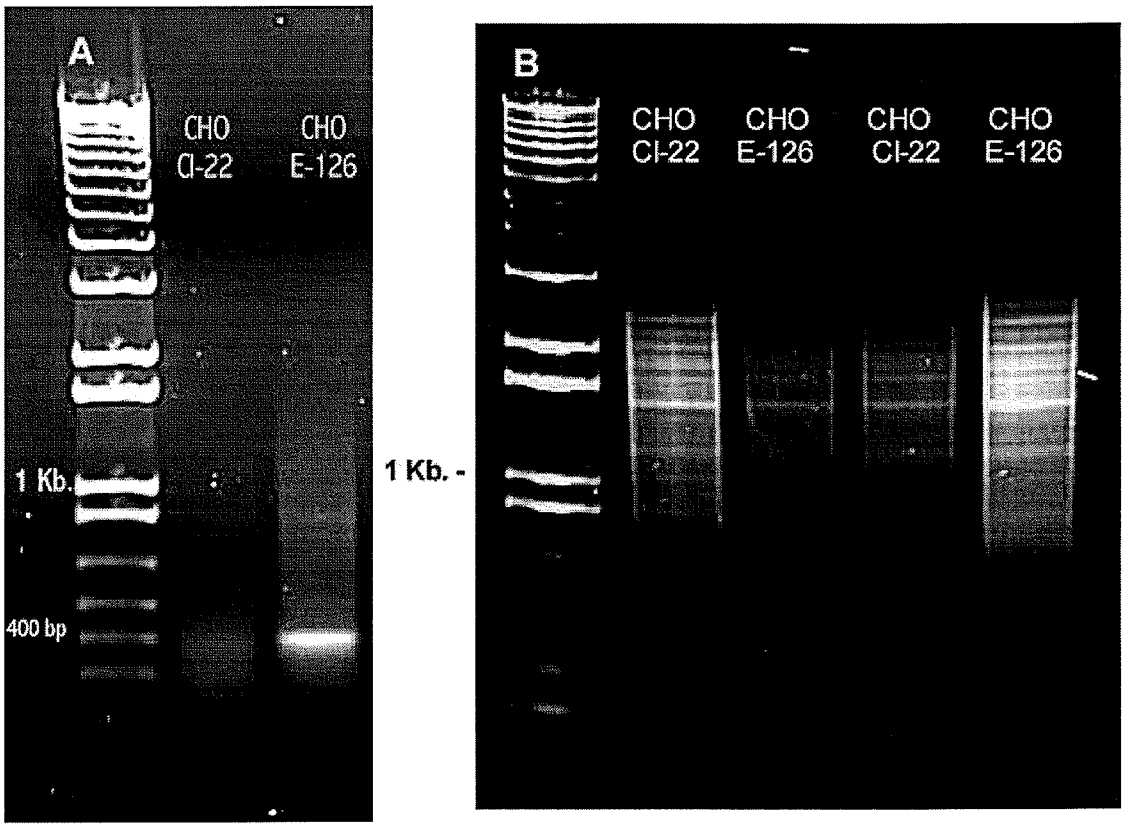

Figure 12: Gel electrophoresis of 5' and 3' RACE-PCR products. **(A)** 5'-RACE-ready cDNA from Cl-22 and E-126 cells was PCR amplified with the Universal Primer Mix and primer NeoES. Lane 1: 1Kb Plus DNA Ladder (Invitrogen), Lane 2: Cl-22 PCR product, Lane 3: E-126 PCR product. **(B)** 3'RACE-ready cDNA from Cl-22 and E-126 cells was PCR amplified with the Universal Primer Mix and primer 3RaceA (Lanes 2+3) or primer 3RaceB (Lanes 4+5). Lane 1: 1Kb Plus DNA Ladder (Invitrogen), Lane 2: Cl-22 PCR product, Lane 3: E-126 PCR product, Lane 4: Cl-22 PCR product, Lane 5: E-126 PCR product.

