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Greggory Bernard Herbert
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RELATIONSHIPS BETWEEN THE EXPRESSION OF GENES INVOLVED IN THE REGULATION OF APOPTOSIS AND CELL DEATH INDUCED BY NITROGEN MUSTARD IN HUMAN COLON CARCINOMA CELL LINES

A DISSERTATION SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

BY GREGGORY BERNARD HERBERT

CHICAGO, ILLINOIS
May 1996
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DEDICATION

To my mother, whose faith, courage, and perseverance have been a lifelong inspiration, and my father, whose memory has carried me through the high and low times of my life. I love you both.
ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Leonard C. Erickson, for his financial support without which this dissertation would not have been possible. I would like to thank the members of my committee, Drs. Thomas Ellis, Mark R. Kelley, Russell O. Pieper, and Israel Hanin for their time and patience. I am extremely grateful to both Patricia Simms and Dr. Thomas Ellis for their guidance and assistance with the use of antibodies and performance of flow cytometric techniques. A large portion of this dissertation would not have been possible without their perpetual support. I would also like to thank Thomas Ellis for putting forth both the time and effort to critically evaluate this dissertation, and more importantly, for forcing me to think scientifically during the early stages of the writing process.

A large debt of gratitude goes to Dr. Roger Kroes for being my scientific role model in the lab. Although thankful for the many technical skills acquired by his tutelage, I can never truly express my gratitude to him for teaching me the intangible skills required to be scientifically successful.

Finally, I would like to thank my wife, Nicky DiRito, D.O., for her unwavering love and patience through this ordeal. I love you man!
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LIST OF ABBREVIATIONS

A<sub>260</sub>  absorbance at 260 nm (280 nm, 595 nm, etc.)
A    Ampere
Ab   antibody
Act D  actinomycin D
bcl-2  B-cell lymphoma/leukemia-2 oncogene
bp    base pair
BCS   bovine calf serum
BSA   bovine serum albumin
cm    centimeter
cDNA  complementary DNA
Ci    curie
°C    degrees celsius
DNA   deoxyribonucleic acid
dATP  deoxyadenosine triphosphate
dCTP  deoxycytidine triphosphate
dGTP  deoxyguanosine triphosphate
dTTP  deoxycytidine triphosphate
dUTP  deoxyuridine triphosphate
DNase  deoxyribonuclease
DEPC  diethylpyrocarbonate
DMSO  dimethyl sulfoxide
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol-bis(ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid</td>
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<tr>
<td>FACS</td>
<td>fluorescence-assisted cell sorting</td>
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<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate-dehydrogenase</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
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<tr>
<td>HSR</td>
<td>homogeneously staining region</td>
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<td>h</td>
<td>hour</td>
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<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid</td>
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<td>Ig</td>
<td>immunoglobulin</td>
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<td>IP</td>
<td>immunoprecipitation</td>
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<td>kb</td>
<td>kilobase</td>
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<td>kDa</td>
<td>kiloDalton</td>
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<td>L</td>
<td>liter</td>
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<tr>
<td>mad</td>
<td>max dimerization gene</td>
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<td>HN2</td>
<td>mechlorethamine, nitrogen mustard</td>
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<tr>
<td>µ</td>
<td>micro</td>
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<tr>
<td>m</td>
<td>milli</td>
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<td>min</td>
<td>minute(s)</td>
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<tr>
<td>M</td>
<td>molar</td>
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<tr>
<td>max</td>
<td>myc-associated factor gene</td>
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<td>myc</td>
<td>(avian) myelocytomatosis virus oncogene</td>
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nano
nanometer
normal
oligodeoxynucleotide
phenol:chloroform:isoamyl alcohol (25:24:1)
phosphate buffered saline
piperazine-N,N’-bis(2-ethane-sulfonic acid)
polyacrylamide gel electrophoresis
polymerase chain reaction
polyoxyethylenesorbitan monolaurate
protein product (53 KDa) of the p53 tumor suppressor gene
revolutions per minute
ribonuclease
ribonucleic acid
Roswell Park Memorial Institute
second(s)
sodium dodecyl sulfate
standard saline citrate buffer
standard saline phosphate EDTA buffer
terminal deoxynucleotidyl transferase assay
times
times gravity
transfer RNA
Tris/borate/EDTA buffer
Tris buffered saline + Tween 20
TE  Tris/EDTA buffer
Tris  Tris (hydroxymethyl) aminomethane
TSA  Tris/saline/azide buffer
UV  ultraviolet
U  unit(s)
V  volts
v/v  volume per volume
W  Watt
w/v  weight per volume
INTRODUCTION

Over the past twenty years researchers have made great strides in advancing our understanding of the biology of cancer. Despite our better understanding of such things as tumorigenesis, DNA repair, and drug resistance, the clinical treatment of cancer has not greatly improved in the past ten years. This outcome is due largely to the unpredictable difficulties encountered in the translation of laboratory science to the clinical arena. A poignant example is drug resistance. Extensive scientific advances have been made in this field with regards to defining the many mechanisms by which a tumor cell can become resistant to chemotherapeutic agents. However, the emergence of tumor cells with a drug resistant phenotype is still the major obstacle encountered in the clinical treatment of cancers.

The discovery of oncogenes and the elucidation of their role in cellular proliferation and tumorigenesis revolutionized the field of cancer research. The long standing tenet that cancer is a disease resulting from deregulated cellular proliferation was developed when activated oncogenes were shown to exist in tumor cells. As a result, the majority of cancer research has been focused on gaining a better understanding of the mechanisms responsible
for cellular proliferation and its regulation in both normal and neoplastic cells. One of the first oncogenes to be characterized was c-myc. That c-myc has a role in cellular proliferation, differentiation, immortalization, transformation, and apoptosis is undisputed. However, the precise mechanism(s) by which c-myc mediates these cellular functions has remained enigmatic despite almost one and a half decades of research.

The second revolution to occur in cancer research was the discovery of tumor suppressor genes. These genes were shown to act as a brake on cellular proliferation and were antagonistic to the function of oncogenes. The removal of these brakes was sufficient to deregulate the growth of cells, therefore, these two classes of genes appeared to regulate cellular proliferation by either pushing down on the accelerator and overriding the brake, or by releasing the brake. As might be expected, the combination of accelerating and releasing the brake synergized the effect on cellular proliferation. This concept has been supported by many studies which have demonstrated the transforming properties of cells possessing an activated oncogene along with a deleted or mutated tumor suppressor gene.

The most studied tumor suppressor gene to date is the p53 gene. p53 is a complex gene in that along with its role as a tumor suppressor, some of its mutated forms possess oncogenic, gain of function, activity. p53 is the most
frequently mutated (or deleted) cancer gene known to date. Mutations of the p53 gene have been shown to occur in approximately 60% of all human tumors and thus its role in tumorigenesis is well supported. Recent findings have added to our current understanding of how p53 functions to prevent the formation of tumors. p53 is thought to maintain genomic stability via its involvement in the G1, G2, and mitotic spindle checkpoints and has been shown to induce apoptosis in certain cell types following DNA damage or activation of certain oncogenes. Thus, p53 suppresses the formation of tumors by preventing the genetic changes that could occur in cells which are allowed to replicate with a damaged genome and more importantly, by inducing apoptosis in those cells that are damaged beyond repair or harbor genetic alterations that are incompatible with its presence.

The third revolution in cancer research, ironically, is the result of work done before the discovery of oncogenes themselves. In the early 1970’s, the role of apoptotic cell death in the kinetics of tissue and tumor growth had been elucidated along with its role in the elimination of tumor cells treated with antineoplastic agents. However, these seminal findings were largely overlooked until recently. The current focus of research on apoptosis as it relates to cancer chemotherapy and tumorigenesis has finally begun to bring these early studies to fruition, and as a result has led to further revisions in the central theory of
Cancer can no longer be looked upon as a disease resulting solely from the deregulation of proliferation. Tumor growth is the balance between cellular proliferation and cellular death, and can be regulated by changes in either component. Therefore, an increase in cellular proliferation coupled with the inhibition of cell death would be synergistic with respect to tumor growth. The inhibition of apoptotic cell death would not, in and of itself, be sufficient to transform a cell since proliferation would still be under tight control and therefore, no real growth advantage would be imparted to the cell: it simply would not die. However, the prolonged survival of a cell that was programmed to die at a certain point in its life span would predispose it to further genetic changes (activation of oncogenes) that could impart a growth advantage to the cell. This would lead to the emergence of a tumor with a fast rate of growth and poor clinical prognosis, both due in part to the inhibition of apoptosis.

The existence of such a "death repressor" was demonstrated by the discovery of bcl-2 which is the prototypical member of a new family of oncogenes that regulate cell survival. The precise mechanism by which bcl-2 inhibits apoptosis is currently unknown, although it involves dimerization with the bax protein. Both in vitro and in vivo studies have verified the co-transforming effects of the bcl-
2 and c-myc proteins, thus supporting the hypothesis that alterations in components of the apoptotic cell death machinery can participate in the transformation of cells. More importantly, the ectopic overexpression of bcl-2 in tumor cell lines has been shown to increase the resistance of many cell types to the majority of chemotherapeutic agents used in the treatment of human cancers.

The next breakthrough in cancer research will possibly be in how cancer is treated. The concept that tumorigenesis is the result of multiple, step-wise, genetic changes is widely accepted and there is compelling evidence supporting the notion that the combinations of these genetic alterations leading to the transformed state may be as variable as the types of tumors encountered. Essentially, each type of cancer is a different disease and needs to be treated as such.

The importance of apoptotic death as a mechanism for tumor regression following chemotherapeutic agent exposure is gaining acceptance. Studies have shown that in many cell types, most, if not all, anti-cancer drugs kill tumor cells by inducing apoptotic cell death. Many oncogenes are known to have dual roles as initiators of cell proliferation and regulators (negative or positive) of apoptosis; c-myc, ras, c-raf, c-abl and rel are just some examples. These genes along with p53, bcl-2 and others have all been shown to be capable of directly or indirectly regulating the induction of
apoptosis in tumor cells following exposure to anti-neoplastic agents. More importantly, the activation of certain oncogenes or loss of tumor suppressor genes can render tumor cells inherently more resistant to a wide variety of chemotherapeutic agents as a result of the inhibition of apoptosis.

The emerging concept is that different tumor cells have different thresholds for the induction of apoptosis in response to anti-neoplastic agents and that these thresholds may be regulated or altered as a result of the genetic changes possessed by the cell. This concept may help to explain the inherent resistance of many tumors to multiple classes of chemotherapeutic agents when classical modes of resistance are insufficient to explain this phenomenon. Therapeutic modulation of genes involved in the regulation of apoptosis may be a potential mechanism for overcoming drug resistance. Therefore, further understanding of the regulation of apoptosis in human tumor cells in response to anti-neoplastic drugs will be essential for the advancement of the clinical treatment of human cancers.

The studies presented in this dissertation attempted to establish a relationship between the expression of oncogenes and tumor suppressor genes and cell death induced by the bifunctional alkylating agent, nitrogen mustard, in human colon carcinoma cell lines. The genes investigated in these studies included c-myc and other members of the myc
transcription factor network, bcl-2, and p53. Initial
studies investigated the role of the proliferative functions
of c-myc in HN2-mediated cytotoxicity and were designed to
test the following hypothesis: bifunctional alkylating
agents, such as nitrogen mustard, inhibit the clonogenicity
of human tumor cells by down-regulating the expression of
critical cellular oncogenes, such as c-myc. The remaining
studies in this dissertation investigated the involvement of
the apoptotic functions of c-myc in the regulation of cell
death induced by HN2. The initial hypothesis was tested:
human tumor cell lines which overexpress c-myc will undergo
an enhanced apoptotic cell death following exposure to
chemotherapeutic agents that cause DNA damage. The remaining
studies attempted to determine how the c-myc enhancement of
apoptosis was being regulated in a subset of human colon
carcinoma cell lines following exposure to nitrogen mustard.

These investigations provide evidence that gene products
involved in the regulation of apoptosis can be modulated by
exposure to HN2. These results are consistent with a
stimulus-response type of model in which the cellular
response to a drug stimulus determines the fate of the cell.
These studies also show that drug-induced apoptosis can occur
in human tumor cells in a wild-type p53-independent manner,
and describe a novel mechanism by which c-myc-enhanced
apoptosis may be regulated in human tumor cells exposed to
HN2.
Nitrogen Mustard

The nitrogen mustards are the most important class of clinically used alkylating agents (1,2). The parent compound, bis (2-chloroethyl) methylamine (mechlorethamine, nitrogen mustard, HN2) was the first clinically effective, non-hormonal anti-cancer agent (3). The current use of HN2 is limited to Hodgkin's and non-Hodgkin's lymphomas because the newer mustard analogs, such as melphalan, chlorambucil, cyclophosphamide, and ifosfamide have a higher therapeutic index, can be administered both orally and intravenously, and cover a wider spectrum of both soft and solid tumors.

Nitrogen mustard is actively transported across the plasma membrane of cells via the low affinity choline transporter (4). Once inside the cell, HN2 binds covalently to cellular macromolecules, including DNA, RNA, protein, amino acids, and nucleotides via two highly reactive, electrophilic, aziridinium intermediates (2,5,6). The bifunctionality of the nitrogen mustards results in the crosslinking of cellular macromolecules, and the anti-tumor and cytotoxic activities of these compounds have been shown
to require both alkylating moieties (7). Brookes and Lawley (8,9) suggested that nitrogen mustard could form DNA-DNA intrastrand and interstrand crosslinks, and that these lesions were responsible for the anti-tumor and cytotoxic effects of this compound. Many sites on DNA are targets for alkylation by HN2, however, the N-7 position of guanine is the most electronegative and therefore, the major site of alkylation (8,10-14). Initial studies showed that crosslinks could be formed on the guanines of DNA (8,15,16) and DNA-DNA intrastrand, DNA-DNA interstrand, and DNA-protein crosslinks were shown to exist in tumor cells treated with therapeutic doses of nitrogen mustard (17-19).

Despite the presence of various types of crosslinking lesions, the cytotoxic potential of HN2 is strongly correlated with the formation of DNA-DNA interstrand crosslinks (15,20). Assuming that the DNA-DNA interstrand crosslink is the cytotoxic lesion, the inhibition of DNA synthesis has been postulated to be the most likely mechanism by which HN2 produces its cytotoxic effects (1,21,22). In this regard, the evidence favors a mechanism involving the inactivation of the DNA template by crosslink damage rather than the inhibition or inactivation of DNA polymerase or other enzymes involved in DNA synthesis (23-27).

Another possible mechanism by which DNA-DNA interstrand crosslinks may exert a cytotoxic effect is the inhibition of gene transcription. Interstrand crosslinks formed by
nitrogen mustards occur in a sequence specific manner, where runs of guanines are the favored sites of alkylation (12,13). Mattes et al. (12) suggested that GC rich areas of the genome would make good targets for alkylation and subsequently showed that GC rich regions in the H-ras promotor are preferentially alkylated by a variety of chemotherapeutic agents. Other studies have shown that nitrogen mustard inhibits transcription in vitro (28-30), and taken together, these studies suggest that nitrogen mustards may preferentially inhibit the transcription of GC rich genes, such as oncogenes, which could contribute to their anti-tumor effects. To this end, studies from this laboratory have shown that c-myc mRNA expression is down-regulated in a human tumor cell line following exposure to various nitrogen mustards (31). However, no evidence exists to suggest that DNA-DNA crosslinks directly inhibit the transcription of human genes in vivo, therefore, the down-regulation of c-myc mRNA may not have been a direct consequence of drug-induced lesions in the c-myc gene. In support of this possibility, other targets for HN2-induced damage have been shown and include the Na⁺/K⁺ ATPase (32), and the plasma membrane of cells (33). Also, many studies have shown that the mechanism by which HN2 exerts its cytotoxic effects must involve lesions other than the DNA-DNA interstrand crosslink (34-39). Despite almost fifty years of research, the exact mechanism by which the nitrogen mustards exert their cytotoxic effects
on human tumor cells remains unknown.

**Apoptosis**

Cell death is an important, evolutionarily conserved, physiological process which is required for the development and maintenance of most, if not all, multicellular organisms (40). Every somatic cell in a multicellular organism contains the genetic blueprint for the death machinery and is thought to be capable of implementing it. The inappropriate execution or inhibition of cell death can be deleterious for the organism and can result in a diseased state (41-45).

The term "apoptosis" (from the Greek: refers to the "dropping off" of leaves from a tree) was first used by Kerr et al. (46) in 1972 to describe a type of cell death that was morphologically distinct from necrosis. Apoptosis is a descriptive term that is applied to a common type of cell death induced by a variety of physiological and non-physiological stimuli under numerous types of circumstances (47 and references within). Although thought to be mediated by a genetic program (40, 46, 48-52), apoptosis is not specifically associated with the element of time and should not be confused with "programmed cell death", a functional term describing the developmental process of cell elimination that is associated with a genetically programmed "cellular clock" (47, 53). Therefore, it must be noted that not all programmed cell deaths occur by apoptosis (54, 55), and
certainly, not all apoptotic cell deaths are programmed with respect to the time of their occurrence (53,56-62). Apoptosis is involved in the removal of cells from both healthy and diseased tissues (46) and the role of this type of cell death is well documented in embryonic development (41,63-69), organogenesis (70-73), tissue and organ homeostasis (46,74-78), development and maintenance of the immune system (79-81,42 and references within), carcinogenesis (42,82-85) and cancer chemotherapy (86-91), AIDS (43,92,93), autoimmune diseases (44), neurodegenerative diseases (45,94,95) and viral infections (42,96).

Apoptosis and necrosis are the two morphologically and biochemically distinct types of cell death recognized by pathologists. Necrotic cell death usually results from severe environmental trauma, such as hypoxia or hypothermia, which directly damages the plasma membrane or interferes with the production of ATP, resulting in the impairment of ion homeostasis across the cell membrane. Consequently, the cell loses the ability to osmoregulate and swells due to the influx of water. Although reversible at this stage, the continued increase in the levels of cytosolic calcium disrupt the cytoskeleton and activate membrane-bound phospholipases and proteases. The lack of ATP forces a switch to anaerobic glycolysis, and leads to a decrease in both intracellular pH and macromolecular synthesis. Finally, the swollen cell ruptures and dies, releasing its contents into the
extracellular compartment. Necrosis is a passive, energy-independent death which elicits an inflammatory response and results in the synchronous death of neighboring cells (48,97). In contrast to necrosis, apoptosis is an asynchronous, energy-dependent process requiring cellular participation in the sense that a molecular death pathway must be activated after death-inducing stimuli are encountered by the cell. The most important aspect of apoptotic cell death is that single cells can be removed from tissues without provocation of an inflammatory response and damage to neighboring cells (48,97).

**Morphology of Apoptosis**

The morphological changes which occur in apoptotic cells have been extensively characterized and reviewed (46,69,98,99). Apoptotic cells lose contact with neighboring cells as a result of cellular shrinkage and loss of specialized cell surface elements such as microvilli and desmosomes. Surface changes occur rapidly and are followed by the fragmentation of the cell into a series of membrane-bound, condensed apoptotic bodies. Most of the organelles, including mitochondria, remain intact and functional. However, the endoplasmic reticulum dilates and fuses with the cell surface resulting in the characteristic membrane blebbing of apoptotic cells (100). The most striking and morphologically characteristic changes in apoptosis occur in
the nucleus. During the early stages of apoptosis, chromatin is fragmented and condenses in the region immediately underlying the nuclear membrane, forming dense, coarse, osmiophilic aggregates, next to which nuclear pores are seldom visible. The nucleus shrinks substantially and chromatin caps are visible at multiple sites around the nuclear periphery. The nucleolus disintegrates and eventually the nuclear membrane becomes discontinuous with chromatin found amongst the cytoplasmic constituents of the cell. As previously mentioned, the cell then fragments into apoptotic bodies which contain a variety of organelles and/or condensed chromatin. During this process, surface markers are expressed which target the apoptotic bodies for phagocytic engulfment by macrophages and neighboring cells, thus ensuring the efficient removal of potentially harmful debris.

**Biochemistry of Apoptosis**

The biochemical hallmark of apoptosis is the non-random fragmentation of chromatin into oligonucleosomal DNA "ladders" (internucleosomal integers of 180-200 bp) (101). The appearance of DNA ladders corresponds with the activation of an endogenous endonuclease that specifically cleaves DNA in the linker region of nucleosomes and is thought to be an early event in the apoptotic process (101,102). The presence of this specific pattern of DNA fragmentation has been documented in a number of cell types using a variety of
apoptotic stimuli (47 and references within).

Despite extensive efforts by many laboratories, the putative apoptotic endonuclease has not been cloned (101). Indeed, current evidence suggests the existence of more than one endonuclease (53). One prospective apoptotic endonuclease is a Ca$^{2+}$/Mg$^{2+}$-dependent endonuclease that was isolated from thymocytes and has been characterized as an anionic protein with a molecular weight greater than 110 kDa. This endonuclease has a double strand cleavage preference for DNA and a pH optimum of 7.5; and its regulation has been extensively reviewed (103). Another candidate for the apoptotic endonuclease is DNase II which is a Ca$^{2+}$/Mg$^{2+}$-independent endonuclease that functions optimally in an acidic environment (pH 6.5-7.0) (104,105). These endonucleases have been shown to be either constitutively expressed, or induced, depending upon the system used in the study (103).

Recent studies have shown that apoptosis can occur in some cell types without the presence of oligonucleosomal DNA ladders (106,107). Further investigation revealed that specific changes in the integrity of higher order chromatin occurred in apoptotic cells before, concomitantly with, or even in the absence of internucleosomal DNA cleavage (107-111). Chromatin changes were manifested as discrete DNA fragments of 300 kbp "rosettes" and 30-50 kbp "chromatin loops" suggesting the cleavage of looped domains of DNA at
the attachment points of the nuclear matrix. Studies using murine thymocytes (112) and isolated rat liver nuclei (113) suggested that internucleosomal cleavage and higher order DNA fragmentation were independent events. The original concept that internucleosomal DNA fragmentation was responsible for the changes in nuclear morphology is now suspect. The fact that higher order chromatin changes occurred before the appearance of DNA ladders and, more importantly, that these changes occurred in the absence of DNA ladders, provides convincing evidence that the changes in higher order chromatin, and not the internucleosomal fragmentation of DNA, were responsible for the condensation of chromatin associated with apoptotic cells (107,114). The 50 kbp chromatin loops are thought to serve as the substrates for endogenous endonucleases that cleave the DNA at internucleosomal sites. Therefore, those cells that undergo apoptosis without the formation of DNA ladders either do not express the proper endonuclease or it simply is not activated. These and other studies suggest that the apoptotic endonuclease is a dispensable component of the apoptotic machinery (107,115).

The role of both divalent cations and protein synthesis is another well studied, albeit poorly understood, area in apoptosis research. The role of Ca$^{2+}$ ions in the induction of apoptosis is well documented in some systems (116-118). The sustained increase in intracellular free Ca$^{2+}$ has been shown to occur prior to the induction of apoptosis by a
variety of stimuli (119-121). In these systems, preventing the increase in intracellular Ca$^{2+}$ inhibits the onset of apoptosis, while treatment with a calcium ionophore, such as A23187, induces apoptosis, suggesting that the influx of Ca$^{2+}$ may be a trigger for the induction of apoptosis (53,119). The potential targets for Ca$^{2+}$ in an apoptotic cell are many and may include the endogenous endonuclease, transglutaminase, cytoskeletal components, and a variety of calmodulin-dependent protein kinases and proteases (53,85,122). However, Ca$^{2+}$ is not an absolute requirement in all apoptotic systems (105,122-126) and further studies need to address the possibility that Ca$^{2+}$ influx is a common consequence, rather than a trigger for apoptosis. The existence of both Ca$^{2+}$/Mg$^{2+}$-dependent and -independent endonucleases may explain why the role of calcium in the apoptotic process can vary between cell types. The possibility that calcium may not be a trigger for the induction of apoptosis should not take away from the importance of this ion as a possible common effector for many of the various morphological and biochemical changes associated with an apoptotic cell (53,85,122).

The requirement for de novo protein synthesis is another complex issue in the regulation of apoptosis. Studies in thymocytes showed a requirement for de novo protein synthesis in the induction of apoptosis since protein synthesis inhibitors were capable of preventing apoptosis (117,127-
129). However, many studies have since shown that de novo RNA/protein synthesis is not a universal requirement and indeed, in many cell systems, inhibitors of RNA and protein synthesis actually induce apoptosis (60,96,97,130,131). This topic has been reviewed elsewhere (132). The bottom line seems to be that apoptosis can occur in either a protein synthesis-dependent or protein synthesis-independent manner depending upon the cell type and the apoptotic stimulus employed in the study.

The activation of tissue transglutaminase is yet another frequent but dispensable feature of apoptosis (53,133,134). Tissue transglutaminase is a calcium-dependent enzyme that catalyzes the crosslinking of appropriate protein substrates forming a protein net that may prevent the leakage of cellular constituents during apoptosis and aid in the formation of apoptotic bodies (53,85). The role of transglutaminases in apoptosis has been reviewed elsewhere (135).

Recent studies demonstrating the solubilization of nuclear matrix proteins in apoptotic cells have provided evidence suggesting a role for nuclear matrix changes in the induction of chromatin degradation and the morphological changes associated with the nucleus of apoptotic cells. The role of nuclear matrix proteins in apoptosis has been recently reviewed (136).

The final stage of apoptosis is the removal of the
cellular debris which is neatly packaged into apoptotic bodies. The apoptotic bodies are either sloughed off into the lumen of tissues or, more commonly, phagocytized by macrophages and neighboring cells. The recognition of the apoptotic bodies by these phagocytic cells is an important step in apoptosis and involves changes in the plasma membrane that facilitate their recognition and removal. These cell surface changes are cell type-dependent and have been reviewed elsewhere (137).

The Role of Apoptosis in Oncogenesis and Cancer Chemotherapy

Cell populations in normal adult tissues are in constant flux responding to autocrine, paracrine, and endocrine signals that regulate their size and function. Tissue size is kept relatively constant by maintaining a balance between cell proliferation and cell death, which occurs almost exclusively by apoptosis (46,77,78,138-140). Any disruption in the maintenance of this balance results in either tissue hyperplasia or atrophy and may have a negative impact upon the organism (40). Cells within many tissues are thought to be under social control and require factors that are supplied by other cells to proliferate and/or survive (85,141,142). These growth factors and survival factors are responsible for maintaining tissue homeostasis by regulating both cellular proliferation and cell death (142).

Carcinogenesis is a multi-step process in which cells
acquire genetic lesions that alter the expression and/or function of a specific subset of cellular proteins (143-146). These genetic lesions are manifested as gene amplifications, chromosomal translocations, deletion mutations, point mutations, and viral insertions, and impart a growth advantage to the cell by freeing it from the constraints of social control. The mechanisms and genes that are involved in the oncogenesis of human tumors are many and have been extensively reviewed (147-152).

The uncontrolled growth of cells has long been attributed to the deregulation of genes involved in cellular proliferation and differentiation (147,149,151). These genes fall into one of two categories: oncogenes or tumor suppressor genes. However, recent studies have reestablished the importance of apoptotic cell death in the maintenance of cell populations. Genetic alterations that inhibit apoptosis or increase the threshold for its induction result in tissue hyperplasia in a similar manner that would occur from an increase in cellular proliferation. Cells in which the inhibition of apoptosis has enhanced their survival, however, are not necessarily tumorigenic since the regulation of their proliferation is still under social control and therefore, they possess no real growth advantage (153). Likewise, a cell that proliferates in a deregulated fashion is not necessarily highly tumorigenic since these cells may show normal or enhanced rates of apoptosis which could prevent
tumor formation (154).

The recent discoveries of genes that are involved in the regulation of apoptosis have provided great impetus in suggesting a role for apoptosis in oncogenesis (reviewed in 42, 82, 85, 86, 155). These genes fall into two classes: 1) inducers of apoptosis whose expression can directly induce cell death or increase the susceptibility of cells to death inducing stimuli; and 2) repressors of cell death whose expression can prevent or delay the onset of apoptosis. The irony of these discoveries was that the majority of these genes had been previously characterized and were well studied because they were the same genes known to be involved in the transformation of normal cells, i.e. oncogenes and tumor suppressor genes. The fact that these genes are now known to be involved in the regulation of both cell proliferation and cell death further supports a role for apoptosis in both the prevention and development of an oncogenic state. In the following sections the major apoptosis regulatory genes will be individually discussed in detail and evidence will be presented which suggests that mutations affecting genes that are either inducers or repressors of apoptosis, coupled with a second mutation which enhances cellular proliferation, may be a common occurrence during the development of malignancy.

The goal of cancer chemotherapy is a simple one, maximize the ablation of cancerous cells while minimizing damage to normal tissues. In the past, the philosophy for
chemotherapy has been "more is better". Current clinical regimens try to maximize the tolerable dose of drug in an attempt to inflict the greatest amount of damage upon cells with the goal of overwhelming the cancer cell, thus causing its demise. The drugs used to fight cancer are by no means selective for tumor cells and it should come as no great surprise that their dosage is limited by the various toxic side effects on normal tissues. Actually it is hard to imagine how a drug which is present at such a high dose can be selective at all since the range of doses at which the drug could show selectivity has been greatly surpassed.

The current resurgence of apoptosis research has had a great impact on the cancer field (86-90). Increasing evidence has shown that low concentrations of chemotherapeutic agents from drug classes with disparate modes of action induce apoptosis in the majority of tumor cells tested (156,157, and 85,86 and references within). Studies have shown that the type of cell death utilized (apoptosis or necrosis) is largely dictated by the level of cellular injury rather than the type of agent used to cause such injury, and that the concentration of drug or duration of exposure required to reach this crossover point is radically different between tumor cell types (158-160). These findings have forced a reevaluation of the current philosophy of chemotherapeutic intervention in the treatment of cancer.
The cytotoxic effect of chemotherapy has long been thought to be due to the direct effect of the various agents on their specific biochemical targets (90,156,161), thus the more damage inflicted upon the target, the greater the chance for cell death. Ironically, the drug/target interactions for the majority of anti-neoplastic agents have been extensively studied, and yet the rationale for how and why such interactions should bring about the death of sensitive cells remains speculative to say the least (156). However, the fact that apoptosis is the common mechanism of cell death in nearly all tumor cells, regardless of the drug/target interaction employed, suggests that the drug/target interaction has an indirect role in cytotoxicity, with the final outcome being determined by the cellular response to a drug-induced change in homeostasis (90,156,161). The cellular response to drug exposure does not appear to be based solely upon the quality or quantity of change or damage induced by the drug (90). This simple pharmacological principle of stimulus-response coupling may go a long way in providing a possible explanation of what has been termed "inherent" drug resistance. Tumors that respond well to therapy are generally capable of readily engaging the apoptotic pathway of cell death, while those tumors which are resistant either do not receive the stimulus (classical drug resistance) or more importantly, do not respond to it, i.e. they have uncoupled the response from the stimulus (90). The
fact that different types of tumor cells have different thresholds for implementing the apoptotic pathway in response to the same drug further supports the concept that the cellular response to drug perturbations is an important determinant in the outcome of drug exposure (90,160,162).

The discovery that apoptosis can be regulated by oncogenes and tumor suppressor genes has taken on even greater importance given the role of apoptosis in the mediation of cancer chemotherapy. Many studies have shown that the apoptotic threshold in tumor cells is directly modulated by many of the oncogenes and tumor suppressor genes that regulate the apoptotic process (reviewed in 85,87,155). Therefore, the inherent drug resistance encountered in many tumors may very well be the result of the oncogenic process itself given the role of apoptosis in both chemotherapy and carcinogenesis (85,88). In support of this concept, the cell death repressor, bcl-2, has been shown to cooperate with c-myc in the oncogenesis of tumors (153,163) and its overexpression in many different tumor cell types imparts resistance to a wide spectrum of anti-neoplastic agents (164,165). Likewise, activating mutations of H-ras and translocation of the c-abl oncogenes have been shown to play a role in cellular transformation (166-168) and these lesions also increase the resistance of cells to chemotherapeutic agents by inhibiting the induction of apoptosis (154,169). Finally, the deletion or mutation of the tumor suppressor,
p53, has been shown to participate in the progression of tumor development (170,171), and cells lacking p53 or possessing a mutant form have been shown to be more resistant to many types of anti-cancer drugs as a result of the inhibition of apoptosis (172-175). The specific roles of apoptosis-regulating oncogenes and tumor suppressor genes in the modulation of neoplastic cell responses to chemotherapeutic interventions will be discussed on an individual basis in detail in the following section.

**Genes Involved in the Regulation of Apoptosis**

**Repressors of Apoptosis**

**bcl-2**

The B-cell lymphoma/leukemia-2 (bcl-2) oncogene was first identified at the t(14;18)(q32;q21) breakpoint (176-178) and constitutes the most common chromosomal translocation in human lymphoid malignancies, occurring in approximately 85% of follicular and 20% of diffuse B-cell lymphomas (179,180). The t(14;18)(q32;q21) translocation occurs when the bcl-2 gene is moved from its normal chromosomal location at 18q21 into juxtaposition with the powerful enhancer elements in the immunoglobulin heavy-chain (IgH) locus at 14q32 and results in the aberrant overexpression of bcl-2 mRNA and protein (181). These findings suggested that bcl-2 possessed oncogenic potential. Vaux *et al.* initially demonstrated that bcl-2 could prolong
the survival of factor-dependent pre-B-cells following the removal of interleukin 3, without any concomitant cell proliferation, suggesting that bcl-2 was blocking the normal apoptotic response of these cells following factor withdrawal (182). The cell death repressor activity of bcl-2 was formally demonstrated by Hockenbery et al. (183) and further supported by other studies (184,185), including antisense studies which showed that reductions in bcl-2 protein levels were insufficient by themselves to cause cell death, but accelerated cell death following survival factor removal (186). The lack of a proliferative effect by bcl-2 cast doubt on its oncogenic potential, however, some studies suggested that bcl-2 could cooperate with a proliferation-inducing oncogene to transform cells (182,187).