A

taa cac ggc ggc atc ag nac agc cgn ntt ntc tag
ttg ngc ctt acc tna gcc gaa tag cct gct gcc acc
caa gcg gcc ggt agb aac cta gcg tcg caa tgc cat
ctt ggt gtn caa tgc atc ttg gtg tnc aat gca tgc
gaa act gat cct cag cta gct tgc caa acc tna cag
gtg ggg tct ttc acc ttg ggt cag tna taa ttt cta
atc tag ttt tta gaa caa tcc ctc att cat cag tgc
aca cac ata ccc aag aga ctg acc cct atc gca gag
tag tca tg

B

```
 Score = 260 (45.1 bits), Expect = 0.00027, P = 0.00027
 Identities = 78/103 (76%), Positives = 78/103 (76%), Strand = Plus / Plus

Query:     3 CTTGGGTCAGTNATAATTTCTAATCTAGTTTTTAGAACAATCCCTCATTCATCAGTGCAC 62
             ||| || |||| | ||||||||||||||| ||| ||| ||||||||  ||| || || ||
Sbjct: 25535 CTTAGGCCAGT-AGAATTTCTAATCTAGATTTCAGAGCAATCCCTTGTTCGTCGGTACAT 25593

Query:    63 ACA-C-ATACCCAAGAGACTGACCCCTATCGCAGAGTAGTCAT 103
             ||| | |||||||||||||    ||||| |  |||  | ||||
Sbjct: 25594 ACATCTATACCCAAGAGACCAGTCCCTA-CATAGAAGA-TCAT 25634
```

Figure 13: Sequence analysis on the sequence obtained following the 5' RACE on CHO E-126 cDNA. **(A)** The un-highlighted sequence corresponds to nucleotides from the U3NeoSV1 *neo* sequence. The sequence highlighted in yellow corresponds to U3NeoSV1 Long Terminal Repeat (LTR) nucleotides. Nucleotides highlighted in grey correspond to unique 5' RACE sequence. **(B)** BLAST sequence alignment of the unique E-126 5' RACE sequence and a homologous region found on chromosome 11 of the mouse genome.

A

naa gcc tcc tca cta ctt ttg gaa tag ctc aga ggc tca tnc
atc ant gca cac acn tac cca aga gac tga ccc cta tgc atg
aga gnc atg atg ggg gaa tat cct tct gta tgc tgt gaa tat
atg gtt tct ctt tat tgg ttg atg aat aaa gct gtg ttg gcc
aat agc cag gca gga agt ata cgc ggg act acc aaa cta caa
gca atc ctg gga agc agg aag gca gag agt gag tcg ntc gag
aga ngc cat gta gct aca gga agg cag aat g

B

C TCA TNC ATC ANT GCA CAC ACN TAC CCA AGA GAC TGA CCC CTA
TGC ATG AGA GNC ATG ATG GGG GAA TAT CCT TCT GTA NNN NNN
NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN
NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN
NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN
NNN NNN NNN NNN NNN NNN NCT ACA GGA AGG CAG AAT G

Figure 14: Sequence analysis from Inverse PCR of the left viral junction (5' end). **(A)** The sequence highlighted in yellow corresponds to the unique 5'RACE sequence. Nucleotides highlighted in grey correspond to nucleotides from the U3NeoSV1 *neo* sequence. Un-highlighted nucleotides are unique to this experiment. **(B)** 5' Inverse PCR non-viral sequence following repeat masking. The sequence highlighted in yellow corresponds to the unique 5'RACE sequence. Nucleotides highlighted in grey correspond to the sequence region representing an area of low complexity and/or repetitive elements.

A

tcc ttg gga ggg gtc tcc tct gan tga ttg act ccc gtc agc ggg
ggt ctt tca aag gag ggt taa aaa cat tca gct aca agc ttc tct
ctc gtc tct cgt ctc tcg tct ctc ccc act ttt tcc tgc ctc ctt
ctc tat ttc tca gac aga ttc tca ctc tgt agt cta ggc tgg ctt
caa att cac ttt gta tcc cag gct gtc ctt gag ctc aca atg atc
ctt ctc cta tct gct ggg gtt aaa aaa ttt taa tat tac tta att
tct ctt ttt ata ttc tag ctg tcc tac att tgg gat taa gag aaa
taa atg ttg tca tgt cta atg atg gca aag atc tat gac ttt tcc
ata gaa aga gtc aat tgc tca gag gta att ttt aaa gtc cct ata
gtc tcc cca caa tct ggg ggt acc acc cat aaa ttc ttg tga gtt
cta aat ctc tgg tct aat tat gtt tgn ttt aaa ac

B

AAG GAG GGT TAA AAA CAT TCA GCT ACA AGC TTC TCT CTC GTC TCT CGT CTC TCG TCT CTC CCC ACT TTT TCC TGC CTC CTT CTC TAN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NNN NAA AAA TTT TAA TAT TAC TTA ATT TCT CTT TTT ATA TTC TAG CTG TCC TAC ATT TGG GAT TAA GAG AAA TAA ATG TTG TCA TGT CTA ATG ATG GCA AAG ATC TAT GAC TTT TCC ATA GAA AGA GTC AAT TGC TCA GAG GTA ATT TTT AAA GTC CCT ATA GTC TCC CCA CAA TCT GGG GGT ACC ACC CAT AAA TTC TTG TGA GTT CTA AAT CTC TGG TCT AAT TAT GTT TGN TTT AAA AC