The subtlety of bcl-2's oncogenic activity was demonstrated in transgenic mice that were engineered to express a bcl-2-immunoglobulin minigene that mimicked the t(14;18) translocation. These mice initially displayed a polyclonal follicular lymphoproliferation and accumulation of B cells due to increased survival (188), but over time developed diffuse large cell immunoblastic lymphomas, half of which possessed deregulating c-myc translocations (189). These results showed that bcl-2 possessed oncogenic potential by prolonging the survival of cells that would normally be eliminated by apoptosis, allowing for the accumulation of secondary genetic lesions that could promote the
proliferation of these cells. The oncogenic cooperativity between bcl-2 and c-myc has been further demonstrated both in vitro (190,191) and in vivo by crossing bcl-2 and c-myc transgenic mice which produced progeny that rapidly developed leukemias (163,192). These findings reclassified bcl-2 into a new category of oncogenes: the death repressor genes.

bcl-2 is a large, three exon gene containing a 225 kb intron 2 that divides the protein coding exons 2 and 3 from the untranslated exon 1 (193). The major promotor, P1, is GC rich and contains multiple SP1 sites, while the minimally used P2 promotor has a classic TATA- and CAAT-box and an SV40 decamer/Ig octamer motif (194,195). Recently, a putative, p53-dependent negative response element has been identified in the bcl-2 promotor (196). The mRNA for bcl-2 is approximately 6 kb, contains a large 3'-untranslated region, and encodes a 239 amino acid, 26 kDa protein (194).

Bcl-2 is an integral membrane oncoprotein that can inhibit cell deaths that are induced by a variety of physiological and non-physiological stimuli (85,197,198). These findings have led to the concept that bcl-2 represses apoptosis by blocking a final, common pathway in the cell death process. That bcl-2 can prevent or delay apoptosis in almost any cellular context is well accepted. However, some pertinent physiological exceptions do exist; namely, complement-mediated lysis, apoptosis induced by cytotoxic T-cells (199), withdrawal of some cytokines (200), and positive
and negative thymic selection (197, 201, 202). Bcl-2 is restricted to tissues in which apoptosis molds developing structures or accounts for the turnover of cell populations; these tissues include thymus, bone marrow, breast, thyroid, prostate, pancreas, GI tract, skin, and nervous system tissue (203). The physiological role of bcl-2 in these tissues is thought to be related to maintaining the progenitor and long-lived cells in these lineages (203). A number of these tissues that express bcl-2 also have a high incidence of cancer, with skin, colon, breast, prostate, and pancreas ranking first, third, fourth, fifth, and ninth, respectively in disease sites (204), suggesting that changes in bcl-2 expression in these tissues may contribute to their oncogenesis.

Despite the resources and effort invested in bcl-2 research, the biochemical mechanism(s) by which this protein represses cell death is unknown. The amino acid sequence of the bcl-2 protein, as predicted from its cDNA, reveals no sequence motifs that would suggest a specific biochemical function for this protein. However, studies on the intracellular localization of bcl-2 might begin to shed some light upon this mechanism or at least help determine where to concentrate future efforts. The bcl-2 protein has been shown by confocal, laser scanning, and electron microscopy to be localized to the nuclear envelope, parts of the endoplasmic reticulum, and outer mitochondrial membrane (205-207). These
findings are consistent with the fact that bcl-2 is posttranslationally anchored to cellular membranes by a single, COOH-terminal stretch of 19 hydrophobic amino acids flanked on both sides by positively charged residues (208,209). Indeed, the insertion of bcl-2 into membranes orients the protein towards the cytosol and has been shown to be important for its death repressor activities (208,210,211).

Recent studies have suggested an antioxidant role for bcl-2 (212,213). The main source of reactive oxygen species in the cell is the mitochondria, with potential production by the endoplasmic reticulum, and nuclear envelope, the main sites of bcl-2 distribution. However, inhibition of apoptosis by bcl-2 has been shown to occur in the absence of a functional electron transport chain (205) and in the absence of a nucleus (214,215) suggesting that the death repressing activity of bcl-2 does not have an absolute requirement for these organelles. The endoplasmic reticulum is a major storage site for intracellular Ca$^{2+}$, a divalent cation with an important role in apoptosis, and implicated as a potential trigger of the process in some cell types. Bcl-2 has been shown to inhibit cell death triggered by Ca$^{2+}$ ionophores without preventing an increase in intracellular Ca$^{2+}$, suggesting that bcl-2 blocks a downstream event in this process (216). Other studies have shown that bcl-2 can prevent apoptosis by inhibiting the efflux of Ca$^{2+}$ through
the endoplasmic reticulum membrane (217). Although a bcl-2/Ca\(^{2+}\) association makes an attractive hypothesis, especially given the importance of Ca\(^{2+}\) in many apoptotic processes, it must be remembered that many of these processes, such as the activation of Ca\(^{2+}\)-dependent endonucleases, transglutaminases, and protein kinases, have been shown to be dispensable for apoptosis and its inhibition by bcl-2 in some systems (53, 107, 122). Such a hypothesis implies that the activation of the initial trigger of apoptosis, or a proximal upstream event, requires an increase or redistribution of intracellular Ca\(^{2+}\) that is inhibited by bcl-2. The identification of the initial trigger(s) of apoptosis, Ca\(^{2+}\)-dependent or otherwise, has eluded researchers to date.

Recent studies have shown that bcl-2 can associate with other proteins and these protein-protein interactions may help elucidate the mechanism(s) by which cell death is inhibited by bcl-2. The discovery of the 21 kDa, bcl-2-associated factor (bax) was a major boost for death repressor research (218). These studies showed that bcl-2 was capable of interacting with a protein partner (bax) through two highly conserved bcl-2 homology domains, BH1 and BH2, which would serve to define the bcl-2 family of proteins (218, 219). More importantly, these studies showed that the overexpression of bax in the presence of a death-inducing stimulus abrogated the death repressing actions of bcl-2, leading to the "rheostat concept" where the ratio between
bcl-2 and bax determines whether a cell is susceptible to apoptosis following a death stimulus, with high levels of bcl-2 favoring survival and high levels of bax favoring cell death (218,220). Bcl-2 homodimers are not capable of repressing death and in fact, bcl-2 was shown to require dimerization with bax to propagate cell survival (219). These results suggested two possibilities, the first being that the bcl-2/bax heterodimer was the functional death repressing complex. In this case, the heterodimer levels would be determined by the relative levels of both bcl-2 and bax proteins, and when bax protein levels were greater than bcl-2 protein levels, bax homodimers would dominate over bcl-2/bax heterodimers and cell death would result. When bcl-2 levels were greater, bcl-2/bax heterodimers would predominate and death would be repressed. The other possibility is that the bax/bax homodimer is a death-inducing complex and bcl-2 promotes survival by heterodimerizing with bax, thus, antagonizing the formation of the bax/bax homodimer. The recent development of a bax knockout mouse unfortunately did not provide a decisive resolution to this question since the loss of bax resulted in hyperplasia or hypoplasia, depending on the cellular context (221).

The discovery of bax prompted the search for other bcl-2 interacting proteins and family members and has uncovered several novel proteins. Another bcl-2 family member which is capable of interacting with bcl-2 is bad, a 22 kDa protein
which binds to both bcl-2 and bcl-x<sub>L</sub> (a novel gene belonging to the bcl-2 family of death repressors) and abrogates the death repressor activity of bcl-x<sub>L</sub> but not that of bcl-2 (222). Other non-bcl-2 family member proteins have also been shown to interact with bcl-2 and include bag-1, an anti-death gene that promotes the survival activity of bcl-2 under some circumstances where bcl-2 alone has no death repressor activity (223). Both p23 ras-related protein (R-ras) and raf-1, an oncogene, have been shown to interact with bcl-2, linking this protein with important signal transduction pathways (224,225).

The remaining bcl-2 family members, bcl-x, mcl-1, and A1 do not specifically interact with bcl-2 but are functional homologs in that they can repress death and heterodimerize with bax (226-230). The most studied of these genes to date is bcl-x which exists in both a short (x<sub>S</sub>) and long (x<sub>L</sub>) form as a result of alternative splicing (226). Bcl-x<sub>L</sub> exhibits death repressing activity that is virtually interchangeable with bcl-2 and has been shown to be expressed in embryonic and postnatal neural tissues where it functions to prevent neuronal cell death (231). Bcl-x has also been shown to be involved in the survival of double-positive thymocytes (232). The short form, bcl-x<sub>S</sub>, lacks the conserved BH1 and BH2 domains and does not dimerize with bax, yet still interacts with bcl-x<sub>L</sub> and bcl-2 (226,230). When bcl-x<sub>S</sub> was overexpressed in the presence of bcl-2 the survival function
of bcl-2 was abrogated and cell death was promoted (226). This observation suggests a function similar to bad in that bcl-x<sub>s</sub> may prevent the formation of bcl-2/bax heterodimers by antagonizing bax-bcl-2 interactions. The multiplicity of these bcl-2 family interactions suggests that the regulation of cell death by these proteins may be dependent upon their relative expression in specific cell types and the regulation of their subsequent interactions. Other posttranslational modifications to these protein family members may also be important for their interaction and/or functional regulation. In support of this concept, studies have shown that the phosphorylation of bcl-2 on serine residues inactivates its death repressor activity (233).

The Role of bcl-2 in Chemotherapy

As described in the previous sections, there is now much evidence supporting the involvement of bcl-2 overexpression in the development of human malignancies. The frequent occurrence of genetic lesions that inhibit the cell death pathway in human tumors underscores their importance in oncogenesis, however, the impact of these types of lesions may go beyond the development of tumors. Many types of tumor cells that overexpress bcl-2 have been shown to be more resistant to a variety of death stimuli, including a large majority of the anti-neoplastic agents used in the treatment of human malignancies (164,165,234-238). The sensitivity of
tumor cells to chemotherapeutic agents can be restored by the targeted depletion of bcl-2 via antisense technology (239,240). More importantly, bcl-2 protected cells from chemotherapeutic agents by suppressing the tendency of damaged cells to induce apoptosis, and this effect was not mediated by alterations in drug efflux, metabolism, or levels of DNA damage (241-243). These results show that the direct suppression of apoptosis represents a discrete mechanism of anti-neoplastic agent resistance. Other components of the death repressor pathway are being discovered and their roles in drug resistance are also being revealed (244-246). The involvement of cell death repressors in the outcome of chemotherapeutic intervention will no doubt be an important area of future research in oncology. The importance of death repressors in the clinical outcome of cancer chemotherapy is still a matter of debate. However, many new studies have shown that these proteins are expressed in clinical tumor specimens, suggesting a role in oncogenesis (247,248).

ras, abl, and raf-1

The GTP-binding protein Harvey ras (H-ras) is frequently overexpressed in primary human neoplasms. The overexpression of wild-type H-ras in rodent fibroblasts led to an elevation in both the mitotic and apoptotic indices. The overexpression of a mutant H-ras also resulted in an elevated mitotic index, however, the apoptotic index remained the same
as, or lower than in the parental cells resulting in a tumor phenotype which was far more aggressive in its growth characteristics than tumors expressing a wild-type H-ras (154).

The role of the c-abl translocation, t(9;22), (Philadelphia chromosome) in chronic myeloid leukemia (CML) has been well characterized (166). Recent studies have shown that v-abl can delay cytokine withdrawal-induced apoptosis of a mast cell line without inducing proliferation (249). Other studies have shown that the resistance of a CML cell line to several normally lethal stimuli can be overcome by downregulating the expression of bcr-abl mRNA with antisense oligodeoxynucleotides (ODNs). These studies suggested that the death resistant phenotype of CML cells was due to enhanced cell survival resulting from the suppression of apoptosis (169).

Raf-1 is a serine/threonine protein kinase that is important in mitogenic signal transduction pathways. Recent reports suggested that raf-1 can synergize with v-myc in the abrogation of IL-3-dependence in myeloid 32D cells by suppressing apoptosis (250), and that raf-1 may suppress apoptosis by promoting the function of bcl-2 (225,251).

**Inducers of Apoptosis**

p53

The p53 tumor suppressor protein was initially
classified as a tumor antigen that could interact with the large T antigen of Simian Virus 40 (SV-40) (252). p53 was reclassified as an oncogene when studies showed that the transfection of some isolated cDNA clones could immortalize cells in culture and transform them in cooperation with another oncogene, such as ras (253, 254). The true nature of p53 as a tumor suppressor was revealed when researchers discovered that the majority of the cDNA clones used in these studies were not representative of wild type p53, but contained missense mutations. Subsequent studies showed that the wild type p53 protein was not capable of transforming cells in culture, and more importantly, was able to prevent the transformation of cells by other oncogenes (255, 256). These findings helped to reclassify p53 from an oncogene to a tumor suppressor gene or anti-oncogene. However, the mutant forms of p53 were still thought to possess gain of function oncogenic activity. These initial studies provided the foundation for future p53 research by hinting at the complex nature of this protein and how its loss/gain of function could be involved in oncogenesis.

p53 is the most frequently mutated cancer gene studied to date, with mutations occurring in approximately 60% of human tumors (257). Missense mutations are the most common, but nonsense mutations and allelic deletions also occur. The p53 gene has been localized to the short arm of chromosome 17 (p13.1) and is comprised of 11 exons, the first of which is
non-coding (258). The 2.5 kb p53 mRNA encodes for a 393 amino acid nuclear phosphoprotein that is present in normal dividing cells at low levels.

The wild-type p53 protein is a 53 kDa protein that functions as a sequence-specific transcriptional activator, the activity of which is essential for its tumor suppressor functions (259). The protein is composed of four functional domains and includes a transcriptional activation domain in the acidic NH-terminus (260,261): a DNA-binding domain (residues 102-290) that binds to two copies of the consensus sequence, 5'-PuPuPuC(A/T)(A/T)GPyPyPyPy-3', which can be separated by as many as 13 bp of random sequence (262,263); an oligomerization domain (residues 319-360) responsible for the formation of p53 tetramers (264); and a basic, COOH-terminus with putative negative-regulatory functions (265). The COOH-terminal end also contains three putative nuclear localization signals (NLS). The predominant nuclear localization signal (NLS) is located adjacent to serine 315, which is an \textit{in vitro} and \textit{in vivo} substrate for p34\textsuperscript{cdk2} kinase (cdk1). It has been postulated that phosphorylation at Ser 315 may alter the cellular localization and/or function of p53 (266).

The wild-type and mutant forms of the p53 protein possess conformational and functional differences that are responsible for their tumor suppressive or oncogenic activities, and have been extensively reviewed (267,268).
The wild-type p53 protein has a very short half-life (less than 30 min) (269), can suppress the growth of tumors in vitro (270,271) and in vivo (272), and prevent the transformation of primary fibroblasts by cooperating oncogenes (256). Evidence suggests that the suppressor function of wild-type p53 requires the transactivation of target genes (259). The wild-type protein can form complexes with several viral oncoproteins, including SV40 large T antigen, adenovirus E1B, HPV16 E6, and others, which either functionally inactivate or enhance the degradation of the p53 protein. Normal p53 has also been shown to complex with the TATA-box binding protein (TBP), the DNA replication factor RPA, SP-1, WT-1 and CCAAT binding factors, single stranded DNA, DNA helicases, and the MDM2 oncoprotein (273 and references within). MDM2 expression is upregulated by wild-type p53 and the protein product of this gene has been shown to bind to and inactivate wild-type p53, suggesting a potential feedback mechanism for p53 regulation (274). Wild-type p53 is capable of suppressing transcription from promoters containing a TATA element via its interactions with TBP (275). Wild-type p53 protein displays a different conformation than most of the mutant forms since the wild-type protein is not recognized by the commercially available PAb 240 monoclonal antibody which is specific for many of the mutant forms of p53 (276). Some studies have shown that wild-type protein can exist in 2 cellular pools, one with a
wild-type conformation and one in which wild-type proteins adopt a mutant conformation (277). One hypothesis suggests that the wild-type p53 protein is conformationally flexible and alters its function as either a growth suppressor (wild-type conformation) or growth promotor (mutant conformation) as needed (278). The relevance of such a theory is unknown but the engineering of p53-/- knockout mice that developed normally did not support a role for wild-type p53 as a growth promotor (279).

Mutant p53 protein exists in many subtle forms which result mainly from missense point mutations. The majority of these point mutations have been mapped by analysis of p53 cDNAs obtained from a large number of tumor specimens. Not surprisingly, these point mutations occurred almost exclusively within the DNA binding domain (257, 280). Of the four known hotspots for point mutations, two are directly involved in contacting DNA, one in the minor groove and the other in the major groove. The third mutated residue bridges the two loops which contain these two residues, aligning them so they can make proper contact with the DNA (281). Missense mutations are often associated with a conformational change in the mutant protein (282). Conformational changes and substitution of important amino acid residues in the DNA binding domain help to explain why the majority of mutant proteins tested can no longer bind to DNA and transactivate genes (283-286). These characteristics are most likely
responsible for the inability of mutant p53 to suppress the
growth of tumor cells, and transformation of primary
fibroblasts (256,270). The half-lives of mutant p53 proteins
vary depending upon the mutation involved, but are several
hours in length (287,288). The posttranslational
stabilization of mutant proteins results in their
overexpression.