Figure 15: Sequence analysis from Inverse PCR of the right viral junction (3' end). **(A)** The sequence highlighted in yellow corresponds to U3NeoSV1 Long Terminal Repeat (LTR) nucleotides. Un-highlighted nucleotides correspond to sequence unique to this experiment. **(B)** 3' Inverse PCR non-viral sequence following repeat masking. Nucleotides highlighted in grey correspond to the sequence region representing an area of low complexity and/or repetitive elements.

```
 Minus Strand HSPs:

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             |||||     | ||   | || || || | | | || ||||||||| ||||||| ||    ||
Sbjct: 24933 ATTTATGTGTAACTGGTAAATTGTGTGATTG-ACACCCCAGATTAGTGGGGAAACCCCAG 24991

Query:   346 G-GACTTTAAAAATTACCTCTGAGCAATTGACTCTTTCTATGGAAAAGTCATAGATCTTT 288
             | |||||| |||||    ||||| |||||||||| |   | |||| ||||| | |||||| |
Sbjct: 24992 GTGACTTTTAAAATGGTCTCTGGGCAATTGACTTTCAC-ATGGGAAAGTGACAGATCTCT 25050

Query:   287 GCCATCATTAGACATGACAACATTTATTTCTCTTAATCCCAAATGTA-GGACAGCTAGAA 229
             || ||||| |||||  |||||||| ||||||| || |  |||||||  |||||||| |||
Sbjct: 25051 GCTATCATCAGACACCACAACATTCATTTCTCCTAGTTACAAATGTGTGGACAGCTGGAA 25110

Query:   228 TATAAAAAAGAGAAAT-TAAGTAATATTAAAATTTTT 194
             | |||||||| |||  | ||||||||||||||||| |
Sbjct: 25111 TGTAAAAAGAAAAAAATTAGTAATATTAAAATTTAT 25146
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Figure 16: BLAST sequence comparison of the Masked 3' Inverse PCR sequence and a homologous region (AL808021) found on mouse chromosome 11.

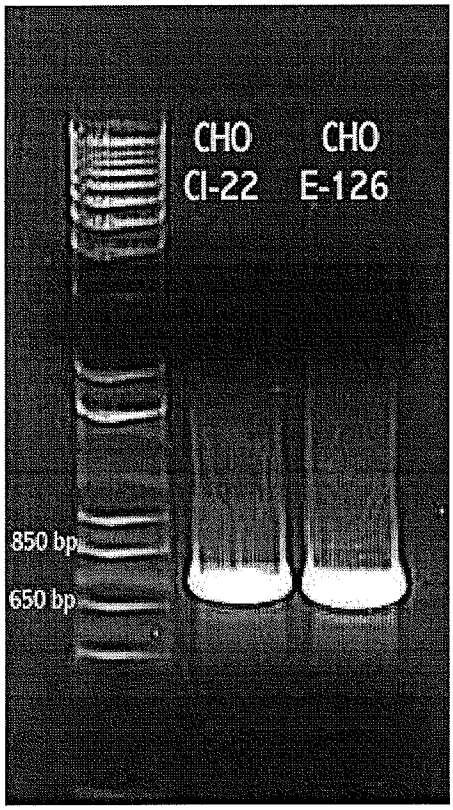

Figure 17: Gel electrophoresis of *gadd7*-based products generated by Reverse-Transcriptase PCR. cDNA was synthesized and PCR amplification performed using total-RNA extracted from Cl-22 and E-126 clones. Lane 1: 1Kb Plus DNA Ladder (Invitrogen), Lane 2: Cl-22 PCR product, Lane 3: E-126 PCR product.

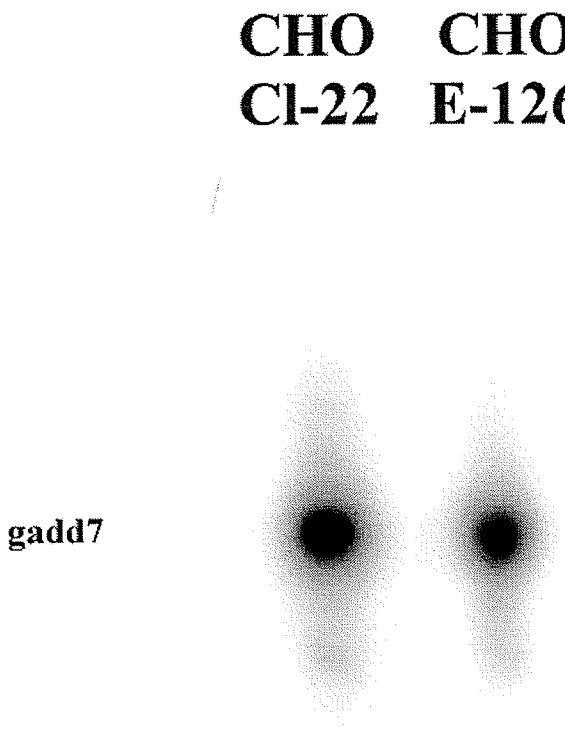

Figure 18: The *gadd7* transcript is present in both CHO Cl-22 and CHO E-126 cells. Total RNA isolated from these clones was separated by electrophoresis, blotted and hybridized to probes specific for 95% of the *gadd7* gene product including the region of potential viral integration.

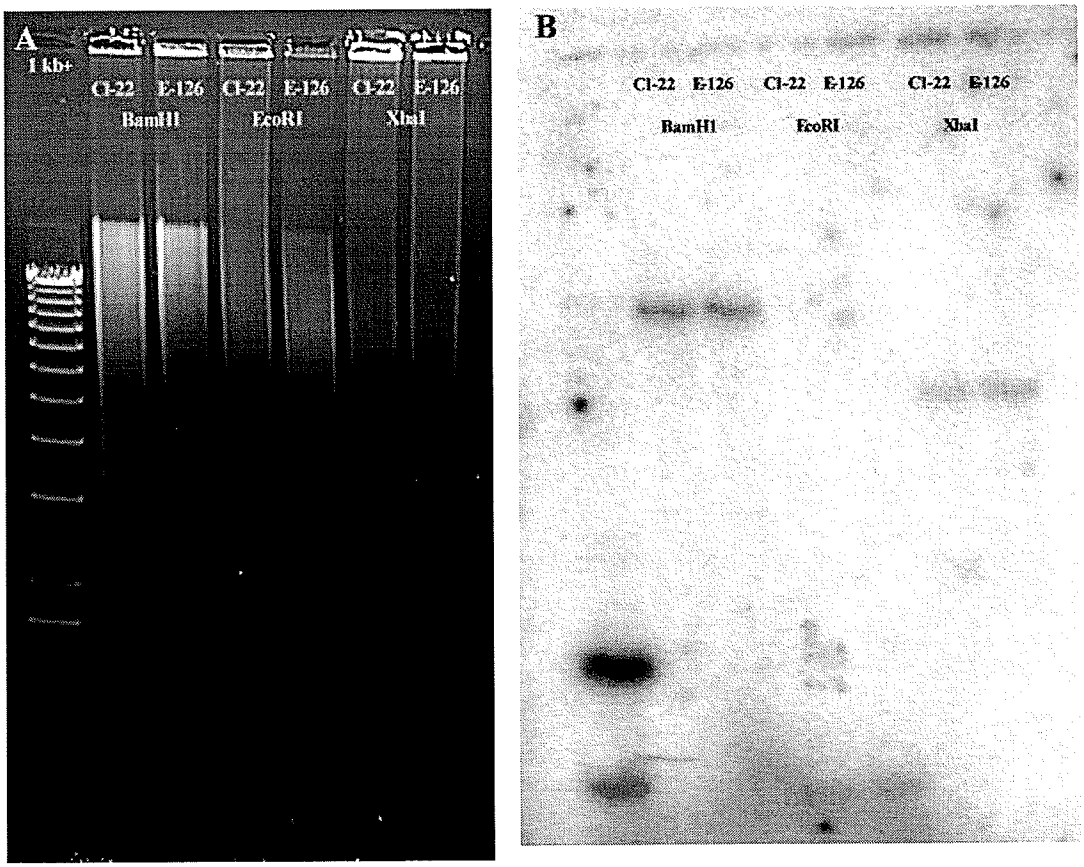

Figure 19: Southern blot of genomic DNA from CHO Cl-22 and E-126 cells digested with *Bam*H1, *Eco*R1, and *Xba*I and probed with the full-length *gadd*7 message. **(A)** Lane 1: 1 kb+ ladder, Lane 2: Cl-22 Genomic DNA digested with *Bam*H1. Lane 3: E-126 Genomic DNA digested with *Bam*H1. Lane 4: Cl-22 Genomic DNA digested with *Eco*R1. Lane 5: E-126 Genomic DNA digested with *Eco*R1. Lane 6: Cl-22 Genomic DNA digested with *Xba*I. Lane 7: E-126 Genomic DNA digested with *Xba*I. **(B)** Transferred gel probed with the full-length *gadd*7 cDNA.

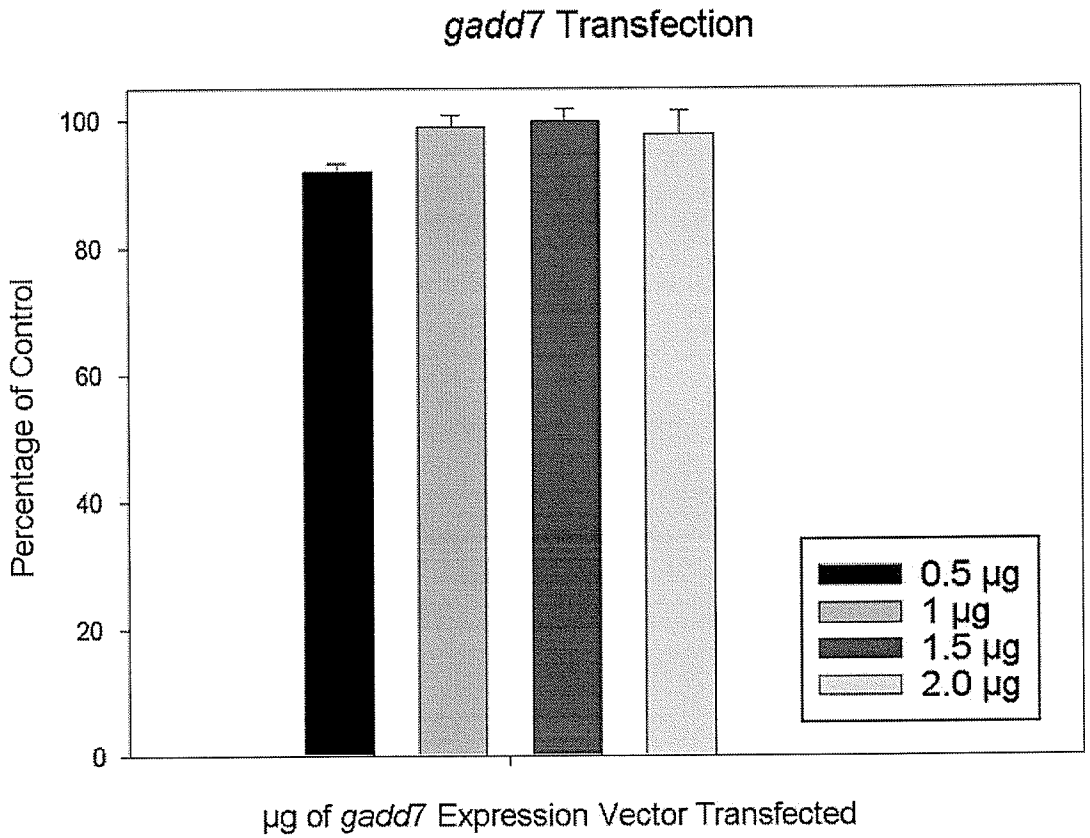

Figure 20: CHO Cl-22 cell survival following transient transfection with various amounts of *gadd7* in pcDNA3.1. CHO Cl-22 cells were transfected with 0.5, 1, 1.5, or 2.0 µg of *gadd7* and cytotoxicity determined 48 hours later by the MTT assay and is presented as the surviving cell fraction (optical density in treated cells as a fraction of the optical density in untransfected cells).

Note: Error bars represent standard deviation.

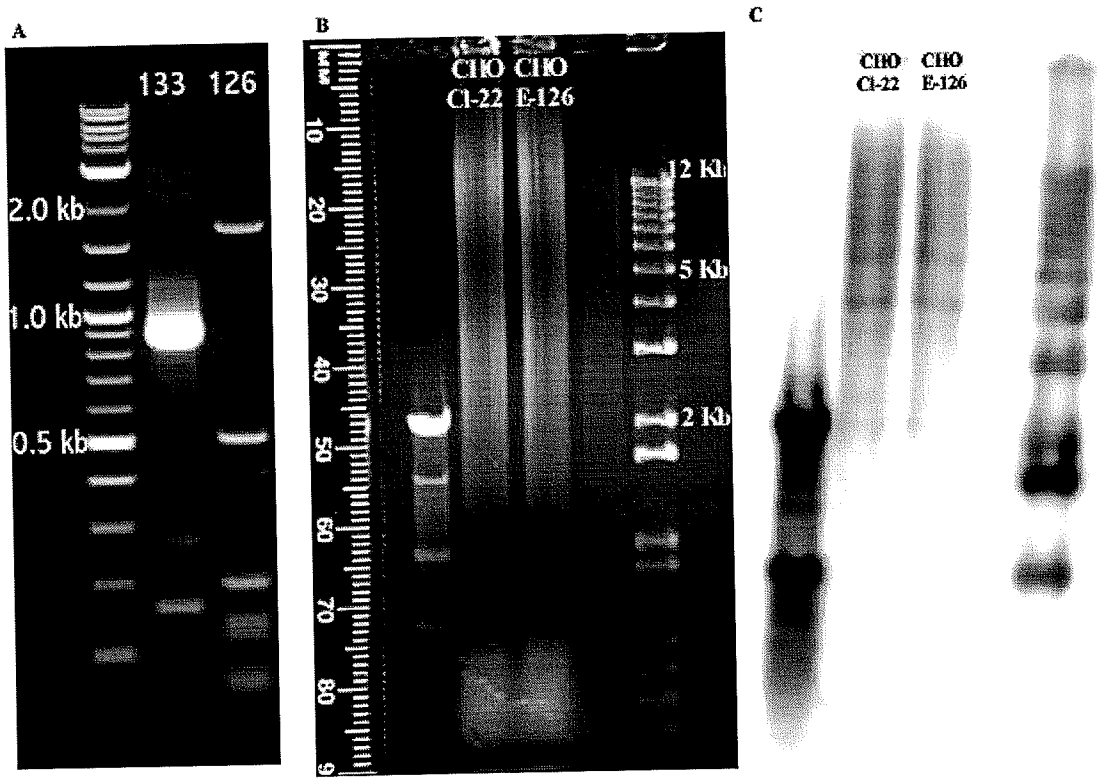

Figure 21: Southern blot of genomic DNA from CHO Cl-22 and E-126 digested with *Eco*RI and probed with the 5' Inverse PCR product. **(A)** Gel Electrophoresis of Inverse PCR from the left viral junction (5' end). The 1.6 Kb band in lane 3 represents the 5' end of the promoter-trap and the flanking genomic DNA. This amplification product was used to probe genomic DNA and total RNA from CHO E-126 and CHO Cl-22 cells. The other bands in lane 3 are non-specific. Lane 2 corresponds to an unrelated Inverse PCR experiment. **(B)** Genomic DNA isolated from CHO Cl-22 and CHO E-126 was digested with *Eco*RI and separated by electrophoresis **(C)** The digested DNA was blotted, and hybridized to a probe consisting of U3NeoSV1 viral sequence (5%) and the genomic DNA flanking the 5' end of the virus (95%).

Table 1. Summary of cross-resistance of etoposide-resistant cell-lines to other apoptosis inducers.

	CHO E-91	CHO E-116	CHO E-126	CHO E-121	CHO E-146	CHO E-210
Camptothecin	-	+	+	+	+	+
Staurosporine	-	+	+	+	+	+
Granzyme B	-	+	+	+	-	+
Granzyme K	-	+	+	+	-	+
TNF-α	ND	+	+	+	ND	+
Fas Antibody	ND	+	+	+	ND	+
Ceramide	+	-	-	-	-	-
γ-irradiation	-	+	-	-	-	-

-: no resistance
+: resistance or reduced apoptosis greater than 3 fold
ND: not determined

Data from Dr. Jeannick Cizeau.

Chapter 4 – Discussion

4.1 Characterization of the cell-line CHO E-126

The subject of this study is the etoposide-resistant cell line CHO E-126, a clone in which we have determined a single U3NeoSV1 promoter trap retrovirus has integrated without any viral rearrangement.