Wild-type p53 is often non-functional when in the
presence of overexpressed mutant p53 proteins (as is the case
in most tumors which contain both alleles). Some mutant
forms of p53 have been shown to exert a trans-dominant
negative effect on the wild-type protein, negating its
functional properties (253,255). The loss of wild-type
function can result from changes in conformation and it has
been shown that mutant protein can force wild-type p53 into
a mutant conformation (289). Also, the presence of mutant
proteins in a p53 tetramer inhibits the DNA binding and
transactivation activities of these heterogeneous oligomers
(285,286). Studies have shown that mutant proteins do not
necessarily aid in transformation by just interfering with
the growth suppressing properties of wild-type p53. Many of
the mutant proteins have been shown to possess gain of
function oncogenic activity in the absence of a trans­
dominant mechanism and this may explain why p53 mutations are
so strongly selected for in tumor cells, and wild-type p53 so
strongly selected against (290). In support of possible gain
of function activities, studies have shown that mutant p53, but not wild-type, can upregulate the expression of the MDR1 drug resistance gene, and synergize with protein kinase C to induce the expression of vascular endothelial growth factor (291,292).

Nonsense mutations result in a truncated protein that is devoid of function because it can no longer oligomerize with other p53 molecules (268). Deletions result in the loss of functional p53 alleles from the genome. The loss of a single allele is not detrimental in the presence of a wild type allele since tumor suppressors act in a dominant/recessive fashion. The loss of both wild type p53 alleles, although not detrimental to the development of the organism, predisposes it to the rapid onset of tumors (279). The most common scenario for the loss of p53’s suppressor functions occurs when one allele sustains a somatic missense mutation. The mutant protein may possess gain of function oncogenic activity and/or exert a trans-dominant negative effect on the wild-type protein. The presence of both a wild-type and mutant p53 allele is usually selected against, resulting in the eventual loss of the remaining wild-type allele (280).

The fact that p53 can suppress the formation of tumors is well accepted, however, the specific pathway(s) by which this occurs is unknown. Other functions for p53 were thought to include the suppression and/or promotion of cellular proliferation during growth and development. The normal
development of homozygous p53-/− knockout mice suggested that p53 was not important for the regulation of normal cell proliferation, differentiation, or arrest. However, greater than 70% of the knockout mice developed sporadic tumors by six months of age which suggested that the normal function of p53 was solely involved in the suppression of tumor formation (279).

One specific pathway by which p53 may suppress tumor formation is through its regulation of cell cycle checkpoints following damage to genomic DNA. One of the hallmarks of tumor cells is their preponderance of genomic aberrations which include translocations, gene amplifications, chromosomal deletions, inversions and insertions, aneuploidy and polyploidy. These aberrations contribute to oncogenes and are thought to occur when DNA synthesis and mitosis are allowed to proceed in the presence of a damaged genome (293). Normal cells arrest at various cell cycle checkpoints which allows for the repair of genomic DNA before DNA synthesis or mitosis can occur, ensuring that genomic fidelity is maintained between cell generations (reviewed in 294).

The role of p53 in the regulation of the G1 checkpoint following DNA damage has been well characterized (295,296, reviewed in 273,297). Ionizing and UV radiation, and most DNA damaging chemotherapeutic agents directly or indirectly cause single and/or double strand DNA breaks, resulting in the G1 arrest of normal cells. The trigger for this arrest
appears to be the presence of DNA strand breaks which are recognized by the COOH-terminus of p53, leading to the stabilization and accumulation of p53 protein in the nucleus, and activation of its sequence-specific DNA binding (296,298-300). The increase in p53 levels leads to the transactivation of the GADD45 (growth arrest DNA-damage) and p21\textsuperscript{WAF1/CIP1} genes, among others. Expression of the GADD45 gene following irradiation is p53-dependent and capable of arresting cells in G1 by an unknown mechanism (301,302). Other types of DNA damage can increase GADD45 expression by p53-independent mechanisms (301,303). GADD45 has been shown to bind the proliferation cell nuclear antigen (PCNA), a regulatory subunit of DNA polymerase δ and ε, and induce DNA excision repair, linking G1 arrest to the repair of genomic DNA (304).

The more probable pathway for the p53-mediated G1 arrest involves the induction of the p21\textsuperscript{WAF1/CIP1} gene (305,306). p21 is a cyclin dependent kinase (cdk) inhibitor with multiple functions. p21 binds to and inhibits the kinase activity of cyclin/cdk complexes which are required for cell cycle progression and also binds to and inhibits PCNA (307,308). The consequences of p21 activation are straightforward: blocking PCNA inhibits DNA replication while the inhibition of specific cyclin/cdk complexes arrests the cell cycle in G1. Unlike GADD45, p21 is also induced by p53-independent pathways during the normal cellular growth response, however,
the response to genotoxic stress requires induction via a p53-dependent pathway (309).

The recent development of a p21-/- knockout mouse has provided direct evidence that p21 is a significant mediator of DNA damage-induced G1 arrest. However, the G1 checkpoint was only partially impaired in response to 7-irradiation suggesting that another p53-dependent mechanism existed that could also induce G1 arrest following DNA damage (310). p21 was not required for the p53-regulated mitotic spindle checkpoint or p53-dependent apoptosis (310). Unlike p53-/- mice, which developed tumors within 6 months of age, p21 deficient mice, which were greater than 7 months old, had not yet developed tumors (310). This finding is quite surprising but consistent with the fact that mutations of p21 are not commonly found in human tumors (311). These results, although preliminary, strongly suggested that the regulation of the G1 checkpoint was not the primary mechanism by which p53 suppressed tumor formation (310). The role of p53 in other cell cycle checkpoints, namely the mitotic spindle checkpoint and possibly the G2 checkpoint, was still maintained in the cells of these mice and may be involved in tumor suppression (312). However, evidence from many studies has directly implicated p53-mediated apoptosis in the suppression of tumor growth and transformation by oncogenes and in the removal of severely DNA-damaged cells (173,272,313-315). Taken together, these results strongly
suggested that apoptosis was the primary mechanism by which
p53 suppressed tumor formation.

Yonish-Rouach and colleagues provided the first evidence
that p53 was a regulator of apoptosis when they showed that
the ectopic overexpression of wild-type p53 directly induced
apoptosis in the non-p53-expressing murine M1 myeloid
leukemic cell line (172). This study utilized a p53
temperature-sensitive mutant to show that the overexpression
of wild-type p53 was sufficient to induce apoptosis, which
was inhibited by interleukin 6, and that apoptosis did not
occur when the mutant form of p53 was overexpressed (172).
Thymocytes readily undergo apoptosis when exposed to DNA-
damaging agents, glucocorticoids, and calcium ionophores
(116,117). Thymocytes from p53-/- knockout mice underwent
apoptosis in response to glucocorticoids and calcium
ionophores in a similar fashion as cells from p53+/+ mice,
but were resistant to apoptosis initiated by DNA-damaging
agents (173,316). Thymocytes from heterozygous p53-/+ mice
showed a response to DNA-damaging agents that was
intermediate between p53-/- and p53+/+ thymocytes (173,316).
Similar findings have been demonstrated in bone marrow-
derived myeloid progenitor cells and embryonic fibroblasts
from p53-deficient mice (175,317). These studies and others
have shown that p53-dependent and p53-independent mechanisms
for the induction of apoptosis exist (reviewed in 318) and
suggested that the apoptotic pathway induced by DNA damage is
p53-dependent (173,316). These results suggested that p53 may function by arresting cells in G1 to allow time for DNA repair, but if the damage is too extreme the cells are triggered to die via apoptosis (85). In support of this concept, p53 has been shown to induce the genetic programs of G1 arrest and apoptosis in the same cell type (319). However, the role of p53 in DNA damage-induced apoptosis may be more complex than previously thought since DNA damage-induced apoptosis has been shown to occur in cycling T lymphoma cells and mitogenically activated T lymphocytes in a p53-independent manner (320).

The mechanism and pathways involved in p53-mediated apoptosis are still a mystery. However, recent studies have started to reveal some of the many possible players, and undoubtedly, the complete story will be complex. The inability of mutant p53 to activate transcription and induce apoptosis suggested that one possible mechanism by which wild-type p53 induced apoptosis was via the transactivation of downstream effector genes. In support of this concept, p53 has been shown to be a direct transcriptional activator of the human bax gene (321) and regulated the induction of apoptosis in M1 leukemic cells by inducing the expression of bax and concomitantly suppressing the expression of bcl-2 through a p53 negative response element (196,322,323). However, other studies have shown that p53-dependent apoptosis can occur in the absence of transcriptional
activation of p53-target genes and suggested that the regulation of apoptosis by p53 occurred via the suppression of genes necessary for survival or a non-transcriptional function of p53 (324). Clearly the pathways involved in the mediation of p53-dependent apoptosis are cell type and species-dependent.

Other possible effectors of p53-dependent apoptosis are the retinoblastoma gene product (Rb) and the transcription factor E2F-1 (325,326). Some studies have suggested that p53 mediated the c-myc enhancement of apoptosis and mutant p53 has been shown to abrogate this process (327,328). c-myc can transactivate p53 expression but the increase in p53 observed during c-myc-enhanced apoptosis resulted from the posttranslational stabilization of the p53 protein (327,329). The alteration in subcellular trafficking and subsequent modulation of p53 function has also been implicated as a mechanism by which p53 regulates apoptosis and both c-myc and bcl-2 are involved in this process (330). Both p53-dependent and independent pathways have been shown to be inhibited by bcl-2 (320,331).

The Role of p53 in Chemotherapy

The role of p53 in carcinogenesis is well established. However, recent evidence has suggested that the status of p53 may also influence the therapeutic response of tumors exposed to anti-neoplastic agents or radiation. In a situation
analogous to bcl-2, p53 mutations that affected the apoptotic functions of the wild-type protein seemed to confer a radiation/chemotherapeutic agent-resistant phenotype to some tumor cells. In vitro and in vivo mouse studies have shown that the presence of wild-type p53 in tumor cells resulted in favorable responses to chemotherapeutic modalities via drug-induced apoptosis, while the presence of a mutant form of p53 rendered these cells inherently more resistant to the same agents (175,332). Similar results have been obtained in some human systems (333,334). However, studies in human systems have also shown that p53 status did not influence the outcome of anti-cancer agent exposure, and that drug-induced apoptosis could occur via p53-independent mechanisms (335-338). The role of p53 in the efficacy of cancer chemotherapy is certainly complex. The existence of p53-dependent and independent pathways for drug-induced apoptosis suggested that the involvement of p53 in tumor cell responses to anti-cancer therapy was cell type and species-specific (339,340). Indeed, what seemed to be important in determining the role of p53 in drug-induced apoptosis was the cellular context in which p53 was expressed (341). Unfortunately, the cellular context of tumor cells is essentially unique for each tumor type, rendering p53 status useless as a prognostic indicator for tumor response.
c-myc

The c-myc proto-oncogene was originally identified as the cellular homolog of the MC29, avian myelocytomatosis retroviral v-myc oncogene (342). The role of c-myc in cellular proliferation, differentiation, and transformation has been well characterized and extensively reviewed (343,344). c-myc is essential for the proliferation of quiescent cells and its expression has been shown to be sufficient, but not necessary, for the transition of cells from G0 into G1 (345,346). c-Myc has been shown to promote the G1-S phase transition via the direct induction of cyclin D1 which can regulate G1 progression and the G1-S phase transition (347). Other evidence has suggested a role for c-myc in the S-G2 transition (348). The deregulated expression of c-myc is sufficient for the immortalization of cells in culture (349) and is capable of transforming cells in cooperation with another oncogene, such as ras or bcl-2 (191,350). Deregulated overexpression of c-myc protein occurs in almost all tumors (except those that overexpress N-myc or L-myc) and has been strongly implicated in the development and progression of malignancies (344,351).

c-Myc belongs to the myc family of highly related oncogenes which includes two other bona fide members, N-myc and L-myc. The myc genes are highly conserved throughout vertebrate evolution and have been isolated in fish (352), amphibians (353), birds (342), and mammals (354). The myc
genes are differentially expressed in mammalian development, with N-myc and L-myc exhibiting a very restricted pattern of tissue and developmental stage specificity (355). c-Myc expression during development is rather generalized and c-myc is the major myc gene expressed in normal adult tissues (355).

The c-myc gene has been localized to chromosome 8q24 and consists of three exons spanning approximately 6 kb, the first of which is essentially noncoding (356,357). The regulation of c-myc expression in both normal and neoplastic cells is extremely complex and has been extensively reviewed (358). c-Myc transcripts can be generated from any of four separate promotors, P₀, P₁, P₂, or P₃. The two major promotors, P₁ and P₂, are located in exon 1 and account for 10-25% and 75-90% of the total c-myc steady-state mRNA, respectively (359,360). The transcription of c-myc mRNA can be regulated at the level of both transcription initiation and elongation (reviewed in 361). The two major c-myc transcripts are approximately 2.4 and 2.2 kb in size and originate from the P₁ and P₂ promotors, respectively (359,360). The half-life of c-myc mRNA is extremely short (10 min to 1 h) (362). The modulation of mRNA stability is another mechanism utilized in the regulation of c-myc expression and is often superimposed on transcriptional controls. The rapid degradation of c-myc mRNA occurs in two steps, the first of which is the translation-dependent
shortening of the poly(A) tail that is inhibitable by cycloheximide. The last step is the rapid degradation of the body of the mRNA which is translation-independent (363,364).

The major c-myc protein (Myc 2) is a 439 amino acid, nuclear phosphoprotein with a molecular weight of approximately 64 kDa which is translated from an AUG start codon located at the 5' end of exon two (365). A less common c-myc species (Myc 1) is the 67 kDa, 453 amino acid protein which is translated from a cryptic CUG initiation codon located at the 3' end of exon 1 (366). The half-life of c-myc proteins is approximately 15-30 minutes (365). The regulation of c-myc translation has also been suggested as a mechanism for the control of c-myc expression, however, this remains controversial (365,367). The c-myc protein has been shown to be involved in the negative autoregulation of c-myc expression in normal cells and some tumor cells. The c-myc protein is capable of inhibiting the transcriptional initiation from endogenous c-myc promoters in a dose-dependent fashion and requires the presence of trans-acting factors (368). This negative autoregulation is lost in many tumor cells and may explain how c-myc is overexpressed in tumor cells in which the more common mechanisms of deregulated c-myc expression are absent (369).

The expression of c-myc in normal cells is tightly regulated at almost every step in the gene expression pathway. Despite this rigorous network of checkpoints, c-myc
protein is overexpressed in almost all tumor cells and, as
might be expected, the mechanisms by which this
overexpression is achieved are quite varied. The most
obvious mechanisms are translocations, gene amplification,
and proviral insertions. Translocations of c-myc are a
common occurrence in many Burkitt's lymphomas and B-ALL in
which the c-myc gene is translocated and placed under
transcriptional control of one of the immunoglobulin loci on
chromosomes 2, 14, or 22 (reviewed in 358,370). Gene
amplifications are also common and can serve as a prognostic
indicator for a number of tumors (358,370). Proviral
insertions are associated with the activation of the c-myc
locus in a number of B- and T-lymphoid tumors (358,370). All
of these mechanisms serve to increase the expression of the
normal c-myc protein and contribute to the oncogenic
progression of tumors. Other oncoproteins have also been
shown to contribute to the transcriptional regulation of c-
myc and include v-abl, p53, fos/jun, adenovirus E1a, v-erb B,
v-raf, and c-myb (371 and references within). Since cancer
is a multi-step process, the oncogenic activation or
overexpression of any of these various oncoproteins could
certainly account for some of the unknown mechanisms by which
c-myc expression is deregulated in tumor cells.

c-myc contains several structural protein motifs which
are important for its functions. Consistent with its role as
a transcriptional activator, the NH-terminal 143 amino acids
of the myc protein are able to transactivate genes (372). Three independent transactivation domains (TADs) can be found within the NH-terminus at residues 1-41, 41-103, and 103-143 (372). A non-specific DNA binding domain can be found between residues 265-318 (373). Two nuclear localization signals (NLS) reside within the myc protein. The first NLS spans residues 320-328 (designated M1) and is sufficient to provide complete nuclear localization (374). The M2 domain (residues 364-374) induces only partial localization and probably does so coincidentally along with its other putative functions (374). The last domain is the COOH-terminal basic region, helix-loop-helix, leucine zipper motif (bHLH-LZ) which is characteristic of many known transcription factors (bHLH or bLZ). The basic region (355-367) defines a specific DNA binding domain and is immediately NH-terminal to the two protein dimerization motifs, the HLH (368-410) and LZ (411-439) (375). The HLH-LZ motifs mediate specific protein/protein interactions with an obligate partner and align the basic regions of the dimerized proteins to define a sequence-specific DNA binding domain.