This cell line had initially shown resistance to the apoptosis inducers Staurosporine, Granzymes B and K, TNF-α, Camptothecin, and Fas Antibody. Research done in this project demonstrates that E-126 also has an increased resistance to hydrogen peroxide and a slight resistance to the drug Cisplatin at higher concentrations. E-126 cells did not display any apparent cross-resistance to the drugs Doxorubicin and Taxol. A review of other research into cell lines resistant to etoposide due to gene mutations reveals that cross-resistance between etoposide and other drugs vary, even when the other agents act specifically against Topoisomerase [see (Fukushima *et al.,* 1999) (Hill *et al.,* 1991) (Lock and Hill 1998)]. Because there is some cross-resistance seen by my research, and a larger amount by preceding studies [Table 1], it seems likely that our trapped gene is related to apoptosis, however its specific role has yet to be determined.

Another approach to determine how E-126 cells are resistant to etoposide involved examination of the protein levels of topoisomerase II alpha in these cells. Decreases in protein and/or mRNA levels of topoisomerase II has been linked to etoposide-resistance [see (Matsumoto *et al.,* 1997) and (Matsumoto *et al.,* 1999)]. In our analysis of the protein levels of topoisomerase II alpha, (the major type II

topoisomerase in a cell), no change was detected in E-126 cells when compared to parental cells. Topoisomerase II activity, regulated by various proteins and phosphorylation states [(Chikamori *et al.,* 2003) (Wells and Hickson 1995) (Kimura *et al.,* 1996)] has also been linked to etoposide resistance but has not yet been examined in E-126 cells. An examination of the activity of topoisomerase would be interesting as E-126 cells have a slower growth rate than Cl-22 cells and lower topoisomerase activities have been linked to this phenotype.

Besides their etoposide resistance and reduced growth rate, E-126 cells display other phenotypic differences from Cl-22 cells. Microscopically, one can see several obvious morphological changes in E-126 cells, including abnormal looking nuclei and increased cellular size. Research has found that a down-regulation of topoisomerase IIa in etoposide resistant cells correlates with the accumulation of polyploidy cells (Melixetian *et al.,* 2000) and etoposide-resistance has been linked with the presence of morphological changes in the cell karyotype (Meliksetian *et al.,* 1999). When the viral integration site and the function of the corresponding gene are known, the question as to whether the morphological changes in E-126 cells are related to etoposide-resistance or are a direct consequence of U3NeoSV1 integration will be better answered.

The 5' RACE experiment has identified the fusion transcript produced in E-126 cells. When the sequence of the 5' RACE was compared with the mouse genome, significant homology was found with the AL808021 region close to the end of chromosome 11. This chromosome region is predicted to produce two different transcripts. We were successful in identifying the flanking genomic DNA at both the

5' and the 3' end of U3NeoSV1 using Inverse PCR. The 5' Inverse PCR sequence contains the expected homology with U3NeoSV1 and with the 5' RACE sequence. In addition, it appears that contained within this region is a large area of repetitive and/or low complexity sequences. The 3' Inverse PCR sequence obtained also contains a repetitive/low complexity element, and the remaining sequence has homology with chromosome 11 of the mouse genome. This homologous region (24933-25146) is close to the homologous region identified in the 5' RACE and 5' Inverse PCR experiments (25535-25634). The 3' RACE experiment did not identify the specific transcript made by our trapped gene in E-126 or the wild-type transcript from Cl-22. Repeating the 3' RACE experiment using primers with increased specificity for the 5' unique sequence on parental CHO cells, or with primers based on the gene-specific region of the 3' Inverse PCR sequence could provide insight into the wild-type transcript produced and the particular gene we have trapped.

Based on results from the Plasmid Rescue technique to determine viral integration, we initially believed that in the E-126 cell-line our promoter-trapping construct had integrated into the *gadd7* gene. Experiments to reproduce these results were not successful. In addition, *gadd7* was not identified in Inverse PCR experiments from either viral junction, nor did the 5' RACE fusion sequence share homology to this gene. The experiments indicating *gadd7* integration suggested that the virus had integrated near the 5' end of that single-copy number gene. Therefore, we would expect a shortened transcript or no *gadd7* message to be produced in E-126 cells. An RT-PCR experiment on the E-126 cell-line detected the full-length *gadd7* message in both Cl-22 and E-126 cells, and a Northern Blot using a probe designed to

detect the majority of *gadd7* including the supposed region of integration resulted in bands of the same intensity and size detected in Cl-22 and E-126 clls. Again, one would expect no transcript or a shortened *gadd7* transcript in E-126 cells if our promoter-trap had integrated into that gene. Finally, a Southern Blot using a full-length *gadd7* probe on Cl-22 and E-126 genomic DNA digested with several restriction enzymes produced identical results. If our trapping construct was in the *gadd7* gene, a different banding pattern would be expected. Combined, this evidence suggests that the results obtained indicating *gadd7* integration were merely an artifact of the Plasmid Rescue experiment and/or the subsequent sequencing reaction.