The c-myc protein is posttranslationally modified by both phosphorylation and glycosylation. The in vivo phosphorylation sites within the c-myc protein include Thr\(^{58}\), Ser\(^{62}\), and Ser\(^{71}\) (376-378), all of which reside within the TAD, and two sequences in the COOH-terminus of myc, one in the acidic region (240-262) and one between residues 342-357
Thr^{58} and Ser^{62} are phosphorylated in vitro by glycogen synthase kinase-3, MAP kinase, and p34^{cd2} kinase (376-378), and their phosphorylation appears to be both mitogen and cell cycle regulated (378). In vivo phosphorylation of Thr^{58} and Ser^{62} residues can alter c-myc function. Ser^{62} phosphorylation has been shown to increase the serum-induced transactivating properties (380,381) and transforming activity (376,377) of c-myc, while phosphorylation of Thr^{58} can inhibit focus formation (377). The COOH-terminal phosphorylation sites are both in vitro substrates for casein kinase II (CK II), although no known in vivo functions have been observed for these two sites (379). Another recently demonstrated posttranslational modification of the c-myc protein is the O-linked N-acetylglucosamine (O-GlcNAc) glycosylation of Thr^{58} within the TAD which may regulate the transactivating activity of the c-myc protein (382,383).

The c-myc protein has been extensively studied and many of the domains responsible for its various functions have been mapped by deletion and mutation analysis. Studies have demonstrated that the amino-terminal 143 amino acids and the carboxy-terminal 89 amino acids are necessary for transformation by c-myc, with an absolute requirement for residues 106-143 (384,385). These areas correspond to the TAD and bHLH-LZ domains, areas responsible for activating the transcription of other genes and mediating protein dimerization and subsequent sequence-specific DNA binding,
respectively, suggesting that c-myc cooperates in the transformation of cells via its ability to induce the expression of transforming gene products. Alterations in the middle portion of the c-myc protein, namely the acidic region, may decrease transformation efficiency but do not abolish transforming activity (384). The areas necessary for the autosuppression functions of myc (386) and the inhibition of cellular differentiation (387) appear to be almost identical to those areas responsible for transformation.

c-Myc has been shown to be both an activator and repressor of transcription (388-390). The dimerization of c-myc with its recently described, obligate protein partner, max, is central to many of myc’s functions, including gene transactivation, and will be described in subsequent sections. Independent of max, the amino-terminal portion of c-myc has been shown to interact \textit{in vivo} with TBP (391,392) and the retinoblastoma-related protein, p107 (393,394), and \textit{in vitro} with the retinoblastoma gene product (Rb) (395). The interaction between c-myc and TBP may facilitate c-myc-mediated transactivation or the inhibition of TFIIA-dependent pre-initiation complexes (372,396). p107-c-myc complexes are capable of suppressing myc-mediated transcriptional activation (393,394). Evidence has recently shown that c-myc can also directly repress initiator element-dependent transcription via its physical interaction with either Yin Yang 1 or TFII-I (396,397). The interaction between c-myc
and these transcription factors requires the presence of the bHLH-LZ of the c-myc protein (396, 397). Therefore, it appears likely that not all of the biological activities of c-myc are dependent upon its interaction with max or its ability to activate transcription.

Many genes have been identified as targets of c-myc-mediated activation or repression. Those genes which are repressed by c-myc include MHC class I (398), collagen (390, 399), neu (400), LFA-1α (401), cyclin D1 (402, 403), C/EBPα (389), and c-myc itself (368). The transcriptional repression of these genes is either direct via inhibition of initiator element-dependent transcription (389) or via indirect mechanisms where c-myc influences the binding activity or abundance of other binding factors (399). Genes that are transactivated by c-myc include ornithine decarboxylase (ODC) (404), α-prothymosin (405), ECA39 (406), eIF4E and eIF2α (407), cyclins A, E, and D1 (347, 402, 408), hsp70 (409), lactate dehydrogenase (LDH) (410), p53 (329), dihydrofolate reductase (DHFR) (411), and carbamoyl-phosphate synthase (glutamine-hydrolyzing)/aspartate carbamoyltransferase/dihydroorotase (cad) (412). The c-myc-mediated activation of eIF4E, eIF2α, hsp70, cyclin A and cyclin E appears to be indirect since no c-myc binding sites have been located in the regulatory sequences of the these genes. Of the remaining genes, ODC, cad, p53, α-prothymosin, and ECA39 all appear to be transactivated via the consensus, c-myc E-
box binding sequence (CACGTG) (329,404-406,412). The DHFR, LDH, and cyclin D1 genes all contain E-box motifs in their regulatory regions, but direct transcriptional activation of these genes via these myc binding sites remains to be demonstrated (347,410,411).

**Myc Transcription Factor Network**

The c-myc protein does not form homodimers in vivo, therefore the transactivation of genes via the binding to a core DNA consensus sequence (CACGTG) requires interaction with an obligate protein partner (413). The discovery of max (414), an obligate partner for c-myc, was a major stepping stone in myc research and led to the establishment of a myc transcription factor network of proteins, which was found to include two other max binding proteins, mad (415) and mxil (416).

Max is a 151 amino acid nuclear phosphoprotein that contains a bHLH-LZ motif (residues 15-99) and an NLS (residues 140-147) (414). Two major forms of max protein, p21 and p22, are found in relatively equal amounts in most cells and result from the alternative splicing of a single max gene which adds a 9 amino acid insert NH-terminal of the basic region (414). Max proteins are capable of forming homodimers and heterodimers with c-myc, L-myc, N-myc (414), mad (415), and mxil (416). Of the four known members of the myc transcription factor network, only c-myc contains a TAD
and is capable of transcriptional activation in concert with max (417,418). Max mRNA and proteins are constitutively expressed at relatively high levels in growing, resting, or differentiating cells and are very stable, with half-lives of greater than 3 h and 18 h, respectively (419-423).

Recently, two novel bHLH-LZ proteins, mad and mxil, have been isolated on the basis of their interaction with max (415,416). Mad and mxil are both 220 amino acid proteins and contain bHLH-LZ motifs between residues 58-150 and 32-112, respectively (415,416). Both proteins contain a potential bipartite NLS and are 43% identical suggesting that they are evolutionarily related (415). The mad protein has been shown to be a 35 kDa, nuclear phosphoprotein with a half-life of 15-30 minutes (422). Mad migrates on SDS-PAGE as a doublet and the faster migrating species is thought to be a precursor of the slower migrating species (422).

Mad and mxil do not form homodimers, nor do they heterodimerize with each other or any of the myc proteins (415,416). When dimerized with max, however, mad and mxil are able to bind the CACGTG E-box motif and antagonize transcriptional activation by competing with c-myc/max dimers for the occupation of these sites (415,416). The affinity of mad and c-myc for max binding is equal, suggesting that the relative levels of these two proteins may act to regulate the levels of each dimer type, and thus the transactivating status of this network (415). This concept has been
demonstrated in differentiating hematopoietic cells in which the levels of c-myc decreased while the levels of mad (or mxil in some cases) increased with a concomitant decline in c-myc/max dimers and increase in mad/max dimers (421,422). The emerging model of this network suggests that max is the central component because of its constitutive expression and ability to interact with all of the other members. Transcription from E-box containing genes would be predicted to occur when c-myc/max dimers predominate while transcription would be predicted to be repressed when max/max, mad/max, or mxil/max dimers predominate.

The functional theme of the myc transcription factor network is the ability of dimerized partners to bind the consensus core hexanucleotide DNA sequence, CACGTG, known as an E-box motif (414,424). As previously discussed, not all of the functions of c-myc appear to require dimerization with max, however, those functions that do require an interaction with max are thought to be mediated by the transactivation of E-box containing genes. Important functions of c-myc that have been shown to require dimerization with max include cell cycle progression, transformation, and growth factor withdrawal-induced apoptosis (425,426). As mentioned before, one mechanism by which this system may be modulated is the independent regulation of max binding partners which determines the relative levels of the various dimers, c-myc/max, max/max, mad/max, and mxil/max. However, another
level of modulation in this network may occur via the phosphorylation of partner proteins which may alter the functional state of the various dimers.

Phosphorylation of both c-myc and max is capable of altering the transactivating and DNA binding activities of these proteins, respectively (380,381,427). The phosphorylation of c-myc does not alter its DNA binding ability when dimerized with max (427). Homodimers and heterodimers of p21 max, p22 max, and c-myc all have subtly different binding specificities in vivo that are regulated by both phosphorylation status and the flanking sequences of the core E-box motif (424,428,429). Heterodimers of c-myc/p21 max and c-myc/p22 max were shown to transcriptionally activate core E-box motifs equally well, however, if the core was flanked 5' by a T and 3' by an A, the activity of c-myc/p21 max dimers was repressed by greater than 30 fold while the activity of c-myc/p22 max dimers was only repressed by 4-5 fold (429). Other studies have shown that phosphorylation of max at Ser\textsuperscript{II} (an in vitro substrate for casein kinase II) inhibits the binding of max homodimers to core E-box sequences (427). More importantly, studies have shown that CKII phosphorylation of Ser\textsuperscript{II} and Ser\textsuperscript{II} of p21 and p22 max enhanced the off rate for DNA binding of max homodimers and c-myc/max heterodimers, with the heterodimers of c-myc/p21 max being more unstable than c-myc/p22 max heterodimers (428). The phosphorylation of max increased the
on rate for DNA binding of c-myc/p22 max heterodimers but not c-myc/p21 max dimers (428). These data suggested that phosphorylation did not alter the affinity for DNA binding of c-myc/p22 max heterodimers but reduced the affinity of c-myc/p21 max heterodimers for DNA binding (428). Therefore, the DNA binding kinetics of c-myc/max dimers depends upon the form of max and its phosphorylation status and is consistent with the possibility that dimers of c-myc/p21 max and c-myc/p22 max may transactivate different subsets of target genes based upon E-box flanking sequences, phosphorylation status, and ratios of heterodimers containing p21 and p22 max (430).

c-myc-Induced Apoptosis

The involvement of c-myc in the induction of apoptosis was first demonstrated in factor-dependent 32D.3 myeloid progenitor cells when Cleveland and colleagues showed that the enforced expression of c-myc accelerated apoptotic cell death following IL-3 withdrawal (431). Evan et al. subsequently showed that the activation of a c-myc/ER chimeric protein could induce apoptosis in Rat1 fibroblasts or primary rat embryonic fibroblasts when cell cycle progression was blocked by various methods including serum-deprivation, isoleucine deprivation, thymidine block, or cycloheximide (432). c-Myc was shown to induce apoptosis in a dose-dependent manner which was cell cycle phase-
independent (432,433). Thus, it seems that the inappropriate expression of c-myc is capable of inducing apoptosis and this appears to be a general function of c-myc. The regions of c-myc required for its apoptotic functions are the same as those necessary for cotransformation, autosuppression, and inhibition of differentiation (432), and an absolute requirement for dimerization with max has been shown (426). These results suggested that c-myc-induced apoptosis is mediated by the transactivation of E-box containing genes.

The deregulated expression of c-myc can induce apoptosis under growth limiting conditions, a scenario which is common to many tumors. Indeed, many studies have shown that deregulated c-myc expression increased the apoptotic index of tumors in vivo suggesting a role for myc-induced apoptosis in oncogenesis (154,434). The fact that c-myc overexpression accelerates proliferation and increases the rate of apoptosis helps to explain why c-myc is not tumorigenic by itself. Many studies have shown that c-myc is only tumorigenic in cooperation with other oncogenes such as bcl-2, ras, raf, and abl, and all of these oncogenes are able to suppress the induction of apoptosis by c-myc (153,154,166,169,250,251). Thus, while the deregulation of c-myc is a common, and apparently important step in oncogenesis, mutations within the myc gene or secondary genetic lesions which function to abrogate the ability of c-myc to induce apoptosis appear to be required for the oncogenic progression to a more
aggressive tumor phenotype (154). This leads to the question: does c-myc-induced apoptosis have a function under normal physiological conditions where c-myc expression is tightly regulated? The answer appears to be yes. A direct role for c-myc in apoptosis has been demonstrated for activation-induced apoptosis in T-cell hybridomas, a model system for the negative selection of thymocytes (435, reviewed in 436). Similar findings have also been found in the TNF-α-induced death of HeLa cells (437).

The fact that c-myc expression is involved in both cellular proliferation and death seems rather ironic. However, upon further consideration it seems appropriate that such a potentially "dangerous" gene product would have a fail-safe mechanism guarding its expression. From the standpoint of a metazoan organism, the uncontrolled proliferation of just one cell is a potentially life threatening situation. Therefore, the emergence of such a risky gene would not be expected to occur unless a mechanism to keep its expression in check was co-evolved. This appears to be the case with c-myc. Two models have been proposed to explain this phenomenon. The first is the "conflict" model (431,432). This model predicts that the expression of c-myc results in cellular proliferation only and when the cellular environment becomes growth restrictive, apoptosis is induced as a result of the "conflict" between growth stimulatory signals (presence of myc) and growth inhibitory signals.
(factor withdrawal, DNA damage-induced cell cycle block, etc.). In essence, the cell becomes a victim of two diametrically opposed cellular signals and dies in what has been described as a "mitotic catastrophe". Evidence exists to support this type of model (432,433).

The second model is the "dual signal" model which predicts that apoptosis is a normal outcome of c-myc expression (438). Thus, the expression of c-myc is like a double-edged sword in that it activates the cellular proliferation pathway and also implements the components of the apoptotic program. Whether a cell will proliferate or die is then regulated by the presence of cell-specific survival factors such as interleukins, IGF-1 or PDGF, which directly suppress the apoptotic pathway (431,433). Evidence also exists to strongly support this type of model (439 and references within). The fact that both models have data to support them may be reconciled by the circumstances under which they were tested. The dual signal model seems more applicable to the normal physiological conditions of cell growth and regulation where c-myc expression is still under tight control, whereas the conflict model may be more appropriate for cells that express c-myc in a deregulated fashion, such as tumor cells. Tumor cells generally overexpress c-myc in an unregulated fashion and are less dependent upon growth factors, therefore, treatment of some tumor cells with a cytostatic agent would seem to create a
situation in which a cellular conflict is created.

Many studies have shown that c-myc-mediated apoptosis is important, however, c-myc is an enhancer of apoptosis and therefore, it is not an obligate component of all apoptotic pathways (431,435). When c-myc is involved in the regulation of apoptosis, it is thought to exert its functions via the transactivation of target genes (426). Several potential target genes for myc-induced apoptosis include cyclin A, p53, and ODC. Overexpression of cyclin A is sufficient for the induction of apoptosis and its induction has been linked to c-myc expression, although the mechanism for this induction is not clear (408). p53 is a direct inducer of apoptosis (172) and its expression can be regulated by c-myc via an E-box motif (329). Indeed, studies have shown that myc-enhanced apoptosis is mediated by p53 (327,440), however, p53 levels were induced by a posttranslational mechanism showing that p53 was not a direct target of c-myc (327). More importantly, other studies have shown that c-myc-enhanced apoptosis can occur in the absence of p53 or without inducing its expression, suggesting that the involvement of p53 in c-myc-enhanced apoptosis is cell type- and species-specific (441-443). The strongest candidate for a mediator of c-myc-induced apoptosis is the ornithine decarboxylase gene (ODC) (444). ODC is a direct transcriptional target of myc/max dimers and is required for cell cycle progression (404). The enforced expression of ODC is capable of inducing apoptosis.
However, ODC was not as effective as c-myc, suggesting that ODC was only a partial mediator of c-myc-induced apoptosis in this system (444). Finally, the promotor of the human bax gene has recently been shown to contain four E-box motifs suggesting that c-myc may be able to directly regulate the expression of the bax gene, a known regulator of apoptotic cell death (321).
CHAPTER 2
MATERIALS AND METHODS

Cell Culture and Drug Treatments

The HT-29 and BE human colon carcinoma cell lines, and the IMR-90 normal human fibroblast cell line were cultured in Eagle’s minimum essential medium (HyClone Laboratories, Logan, UT) supplemented with 10% BCS (HyClone Laboratories), 1 mM L-glutamine (JRH Biosciences, Lenexa, KS), 1 mM sodium pyruvate (JRH Biosciences), 20 mM HEPES (JRH Biosciences), 1X non-essential amino acids (JRH Biosciences), 1X biotin/vitamin B₁₂ (1000X is 0.1 mg/ml d-biotin, 1.36 mg/ml vitamin B₁₂) (Sigma, St. Louis, MO), and 50 µg/ml gentamycin (JRH Biosciences) at 37°C, in closed flasks.

The human colon carcinoma cell line Colo320HSR was cultured in RPMI 1630 (JRH Biosciences) supplemented with 15% heat inactivated (56°C for 1 h) BCS (HyClone Laboratories), 2 mM L-glutamine, 50 U/ml of penicillin, and 50 µg/ml of streptomycin (JRH Biosciences) at 37°C in closed flasks.

The human cervical carcinoma cell line, HeLa, was cultured in Dulbecco’s modified Eagle’s essential media (Sigma) supplemented with 10% BCS (HyClone Laboratories), and 50 µg/ml of gentamycin at 37°C in a humidified atmosphere.
consisting of 92% air/8% CO₂.

The human promyelocytic leukemic cell line, HL-60, and the human Epstein Barr virus-infected B-cell line, LM-EBV, were cultured in RPMI 1640 (JRH Biosciences) supplemented with 10% BCS, 2 mM L-glutamine, 50 U/ml of penicillin and 50 µg/ml of streptomycin at 37°C in a humidified atmosphere consisting of 92% air/8% CO₂.

All cell lines were subcultured every 4-5 days and maintained as exponentially growing cultures. Cell counts for subculturing and experiments were determined by counting cells either on a Coulter Counter, Model ZBI (Coulter Electronics, Inc., Hialeah, FL) or a bright-line hemacytometer (Reichert Scientific Instruments, Buffalo, NY).

Nitrogen mustard (Sigma) was dissolved in sterile filtered 0.1 N HCl and stored as a 10 mM stock solution at -20°C. Actinomycin D (Act D; Boehringer Mannheim, Indianapolis, IN) was dissolved in 100% ethanol and a 1 mg/ml stock solution was wrapped in aluminum foil and stored at -20°C. Exponentially growing cells (2.5 X 10⁵ cells/ml for Colo320HSR and 6-8 X 10⁶ cells per T-75 flask for HT-29 and BE cell lines) were exposed to HN2 for 1 h at 37°C. Cells were then washed free of drug, refed with fresh medium, and cultured at 37°C until lysed at various time points. Cells treated with Act D were continuously exposed to drug until lysed at various time points.
Macromolecular Isolation

Total RNA Isolation

Total cellular RNA was isolated essentially by the method of Chirgwin et al. (445). Briefly, 2-10 X 10^6 cells were washed 2X in ice cold 1X Dulbecco’s phosphate buffered saline (pH 7.4) (Gibco BRL, Gaithersburg, MD), and lysed in 1.8 ml of RNA lysis buffer (4 M guanidium isothiocyanate (Gibco BRL), 20 mM sodium acetate, 10 mM DTT, and 0.5% N-lauroyl sarcosine (w/v) (Sigma)). Chromosomal DNA was sheared by repeated passage of the lysate through a 20 gauge syringe needle. At this stage RNA samples could be frozen and stored at -70°C. RNA was recovered as follows: 1.8 ml of lysate was layered on top of a 400 µl 5.7 M CsCl, 100 mM EDTA cushion in an 11 X 34 mm Ultra-Clear centrifuge tube (Beckman Instruments, Inc., Palo Alto, CA) and centrifuged at 49,000 rpm for 2.5 h at 20°C in a Beckman TL-100 ultra-centrifuge using a TLS-55 rotor.

Following sedimentation, the supernatant was aspirated and the tubes were inverted for 10-15 min to drain the excess liquid. The RNA pellet on the bottom of the tube was resuspended in DEPC-treated (Sigma) distilled/de-ionized water for 15 min and transferred to an autoclaved 1.5 ml microfuge tube. RNA was precipitated 2X with 1/10 volume of 3 M sodium acetate (DEPC-treated) and 2.5 volumes of 95% ethanol for at least 30 min at -70°C. RNA was pelleted by microcentrifugation (12,000 X g at 4°C), air dried,
resuspended in DEPC-treated distilled/de-ionized water, quantitated by $A_{260}$, and stored at -70°C.

**Genomic DNA Isolation**

Genomic DNA was isolated as follows: 10-20 X 10^6 cells were washed 2X in ice cold PBS (pH 7.4) and lysed in a solution (3 X 10^6 cells/ml) consisting of 50 mM NaHCO$_3$/Na$_2$CO$_3$, 1 mM EDTA, 0.5% N-lauroyl sarcosine (w/v), and 300 µg/ml proteinase K (pH 10.4). The cell lysate was incubated at 37°C for 3-6 h with occasional gentle mixing. The lysate was deproteinized by extraction with an equal volume of phenol:chloroform:isoamyl alcohol (PCI) (25:24:1) 2X and once with chloroform:isoamyl alcohol (24:1). The lysate was gently mixed with the organic phase until an emulsion was formed (5 min). The phases were separated by centrifugation in a Beckman J6-M centrifuge at 2370 rpm (1600 X g) for 5 min at 22°C. After the last extraction, the aqueous phase was transferred to a 25 ml Erlenmeyer flask and the DNA precipitated with 0.2 volumes of 11 M ammonium acetate and 2.5 volumes of 95% ethanol. The DNA was spooled out with a glass rod, washed by dipping into 70% ethanol, transferred to a 1.5 ml microcentrifuge tube, resuspended in 400 µl of milli-Q water, and treated with RNase A (10 µg/ml) for 30 min at 37°C.

Following RNase digestion, the DNA solution was extracted 2X with an equal volume of PCI and once with
chloroform:isoamyl alcohol. Emulsions were formed by gentle mixing and phases were separated by centrifugation in a Beckman Microfuge 11 at 500 X g for 5 min using a fixed angle rotor. The DNA was recovered by precipitation with 1/10 volume of 3 M sodium acetate and 2.5 volumes of 95% ethanol (≥ 30 min at -70°C), pelleted by microcentrifugation (12,000 X g at 4°C), washed in 70% ethanol, and resuspended in 400-800 µl of TE (10 mM Tris, 1 mM EDTA, pH 8).

The DNA was digested to completion with a restriction enzyme of choice (5 U/µg DNA for 1-3 h) at 37°C, PCI extracted, ethanol precipitated, washed with 70% ethanol (37°C for 30 min), resuspended in milli-Q water, quantitated by A260, and stored at -70°C.

**Protein Isolation**

Whole cell protein isolation for western blot (immunoblot) analysis was performed as follows: cells were counted and a specified number of cells was washed 2X in ice cold PBS (pH 7.4). The cell pellet was broken up and then lysed by the addition of an appropriate volume of sample buffer (100 mM DTT, 2% SDS, 80 mM Tris (pH 6.8), 15% glycerol, and 0.1% bromophenol blue) which gave a concentration of 2 X 10^4 cells/µl of lysate. The sample lysates were boiled for 10 min and then passed repeatedly through a 20 gauge syringe needle to shear chromosomal DNA and reduce the viscosity of the lysate. Samples were then
stored at -70°C until analysis. Prior to electrophoresis, samples were boiled for 10 min to solubilize any precipitated proteins.

Quantitation of Macromolecules

Quantitation of DNA and RNA was performed by UV spectrophotometry. Absorbance readings were taken on 1 µl aliquots of nucleic acids (1 ml total volume) at 325 nm (background absorbance), 280 nm (protein detection), and 260 nm (nucleic acid detection). An $A_{260}$ of 1.0 corresponds to 50 µg/ml of double stranded DNA, 37 µg/ml of single stranded DNA, and 40 µg/ml of RNA. The purity of nucleic acid samples was determined by $A_{260}/A_{280}$ ratios where pure preparations of DNA and RNA have $A_{260}/A_{280}$ ratios of 1.8 and 2.0, respectively. UV absorbance readings were taken on either an LKB Ultrospec 4050 (LKB Biochrom Ltd., Cambridge, England) or a GeneQuant RNA/DNA Calculator (Pharmacia LKB Biochrom Ltd., Cambridge, England) using 1 ml quartz cuvettes with a 1 cm path length (Hellma).

Protein quantitation was performed by the method of Bradford (446) using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). Briefly, 1 µl of cell lysate was added to 999 µl of dye reagent mix (1 part dye reagent concentrate and 4 parts milli-Q water) in a disposable Ultra-VU 2.9 ml cuvette (Baxter Scientific Products, McGaw Park, IL) and mixed by inversion. Samples were allowed to sit for
2 min and then $A_{395}$ readings were recorded. Protein concentrations were determined from a standard curve generated with known concentrations of BSA ranging from 0.5-10.0 µg/ml.

**Southern Blot Analysis**

DNA samples containing 1/6 volume of sample loading dye (30% glycerol, 0.25% bromophenol blue) were electrophoresed on 0.5-0.8% neutral agarose gels (Seakem GTG, FMC, Rockland, ME) containing 0.5 µg/ml of ethidium bromide at 1-1.5 V/cm for 16-24 h in 1X TBE (90 mM Tris base, 90 mM boric acid, 2 mM EDTA (pH 8.0)). Following electrophoresis, to facilitate the transfer of high molecular weight DNA, the gels were soaked in 0.25 N HCl for 15 min to depurinate the DNA (bromophenol blue dye turns yellow when acid treatment is complete). Gels were then soaked in 0.5 N NaOH, 1.5 M NaCl for 30 min to cleave the depurinated sites on the DNA (tracking dye turns blue when alkali treatment is complete). Finally, gels were soaked in 500 mM Tris, 3 M NaCl for 45 min to equilibrate the gels for transfer.

The DNA was transferred to a nylon membrane (GeneScreenPlus, New England Nuclear, Boston, MA) by capillary blotting with 10X SSC (1X SSC is 150 mM NaCl, 15 mM trisodium citrate, pH 7.0) for 18-24 h. Following transfer, the nylon membrane was sequentially soaked in 0.4 N NaOH (45 sec) and 200 mM Tris, 2X SSC (2 min). The membrane was then
UV cross-linked (Stratalinker UV Crosslinker, Stratagene, LaJolla, CA), air dried and stored in a sealed plastic hybridization bag (Oncor, Gaithersburg, MD).

**Slot Blot Analysis**

DNA (1 µg or 5 µg) was added to 50 µl of TE (pH 8.0). DNA was denatured by incubating with NaOH (final concentration 0.25 N) at 65°C for 1 h. Samples were chilled on ice, diluted with 1 volume of 0.125 N NaOH, 0.125X SSC, and loaded onto a slot blot apparatus (Hybri-Slot Manifold, Gibco BRL) containing a presoaked (400 mM Tris (pH 7.5) for at least 30 min) nylon membrane (GeneScreenPlus) and two pieces of Whatman 3MM chromatography paper (Whatman International Ltd., Maidstone, England). Samples were allowed to sit in the apparatus for 30 min, after which time vacuum was applied for 30-60 sec. The membrane was removed, UV cross-linked and air dried. Filters were hybridized with a GAPDH probe to assess loading, stripped, and rehybridized with a c-myc probe.

**Northern Blot Analysis**

Total RNA was denatured in 50% DMSO, 1 M glyoxal (deionized with Bio-Rad RG 501-X8 mixed bed resin), 10 mM NaH$_2$PO$_4$/Na$_2$HPO$_4$ (pH 6.8), at 50°C for 1 h. Samples (21 µl total volume) were chilled on ice for 5 min and then mixed with 1/6 volume of Type IV loading dye (6X is 40% sucrose,
0.25% bromophenol blue). RNA was electrophoresed through 1% agarose gels at 100 V for 4-5 h in a recirculating 10 mM NaH$_2$PO$_4$/Na$_2$HPO$_4$ (pH 6.8) electrophoresis buffer.

Following electrophoresis, RNA was transferred to a nylon membrane (GeneScreenPlus) by capillary blotting with 10X SSC for 18-24 h. The glyoxal reaction was reversed and membranes neutralized by sequential soaking in 0.05 N NaOH for 15 sec, and 200 mM Tris (pH 7.5), 1X SSC for 35 sec. Membranes were UV cross-linked, air dried, and stored in sealed hybridization bags. The integrity of RNA on membranes was verified by the visualization of 18S and 28S ribosomal RNA bands with a UV light source. These ribosomal bands were marked with pencil and later used to determine the size of specific RNA hybridization signals (18S and 28S ribosomal RNA bands run at a size of ~ 1.9 and 4.8 kb, respectively).

**Membrane Hybridization**

Prehybridization of all nylon membranes was done in 50% formamide (deionized with Bio-Rad RG 501-X8 mixed bed resin), 10% dextran sulfate, 5X SSPE (1X SSPE is 150 mM NaCl, 10 mM NaH$_2$PO$_4$/Na$_2$HPO$_4$, 1 mM EDTA, pH 7.4), 1% SDS, 1X Denhardt’s solution (0.02% ficoll 400, 0.02% polyvinylpyrrolidone, 0.02% BSA Pentax Fraction V), and 250 µg/ml of denatured salmon sperm DNA at 42°C for 6-24 h in a shaking water bath. Hybridization with a denatured $\alpha^{32}$P dCTP-labeled probe (boiled for 5 min and snap cooled on ice for 5 min) was performed in the same
prehybridization solution at 42°C for at least 24 h.

Following hybridization, membranes were washed in 500 ml of 2X SSPE, 0.5% SDS at room temperature for 15-30 min with agitation, followed by a 6 min wash in 7 L of 0.1X SSPE, 0.1% SDS at 60°C in a Disk Wisk apparatus (Schleicher and Schuell, Inc., Keene, NH). After blotting with Whatman 3MM chromatography paper, the damp membranes were sealed in plastic hybridization bags for autoradiographic analysis. The membranes were exposed to X-ray film (X-OMAT AR, Eastman Kodak Co., Rochester, NY or Fuji RX, Fuji Photo Film Co. Ltd., Japan) at -70°C with an intensifying screen (Lightning Plus, E.I. du Pont de Nemours and Company, Inc., Wilmington, DE). Films were developed in a Kodak m35A X-OMAT autoprocessor.

Membrane Stripping and Reprobing

Membranes were stripped of probe by boiling in a solution of TE (pH 7.5), 1% SDS for 30-60 min with constant stirring. The complete removal of probe was verified by autoradiography and reprobing of the membrane was performed as previously described.

S1 Nuclease Protection Assay

RNA (8 µl total volume) was added to a reaction mix (100 µl total volume) consisting of 80% formamide (deionized), 1X S1 hybridization buffer (10X S1 hybridization buffer is 4 M NaCl, 400 mM PIPES (pH 6.8), 20 mM EDTA (pH 8.0)), excess 5'
\( ^{32} \text{P} \) end-labeled probe, and 100 \( \mu \text{g/ml} \) of tRNA in a sterile 1.5 ml microfuge tube. Samples were heated at 68°C for 15 min to loosen DNA/RNA complexes, and transferred to a 53°C water bath for at least 12 h. After hybridization of probe to target RNA sequences, each tube was removed from the water bath and 300 \( \mu \text{l} \) of ice cold S1 digestion buffer (66 mM sodium acetate, 300 mM NaCl, 4 mM ZnSO₄) and 1 \( \mu \text{l} \) of S1 nuclease (400 U/\( \mu \text{l} \)) were added to each sample, which was mixed and placed on ice. S1 nuclease digestion was performed at 37°C for 1 h.

S1 digestion was stopped by extraction of the sample 1X with an equal volume of PCI. Phases were separated by microcentrifugation for 10 min and then the aqueous phase was transferred to a sterile microfuge tube. RNA was precipitated for at least 30 min at -70°C by the addition of 1 ml of 95% ethanol, pelleted by centrifugation at 12,000 \( X \) g at 4°C, air dried, and resuspended in 5 \( \mu \text{l} \) of DEPC treated milli-Q water. Samples were denatured in 15 \( \mu \text{l} \) of formamide dye (90% formamide, 0.5X TBE, 0.1% bromophenol blue, 0.1% xylene cyanol) at 95°C for 5 min, chilled on ice, loaded onto a 0.75 mm thick, urea/polyacrylamide gel (4%) and electrophoresed at 25 mA/gel in 1X TBE (chilled to 4°C) until the bromophenol blue dye front ran off the gel. Gels were then fixed in 10% acetic acid for 15 min, rinsed with milli-Q water for 15 min, placed on Whatman 3MM paper and dried on a Drygel Jr. model SE 540 (Hoefer Scientific Instruments, San Francisco, CA) for 1 h at 80°C with vacuum, wrapped in plastic wrap and autoradiographed.
probe excess was determined by running a 1:100 dilution of undigested probe on each gel and comparing the intensity of this band to the intensity of the bands generated by the protected fragment.

Protected fragments were sized by comparison to end labeled DNA size markers which were run on each gel. The DNA size markers were generated by HinfI restriction endonuclease digestion of pBR322 plasmid (Promega, Madison, WI) and resulted in size markers of 1632, 506, 517, 396, 344, 220, 221, and 154 nucleotides.

**DNA Probes**

DNA probes used in hybridization studies included the following: the 420 bp PstI exon 2 fragment of the human c-myc gene which was derived from the genomic c-myc, pHSR-1, (American Type Culture Collection, Rockville, MD) and subcloned into a pGEM-3Z vector (Promega); a PCR generated, 215 bp fragment of the Histone H3.3 gene; a 400 bp (-116 to -516) 5' flanking region fragment of the human GAPDH gene which was generously provided by Dr. Maria Alexander-Bridges (Harvard Medical School, Boston, MA); a PCR generated, 447 bp exon 1/2 fragment of the human bcl-2 cDNA; and a PCR generated, 790 bp fragment of the human p53 cDNA spanning exons 3-7.

The c-myc probe used in S1 nuclease protection assays was derived from a pGEM-3Z -8 plasmid vector containing the 420 bp PstI fragment of exon 2 of the human c-myc gene and resulted in the protection of a 186 bp S1 nuclease resistant fragment.
The c-myc, histone H3.3, and GAPDH probes have been previously characterized and used extensively in our laboratory. The bcl-2 and p53 cDNA probes were designed using OLIGO 5.0 primer Analysis Software (National Biosciences, Inc., Plymouth, MN) and the identity of the PCR generated fragments was verified by restriction endonuclease analysis.