4.2 Significance of results

Gene-trapping has proved effective as a means to identify genes with apoptotic roles including transiently induced survival genes related to apoptosis (Wempe *et al.,* 2001). We hope that promoter-trapping in the E-126 cell-line and the subsequent characterization of that cell-line will lead to the identification of a novel gene with a significant role in apoptosis.

Within the suspected region of integration on chromosome 11 of the mouse genome is a predicted transcribed region (by database comparisons to protein sequences and ESTs) and a mouse cDNA clone with identity to this region (Clone ID: 4972349 openbiosystems, Huntsville, AL). Our sequence data suggests intronic integration into that gene. The predicted transcript has 86% amino acid identity (271/315) with the protein sequence of the cloned cDNA. When the 315 residues of protein sequence from the cloned cDNA was examined for conserved domains

(http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) 70% of 178 base pairs (expect: 1e-11) had identity with the alpha subunit of the Prolyl 4-hydroxylase gene.

Prolyl 4-hydroxylase (P4-H), an enzyme conserved in mice and humans (Nokelainen *et al.,* 2001) plays a role in normal metazoan development, extracellular matrix formation, catalyzes the hydroxylation of proline residues and functions as a chaperone during collagen synthesis (Winter and Page, 2000). There are two isoenzymes of the vertebrate P4-H enzyme with they Type I isoenzyme expressed in developing and malignant tissues and Type II isoenzyme expressed in more differentiated cells (Niss *et al.,* 2001).

Family members of Prolyl hyrdoxylases have been implicated in the posttranslational modification of the hypoxia-induced factor (HIF) protein. These modifications signal that protein for degradation under normoxic conditions (Bruick and McKnight, 2001). Suppression of this family member in *Drosophophilia melanogaster* cells led to an accumulation of the hypoxia-induced gene lactate dehydrogenase implicating HIF prolyl hydroxylase enzymes in sensing cellular oxygen levels (Bruick and McKnight, 2001). In addition, when the alpha subunit of P4-H is knocked-out in *C. elegans*, "extensive embryonic lethality" occurs [(Hill *et al.,* 2000) (Friedman *et al.,* 2000)].