**cDNA Synthesis and PCR**

First strand cDNA synthesis was performed as follows: 1 µg of total RNA (8 µl volume) was denatured for 6 min at 65°C, snap chilled on ice, and added to a reaction mix (20 µl total volume) containing 500 µM each of dGTP, dATP, dTTP, and dCTP, 1X reverse transcription buffer (Gibco BRL) (5X reverse transcription buffer is 250 mM Tris (pH 8.3), 375 mM KCl, and 15 mM MgCl$_2$), 10 mM DTT, 10 µg/ml oligo (dT)$_{12-18}$ (Gibco BRL), and 20 U of RNasin (Promega). The reaction was started by the addition of 400 U of Moloney murine leukemia virus reverse transcriptase (Gibco BRL) and performed at 37°C for 1.5 h. After reverse transcription was complete, the reaction was terminated by heating at 95°C for 5 min and then chilled on ice. Reactions were now ready for PCR amplification of primer specific cDNA fragments.

All PCR reactions were set up in 0.5 ml GeneAmp reaction tubes (Perkin Elmer Cetus, Norwalk, CT) and performed on a Perkin Elmer Cetus DNA Thermal Cycler. The template for PCR amplification (reverse transcription reaction or DNA) was added
to a reaction mix (100 µl total volume) consisting of the following: 1X PCR buffer (10X PCR buffer is 15 mM MgCl₂, 500 mM KCl, and 100 mM Tris (pH 8.3)), 200 µM each of dATP, dCTP, dGTP, and dTTP, 200 nM of each primer, and 2 U of AmpliTaq DNA polymerase (Perkin Elmer Cetus). Reactions were overlaid with 75 µl of mineral oil (Sigma) and subjected to PCR.

The primer pair used for histone 3.3 amplification was as follows: upper primer = 5'CCACTGAACCTCTGGATGCGC 3', lower primer = 5'GCGTGCTAGCTGGATGTCTT 3'. Reactions were heated to 96°C for 5 min and then subjected to 30 cycles of PCR amplification using the following conditions for primer denaturation, annealing, and extension: 30 sec at 95°C, 30 sec at 55°C, and 45 sec at 72°C, respectively. After PCR, the reactions were heated for 5 min at 72°C to complete the extension of primers and then the mineral oil was removed by extracting the reaction with 2 volumes of chloroform:isoamyl alcohol. PCR products were recovered by PCI extraction and ethanol precipitation, and then agarose gel purified.

The primer pair used to amplify the 447 bp fragment of the bcl-2 cDNA consisted of the following upper and lower primers: 5'GGTGAACCTGGGAGGATTGT 3' and 5'GTGCTGTCTGTGTGTGAT 3', respectively. Thirty cycles of PCR amplification were performed as previously described except that the bcl-2 primers were denatured for 1 min at 94°C, annealed for 1 min at 55°C, and extended for 1 min at 72°C.

The 26-mers 5'CGTCCCAAGCAATGGATGATTTGATG 3' and...
5′AGCTCGTGGTGAGGCTCCCCTTTCTT 3′ were used to amplify the 790 bp fragment of the human p53 cDNA using the following PCR conditions for 30 cycles: denature for 1 min at 95°C, anneal for 1 min at 62°C, and extend for 1 min 45 sec at 72°C.

**32p Labeling of DNA Probes**

DNA probes for hybridization studies were labeled to high specific activity using the random primer protocol of Feinberg and Vogelstein (447) with a Prime-a-Gene labeling system (Promega). Briefly, 25 ng of denatured DNA template (boiled for 5 min and snap cooled on ice) were added to a reaction mixture containing 1X labeling buffer (5X labeling buffer is 250 mM Tris (pH 8.0), 25 mM MgCl₂, 10 mM DTT, 1 M HEPES (pH 6.6), and 26 A₂₆₀ U/ml random hexadeoxyribonucleotides), 20 mM each of unlabeled dATP, dTTP, and dGTP, 50 µCi of α³²P-labeled dCTP (3000 Ci/m mole, Amersham, Arlington Heights, IL), 400 µg/ml nuclease free BSA, and 5 U of the Klenow fragment of DNA polymerase I. The reaction was allowed to proceed for at least 2 h (usually overnight) at room temperature.

Unincorporated nucleotides were removed by size exclusion chromatography on a Sephadex G-50 spin column (Boehringer Mannheim). Probes were denatured prior to being added to hybridization bags by boiling for 5 min and then snap cooling on ice.

The c-myc probe for S1 nuclease protection assays was 5′ end labeled with T4 polynucleotide kinase. Briefly, the
pGEM-3Z -8 vector (10 µg) containing the PstI exon 2 fragment of the human c-myc gene was digested with BstE II (1 U/µg of DNA, Gibco BRL) in 100 µl (total volume) of restriction endonuclease REact 2 buffer (1X is 50 mM Tris (8.0), 10 mM MgCl₂, and 50 mM NaCl) for 1 h at 37°C. BstE II linearizes the plasmid by cutting the DNA at one site within the c-myc insert leaving a 5' overhang and results in a 186 bp, RNA hybridizable probe. Following digestion with BstE II, 5' phosphates were removed with bacterial alkaline phosphatase (100-200 U, 65°C for 1 h) (Gibco BRL). The sample was then treated with proteinase K (2.8 mg/ml) in 1X proteinase K buffer (6X buffer is 60 mM Tris (pH 7.8), 30 mM EDTA, and 3% SDS) for 1 h at 45°C, extracted 2X with an equal volume (180 µl) of PCI, precipitated with 1/10 volume of sodium acetate, 2.5 volumes of 95% ethanol at -70°C for at least 1 h and microcentrifuged for 30 min at 4°C. The pellet was air dried, reconstituted in 100 µl of TE (pH 8.0) and purified on a Sephadex G-50 spin column.

The probe was then 5' end-labeled as follows: 9 µg of linearized plasmid DNA was added to a reaction (100 µl total volume) containing 1X kinase buffer (10X kinase buffer is 700 mM Tris (pH 7.6), 100 mM MgCl₂, and 50 mM DTT), 10 µCi of ³²P-labeled dATP (6000 Ci/mmmole, Amersham), and 5-10 U of T4 polynucleotide kinase (Promega). DNA was labeled for 30 min at 37°C and then the reaction was stopped by the addition of EDTA (to 10 mM). The labeled DNA was extracted 1X with an equal volume of PCI, ethanol precipitated (-70°C for at least 1 h),
pelleted, resuspended in TE (pH 8.0), purified over a Sephadex G-50 column and stored at -70°C.

Quantitation of Hybridization Studies

Autoradiographs of northern blots were quantitated on an LKB Ultrascan XL laser densitometer. Sl gels, Southern and slot blots were quantitated on a Betagen Betascope 603 Blot Analyzer.

Western Blot Analysis

Proteins (less than 100 µg/lane) were fractionated by discontinuous SDS-PAGE (10 or 12.5% separating gel, 5% stacking gel) in a Tris/glycine electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) at 15 mA/gel for 5-6 h. Proteins were transferred to nitrocellulose (S&S NC, 0.2 µm, Schleicher and Schuell) in a buffer consisting of 25 mM Tris (pH 8.3), 192 mM glycine, 1.3 mM SDS, and 20% methanol, for 1-2 h at 100 mA using a Hoefer Semiphor semi-dry transfer unit (Model TE-70). The nitrocellulose membrane was blocked overnight at 4°C in blocking buffer (10 mM Tris (pH 8.0), 150 mM NaCl, 0.2% Tween 20 (Sigma), 0.2% Nonidet P-40, 0.02% SDS, 0.02% NaN₃, and 5% Carnation nonfat dry milk (Nestle Food Company, Glendale, CA)).

After blocking, the membrane was incubated with a primary Ab (in block buffer) for 1 h at room temperature with agitation and then washed (10 min at room temperature with agitation) 3X in TBST (10 mM Tris (pH 8.0), 150 mM NaCl, 0.2% Tween 20,
0.2% Nonidet P-40, 0.02% SDS). The membrane was then secondarily incubated with a horseradish peroxidase-linked whole Ab (either a sheep anti-mouse Ig or a donkey anti-rabbit Ig (Amersham), diluted 1:5000 in TBST) for 1 h at room temperature and again washed 3X with TBST. Specific proteins were detected by enhanced chemiluminescence (Amersham) and visualized on X-ray film (X-OMAT AR or Fuji RX) with exposure times ranging from 2 sec to 45 min.

Western blots were often stripped of a specific primary Ab and reprobed with a different Ab. Immunoblots were stripped by immersing the membrane in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris (pH 6.7)) for 30 min at 50°C with occasional agitation. The membrane was washed 2X in a large volume of TBST for 10 min, blocked for 1 h at room temperature and then immunoblotted as previously described using a different primary Ab.

The molecular weight of a specific protein was determined by comparison to low range molecular weight standards (Bio-Rad) which were co-electrophoresed with cell lysates. After protein transfer, the portion of nitrocellulose containing the molecular weight markers was removed and stained with amido black (naphthol blue black, Sigma) (0.1% amido black, 10% methanol, 2% acetic acid) for 5-10 min and then destained in 50% methanol, 7% acetic acid for 30 min. The log₁₀ of the molecular weight of the standards was plotted as a function of their R₉ values (distance of protein migration/distance of tracking dye migration) and
used to calculate the molecular weight of an unknown protein.

Electrophoretic transfer of proteins was verified by the staining of post-transferred gels. Briefly, gels were stained with Coomassie Brilliant Blue R (Sigma) (0.25% Coomassie BB in 50% methanol, 10% acetic acid) for 20-30 min at room temperature with agitation and then destained in 20% methanol, 7% acetic acid overnight at room temperature.

**Immunoprecipitation**

Specific proteins were immunoprecipitated using either a denaturing lysis buffer (25 mM Tris (pH 8.0), 50 mM NaCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 0.1% NaN₃) or a moderately denaturing lysis buffer (20 mM Tris (pH 8.0), 150 mM NaCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% NaN₃). Coimmunoprecipitation of proteins was done using a non-denaturing lysis buffer (20 mM Tris (pH 8.0), 150 mM NaCl, 0.2% Nonidet P-40, and 0.1% NaN₃).

All steps in the immunoprecipitation protocol were done either at 4°C or on ice to reduce endogenous protease activity. Briefly, a specified number of cells (up to 50 X 10⁶) was washed 2X in ice cold PBS (pH 7.4). The cells were lysed in 1 ml of ice cold IP lysis buffer containing 1 mM phenylmethylsulfonyl fluoride (Sigma), which had been added to the lysis buffer just prior to use, vortexed, and placed on ice for 1 h. The viscosity of the lysate was reduced by repeated passage through a 20 gauge syringe needle and then genomic DNA and cellular debris were
cleared from the lysate by microcentrifugation for 30 min at 16,000 X g.

The supernate was transferred to a new microfuge tube and precleared with 25 µl of a 1:1 slurry of GammaBind G Sepharose (Pharmacia LKB Biotechnology, Uppsala, Sweden):TSA (10 mM Tris (pH 8.0), 140 mM NaCl, and 0.25% NaN₃) for 1 h with agitation. The sepharose was pelleted (16,000 X g for 3 min) and the lysate again transferred to a new microfuge tube. The primary immune complex was formed by incubation of the lysate with a specific Ab for 1 h with agitation, after which time 25 µl of GammaBind G Sepharose:TSA (1:1) was added to the lysate and a precipitable, secondary immune complex allowed to form for 1 h with agitation.

Immune complexes were precipitated by microcentrifugation at 16,000 X g for 3 min, and then washed 3X in 1 ml of ice cold wash buffer (wash buffer = lysis buffer). Following the final wash, immune complexes were reduced by adding 40 µl of western sample buffer to the sepharose pellet. The samples were boiled for 10 min and stored at -70°C until analyzed by SDS-PAGE and western blotting as previously described.

**Antibodies**

All of the antibodies used in these studies were commercially available. Concentrated antibody stocks (1 µg/µl) were stored at -20°C until diluted in PBS (pH 7.4) + 0.1% NaN₃ to a working stock (0.1 µg/µl) which was then stored at 4°C for up to 1 year.

The p53 antibodies Ab-3 and Ab-6 were obtained from Oncogene
Ab-3 (clone PAb 240) is a mouse monoclonal antibody (IgG1) raised against a human epitope (residues 212-217) and is specific for the mutant form of mammalian p53. This antibody will also recognize denatured wild type p53 protein when the exposed epitope is identical to the mutant epitope. Ab-6 (clone DO-1) is also a mouse monoclonal antibody (IgG2a) which was raised against a human NH-terminal epitope and recognizes both mutant and wild type p53 protein. The Ab-6 antibody was used as the primary Ab in p53 related western blot studies at a working concentration of 2 µg/ml. The concentration of Ab-6 and Ab-3 used in immunoprecipitation studies was 0.1 µg/ml and 1.5 µg/ml, respectively.

The mouse anti-human bcl-2 monoclonal antibody (clone 124, IgG1) was raised against an NH-terminal epitope (residues 51-54) and recognized the 26 kDa human bcl-2 protein. This antibody was obtained from Cambridge Research Biochemicals (Wilmington, DE) and used in immunoblot studies at a working concentration of 5 µg/ml.

Two c-myc antibodies were used in these studies and were obtained from Cambridge Research Biochemicals. The mouse monoclonal antibody (IgG1, clone 6E10) was raised against an epitope spanning amino acid residues 171-188 of human c-myc and recognized only the human c-myc protein. The rabbit polyclonal antibody (IgG) was raised against the NH-terminal residues [44-55] + Cys of human c-myc and recognized all of
the human myc proteins (pan myc). The rabbit polyclonal Ab was used as the primary Ab in western blot studies at a concentration of 5 µg/ml. A cocktail of the monoclonal and polyclonal Abs (1 µg/ml of each) was used in immunoprecipitation studies of c-myc.

The anti-mad and anti-max rabbit polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-mad Ab was raised against a COOH-terminal epitope spanning residues 203-221 and recognized the human mad protein. This antibody was used in western blot studies at a concentration of 4 µg/ml. The anti-max Ab was raised against a COOH-terminal epitope that spanned from residues 135-151 of the p21 form of the human max protein and recognized both the p21 and p22 human max proteins. The max antibody was used at a concentration of 1 µg/ml in western blot studies and 0.5 µg/ml for coimmunoprecipitation analysis of c-myc and max proteins.

All antibodies used in the studies presented in this dissertation were titrated to maximize the signal to noise ratio. The apparent molecular weight of specific antigens was determined, and the specificity of antigenic bands was verified prior to the use of any antibody in an experiment. Specificity controls for each Ab varied as follows: for anti-c-myc, a classical serum stimulation, cellular differentiation experiment was performed in HL-60, IMR-90, and HeLa cells; for anti-p53 Ab, the HL-60 (p53 -/-) cell line was used as a negative control, while IMR-90 and HeLa cells (p53 wt/wt) were used as positive controls.
Also, isotype controls were used (MOPC21/IgG₁ and UPC10/IgG₂a). For anti-bcl-2 Ab, the MOPC21 isotype control was utilized along with a positive control cell line, LM-EBV. The specificity of the anti-max and anti-mad Abs was demonstrated by antigen block experiments with the respective peptide antigens.

### Apoptotic DNA Isolation and Analysis

Low molecular weight DNA for nucleosome laddering gels was isolated as follows: approximately 10x10⁶ cells were washed 2X in ice cold PBS (pH 7.4) and lysed in 1 ml of lysis buffer (10 mM EDTA (pH 8.0), 50 mM Tris (pH 8.0), and 0.5% N-lauroyl sarcosine, w/v), treated with RNase A (250 µg/ml) for 1 h at 37°C and with proteinase K (500 µg/ml) for 2 h at 50°C. The lysates were microcentrifuged (13,000 X g) for 30 min at 4°C. High molecular weight DNA was removed and the supernates were extracted 2X with PCI, once with chloroform:isoamyl alcohol, and ethanol precipitated with 1/10 volume of 3 M sodium acetate, 2.5 volumes of 95% ethanol, overnight. Samples were pelleted, washed in 70% ethanol, air dried, resuspended in TE (pH 8.0), quantitated by A₂₆₀, and stored at 4°C. One fifth the volume of loading dye (5X loading dye is 10 mM EDTA (pH 8.0), 0.25% bromophenol blue and 50% glycerol) was added to each 10 µg sample, which was heated at 65°C for 10 min, loaded onto a 2% agarose mini-gel (Seakem GTG) containing 0.5 µg/ml ethidium bromide, and electrophoresed at 6 V/cm until the tracking dye ran off the bottom of the gel (2-3 h). The gel was photographed using
a Fotodyne UV-440 transilluminator system (Fotodyne Inc., New Berlin, WI) with Polaroid Type 57 film (Polaroid Corporation, Cambridge, MA).