Besides the relationship between members of the prolyl hydroxylase gene family and oxygen radicals in the cell, there is nothing in the literature to linking these genes with apoptosis resistance. If the gene into which our trapping-construct has integrated is a member of the prolyl hydroxylase gene family, knocking-out the activity of this gene could explain the resistance of E-126 cells to hydrogen peroxide.

The mechanism by which E-126 cells are resistant to other apoptosis inducers would require some related pathway not dependent on free-radicals in the cell.

The 5' RACE experiment resulted in am amplification product of 400 base pairs with 300 nucleotides of sequence obtained. Of these, only 100 base pairs were non-viral. The 5' RACE fusion product failed to show homology with cDNA sequence from chromosome 11 of the mouse genome. This does not exclude the possibility that the U3NeoSV1 promoter-trap is affecting that transcript. A change in splicing could explain this result, or the virus may be integrated in the antisense strand of the gene.

4.3 Future directions

The cDNA sequence corresponding to the region of homology on chromosome 11 has been obtained. A Southern Blot with this sequence as probe on the genomic DNA from Cl-22 and E-126 cells should confirm whether the site of U3NeoSV1 integration is related to this gene. If so, a Northern Blot on the RNA from Cl-22 and E-126 cells with the cDNA sequence as probe will indicate whether we have a true knock-out or not.

Another interesting item for examination in E-126 cells is an analysis of their topoisomerase II activity. This may be accomplished in several ways, including the reconstitution of reverse gyrase assay or analysis of Topoisomerase II activity based on the decatentation of kinetoplast DNA (TopGEN, Columbus, Ohio). Topoisomerase II activity is of interest in E-126 as some of the phenotypic characteristics in this cell-line (reduced growth rate and morphological changes) are linked with a reduction in the activity of this protein in etoposide-resistant cells.

If the gene into which U3NeoSV1 has integrated has a prolyl hydroxylase-like domain, examination of the sensitivity of E-126 cells to agents that generate free-radicals in a cell would be interesting. Since there is evidence that this gene-family may be involved in cellular oxygen sensing (Bruick and McKnight 2001), and our cell line appears to have an increased resistance to hydrogen peroxide based apoptosis, E-126 cells may show an increased resistance to other agents generating free-radicals in a cell.

Chapter 5 – References Cited

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