**Terminal Deoxynucleotidyl Transferase Assay (TDT A)**

Apoptosis-induced DNA fragmentation was assessed in situ by means of the TDT A. Following drug treatments, approximately 10x10^6 cells were washed 1X in ice cold PBS (pH 7.4) and fixed in 2 ml of ice cold 1% formaldehyde (Mallinckrodt) in PBS for 15 min on ice. Cells were then washed 3X in cold PBS, resuspended in 70% ethanol and stored at -20°C overnight. The cells were rehydrated in PBS for 15 min on ice, aliquoted into Falcon 2054 12 X 75 mm round bottom polystyrene tubes (Becton Dickinson, Lincoln Park, NJ) (1-1.5x10^6 cells/tube), and pelleted (500 X g for 5 min at 4°C). The supernatant was removed and the inside walls of the tubes were thoroughly dried with a Kimwipe (Kimberly-Clark Corporation, Atlanta, GA). The cells were resuspended in 40 µl of TDT solution (200 mM potassium cacodylate, 25 mM Tris, 0.25 mg/ml BSA, 1 mM CoCl₂, 25 µM biotin-16-dUTP (Boehringer Mannheim), and 25 U of terminal transferase (Boehringer Mannheim) ) and incubated at 37°C for 30 min. The cells were then washed 3X with 2 ml of cold PBS + 2% BCS, resuspended in 50 µl of staining buffer (4X SSC, 0.1% Triton X-100, 5% Carnation nonfat dry milk, and 0.75 µg/ml fluorescein isothiocyanate-conjugated avidin (Sigma)) and incubated at room temperature for 30 min in the dark. The cells were washed 3X
in 2 ml of cold PBS + 2% BCS, resuspended in 1 ml of PBS + 2% BCS and stored in the dark on ice until subjected to FACS analysis. Ten thousand events were recorded using a Becton Dickinson FACStarPlus (San Jose, CA) and analyzed using LYSIS II software. A 5 W argon ion laser tuned to 488 nm and emitting at 200 mW, was used for fluorescein excitation and emissions were measured using a 530/30 band pass filter. Each sample was split into 2 tubes, one with terminal transferase and one without terminal transferase, therefore, each sample was run with its own negative control.

**Morphological Analysis of Apoptotic Nuclei**

The percentage of apoptotic and necrotic cells occurring in a nitrogen mustard treated population was quantitated by the morphological analysis of propidium iodide stained nuclei. Briefly, 1-2 X 10^6 cells were isolated at various times following exposure to HN2, washed 1X with ice cold PBS (pH 7.4) and fixed in 1% formaldehyde (in PBS) for 15 min on ice. The cells were washed 1X with ice cold PBS, permeabilized with 0.1% Triton X-100 for 3 min on ice and then treated with RNase A (1 mg/ml) for 30 min at 37°C. RNase was removed and the cells incubated with propidium iodide (50 µg/ml in PBS) (Sigma) for at least 1 h at 4°C in the dark. The cells were then analyzed by fluorescence microscopy on a Nikon DIAPHOT-TMD inverted microscope (Nippon Kogaku K.K, Tokyo, Japan) with a Nikon mercury lamp power supply (HB0-100W/2). Photomicrographs were taken using
a Nikon N6006 35 mm SLR camera and Tmax 100 black and white negative film (Kodak).

**Cellular Viability Assay**

Cellular viability was assessed based upon membrane integrity and cellular enzyme function using a propidium iodide/fluorescein diacetate (Sigma) based flow cytometric assay. The nucleic acid intercalating dye propidium iodide is impermeable to the intact cellular membrane of viable cells but stains the nuclei of non viable cells because the cell membrane of non viable cells becomes leaky as integrity is lost. Fluorescein diacetate is membrane permeable and once inside the cell is cleaved by general cellular esterases, generating a non-permeable fluorochrome, fluorescein, which becomes trapped inside viable cells.

Viability of cells was determined as follows: 1-2 X 10^6 cells were aliquoted into 12 X 75 mm Falcon round bottom polystyrene tubes and washed 2X in ice cold HBSS (pH 7.4) (Gibco BRL). The cells were resuspended in 1 ml of HBSS containing fluorescein diacetate (0.1 ng/ml) and propidium iodide (50 µg/ml), incubated at room temperature for 10 min in the dark, and washed 1X with HBSS. The cells were then resuspended in 1 ml of HBSS and stored in the dark on ice, until analyzed on a Becton Dickinson FACStarplus. Ten thousand events were recorded for each sample. Fluorochromes were excited at 488 nm and emissions measured using a 530/30 band pass filter (fluorescein) and a
Antisense ODN Studies

Gene specific antisense ODNs were used in an attempt to modulate the expression of different genes. Two methods of ODN delivery were utilized. The first method involved the delivery of antisense ODNs to membrane permeabilized cells. Briefly, 1 X 10^6 cells were washed 1X in HBSS (pH 7.4), resuspended in 1 ml of permeabilization buffer (137 mM NaCl, 100 mM PIPES (pH 7.4), 5.6 mM glucose, 2.7 mM KCl, 2.7 mM EGTA, 1 mM Na-ATP, 0.1% BSA, and 1.5 U/ml of Streptolysin O (Sigma)) containing a specified concentration of either sense or antisense ODNs, and incubated for 5-10 min at room temperature. Following incubation with ODNs, the permeabilization of cells was reversed by pelleting the cells, decanting the buffer and washing the cells 1X in 5 ml of culture media. The cells were resuspended in culture media, incubated at 37°C, and whole cell proteins isolated at various time points for western blot analysis of specific proteins. This method exposed cells to ODNs for a very short time (5-10 min).

The second method of antisense delivery involved the continuous exposure of cells to ODNs in the culture medium. Approximately 1 X 10^6 cells were resuspended in 3-5 ml of culture media containing 5% or 10% DNase inactivated BCS (heated at 65°C for at least 30 min) and then treated with the appropriate concentration of either sense or antisense ODNs. Cells were
incubated with ODNs for various times and then whole cell proteins were isolated for western blot analysis. In the longer time course studies, ODNs were reapplied every 12 or 24 h.

Three different antisense ODNs targeted against human c-myc were utilized in antisense studies. The first anti-c-myc ODN was the anti-gene, triplex forming, unmodified ODN, 5’ TGGGGAGGTTGGGAGGTTGGGAAGG 3’, which was targeted against the p1 promoter of the c-myc gene (448). The control sense ODN was 5’ CCTTCCCCACCCCTCCCCACCTCCCCCA 3’. The two antisense c-myc ODNs were previously characterized by Bacon and Wickstrom (449). The first of these antisense ODNs was targeted against the AUG initiation codon of the human c-myc mRNA (designated 559-5730). The sequence of the 559-5730, unmodified antisense ODN was 5’ AACGTTGAGGGGCAT 3’. The second antisense c-myc ODN (1-14F) was targeted against the 5’ cap sequence of the human c-myc mRNA and was synthesized as either an unmodified or phosphorothioated ODN. The 1-14 F antisense and sense sequences were 5’ GCACAGCTCGGGGT 3’ and 5’ ACCCCCGAGCTGTGC 3’, respectively.

The antisense p53 ODN, 5’ CTGCGGCTCCTCCATGGCAGT 3’, was targeted to a flanking region of the translation initiation site of the human p53 mRNA (450). This antisense ODN was unmodified for these studies.

All unmodified ODNs were synthesized on an Applied Biosystems 391 PCR-MATE EP DNA Synthesizer (Foster City, CA), using standard phosphoroamidite chemistry. Oligos were cleaved from the
substrate, phosphate deprotected, and base deprotected in concentrated ammonium hydroxide for 8 h at 55°C. Deprotected oligos were desalted and purified on Oligo Purification Cartridges (OPC, Applied Biosystems) following the manufacturer’s directions. Phosphorothioated antisense c-myc ODNs (1-14 F) were purchased from Oligos Etc., Inc.
CHAPTER 3

RESULTS

The involvement of c-myc in cellular proliferation, differentiation, transformation, and apoptosis has been extensively documented (343,344). Deregulated overexpression of c-myc occurs in a majority of human tumors and is involved in the progression of the tumorigenic state (344,351). Studies utilizing antisense ODNs targeted against c-myc mRNA have shown that the clonogenicity of human tumor cell lines can be inhibited by the specific reduction in c-myc expression (451-454). Bifunctional alkylating agents such as nitrogen mustard inhibit the clonogenicity of human tumor cells. Our laboratory has previously shown that various mustard analogs inhibit c-myc transcription in vitro (29) and down-regulate the expression of c-myc mRNA in the human colon carcinoma cell line, Colo320HSR, in a concentration and time-dependent manner (31). Based upon these observations, the following hypothesis was investigated: Bifunctional alkylating agents, such as HN2, inhibit the clonogenicity of human tumor cells by down-regulating the expression of critical cellular oncogenes, such as c-myc.
COMPARISON OF C-MYC GENE COPY NUMBER AND EXPRESSION IN THE COLO320HSR, HT-29, AND BE HUMAN COLON CARCINOMA CELL LINES

The three human colon carcinoma cell lines, Colo320HSR, HT-29, and BE, were chosen to address this question because of their varying levels of c-myc mRNA expression. These cell lines were characterized with respect to c-myc gene copy number and expression. Southern blot analysis revealed that the c-myc gene was highly rearranged and amplified in the Colo320HSR cell line while no gross alterations were observed in the HT-29 or BE cell lines (Figure 1A). The Colo320HSR cell line was shown by slot blot analysis to possess 26-30 copies of the c-myc gene when compared to the BE cell line, while the HT-29 cell line contained approximately twice the number of c-myc alleles relative to BE cells (Figure 1B). BE cells were shown to be single copy for the c-myc gene (i.e. possess two alleles) by Southern blot comparison with a panel of cell lines containing a single copy of the c-myc gene (data not shown).

The steady state expression of c-myc mRNA was determined in asynchronous log phase cells by S1 nuclease protection analysis. These studies showed that c-myc mRNA levels in Colo320HSR and HT-29 cells were 10-12 and 1.5-2 fold higher, respectively, relative to BE cells (Figure 2A). Similar results were obtained by northern blot analysis (data not shown). BE and HT-29 cells expressed similar levels of c-myc protein. However, p64 c-myc protein levels were
Figure 1. c-Myc gene analysis of the Colo320HSR, HT-29, and BE human colon carcinoma cell lines.

(A) Southern blot analysis. EcoRI restriction endonuclease digested genomic DNA from Colo320HSR cells (5 or 10 µg), or HT-29 and BE cells (5 µg, in duplicate) was fractionated on a 0.5% agarose gel, Southern blotted, and hybridized with a ^32P-labeled, 420 bp human c-myc exon 2 probe. The membrane was stripped and rehybridized with a human GAPDH probe to assess sample loading (data not shown).

(B) Slot blot analysis of c-myc gene copy number. EcoRI digested genomic DNA (1 or 5 µg) from each cell line was spotted onto a nylon membrane and hybridized with a ^32P-labeled human c-myc exon 2 probe to determine the relative gene copy number. The membrane was stripped and rehybridized with a human GAPDH probe (a single copy gene) to normalize for sample loading. A representative blot of two independent isolations, each repeated four times, is shown. Bands were quantitated on a Betagen Betascope 603 blot analyzer.
**Figure 2.** Comparison of c-myc mRNA levels in Colo320HSR, HT-29, and BE cell lines.

(A) RTPCR analysis of c-myc levels in the different cell lines. Total RNA was extracted and reverse transcribed. Primers specific for c-myc were used to amplify a 12.5 kb fragment from cell line RNA. The PCR products were resolved on a 1% agarose gel and visualized by ethidium bromide staining.

(B) Western Blot analysis of c-myc and GAPDH protein levels. Equal amounts of protein were loaded and subjected to SDS-PAGE. The blots were then probed with rabbit polyclonal anti-human c-myc Ab and mouse monoclonal anti-human GAPDH Ab. The bands were visualized by enhanced chemiluminescence and quantitated by densitometric analysis of autoradiographs. The gel shown is representative of 4 independent experiments. The upper bands are due to the c-myc protein, and the lower bands are due to the GAPDH protein.
Figure 2. Comparison of the relative c-myc mRNA and protein levels in the human colon carcinoma cell lines Colo320HSR, HT-29, and BE.

(A) S1 nuclease protection analysis of c-myc mRNA levels in the 3 human colon carcinoma cell lines. A $^{32}$P-5' end labeled c-myc exon 2 probe was hybridized to 20 µg of total RNA from each cell line and resulted in a 186 bp protection. Samples were done in triplicate and quantitated on a Betascope 603 blot analyzer. (B) Western blot analysis of p64 c-myc protein levels. Whole cell protein extracts from 4 X 10^5 cells were fractionated on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose with a Semiphor semi-dry transfer unit, and immunoblotted with a rabbit polyclonal anti-human myc Ab (5 µg/ml). c-myc protein was visualized on autoradiographic film by enhanced chemiluminescence detection. c-myc protein levels were quantitated by densitometric analysis of autoradiographs. The gel shown is representative of at least five independent experiments. (C) Immunoprecipitation analysis of p64 c-myc protein levels. c-myc protein was immunoprecipitated from 5 X 10^6 cells with 1 µg each of a rabbit polyclonal anti-human myc Ab and a mouse monoclonal anti-human c-myc Ab. Immunoprecipitates were electrophoresed on a 10% SDS-polyacrylamide gel and immunoblotted with a pan-myc rabbit polyclonal Ab. c-myc protein was detected by enhanced chemiluminescence and quantitated by densitometric analysis of autoradiographs. The gel shown is representative of 4 independent experiments. Specific bands were sized by comparison to protein molecular weight standards which were co-electrophoresed and transferred with the samples, cut from the rest of the membrane and stained with amido black. Unknown molecular weights were determined by $R_f$ values. The large band below the specific c-myc band in the IP analysis is the heavy chain of the rabbit IgG Ab used in the immunoprecipitation. The heavy chain is detected because of the subsequent immunoblotting step used to detect the specific protein. Since a rabbit primary Ab was used in the immunoblotting procedure to detect c-myc protein, the secondary Ab is an anti-rabbit IgG Ab and will bind to both the rabbit polyclonal Ab which is bound specifically to c-myc protein, and also the rabbit IgG heavy chain which is present on the membrane. Thus both are visualized following enhanced chemiluminescence detection.
A Colo320HSR HT-29 BE

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c-myc

B Colo320HSR HT-29 BE

C Colo320HSR HT-29 BE

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p64 c-myc

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approximately 7 fold higher in Colo320HSR cells relative to HT-29 and BE. This was determined by either western blot analysis (Figure 20) or RT-PCR (Figure 21).

Experiments to determine if exposure to HN2 would reduce the expression levels of c-myc mRNA in Colo320HSR, HT-29, and BE cells. If the working hypothesis is viable then c-myc mRNA levels should be decreased in all three cell lines following drug treatment. Cells were treated with concentrations of HN2 that inhibited their growth by either 90% (1 log) or 99.9% (3 logs). These concentrations were 0.1 μM and 6 μM, 4 μM and 10 μM, and 10 μM for Colo320HSR, HT-29, and BE cell lines, respectively. Real-time RT-PCR was performed on the treated and untreated cells to determine the expression level of c-myc mRNA in each cell line. The results showed that c-Myc message levels at the 0.1 μM and 4 μM concentrations were similar to those of the untreated cells, while the 6 μM and 10 μM concentrations showed a substantial recovery except in the HT-29 cell line treated with 6 μM HN2, in which mRNA levels began to return to control levels.
approximately 7 fold higher in Colo320HSR cells relative to HT-29 and BE cells, as determined by either western blot analysis (Figure 2B) or immunoprecipitation analysis (Figure 2C).

### Nitrogen Mustard Causes a Concentration and Time-Dependent Decrease in C-Myc mRNA Levels in Human Colon Carcinoma Cell Lines

Experiments were designed to determine whether exposure to HN2 would reduce the expression levels of c-myc mRNA in Colo320HSR, HT-29, and BE cells. If the working hypothesis is viable then c-myc mRNA levels should be decreased in all three cell lines following drug treatment. Cells were treated with concentrations of HN2 that inhibited their clonogenicity by either 90% (1 log) or 99.9% (3 logs). These concentrations were 2 µM and 6 µM, 4 µM and 10 µM, and 10 µM and 30 µM for Colo320HSR, HT-29, and BE cell lines, respectively (31,455). S1 nuclease analysis showed that c-myc mRNA levels in all three cell lines were reduced in a concentration and time-dependent manner over a 24 h time course following exposure to HN2 (Figure 3A, B, and C). The patterns of c-myc mRNA reductions were similar in all three cell lines, with the nadir occurring at 12 h (Figure 4A and B). c-Myc message levels at the 24 h time point showed no substantial recovery except in HT-29 cells treated with 4 µM HN2, in which mRNA levels began to return to control levels.
in c-myc mRNA levels in cells treated with HN2 were 60% ± 2, 66% ± 3, and 26% ± 1 for the Colo320HSR, HT-29, and BE cell lines, respectively (Figure 4B). These studies were repeated by northern blot analysis with similar results (data not shown).

Cell lines were treated with an equi-molar concentration of HN2 to further confirm the relationship between the decrease in c-myc mRNA and the inhibition of clonogenicity. Colo320HSR, HT-29, and BE cells were exposed to a 6 µM concentration of HN2 which inhibited clonogenicity in these cell lines by 3 logs, 1.5-2 logs, and less than 1 log, respectively. If the inhibition of cell growth after HN2 exposure was linked to the effect on c-myc mRNA levels, then Colo320HSR cells should have the greatest reduction in c-myc message, followed by HT-29 cells. BE cells should be essentially unaffected at a 6 µM concentration. The results from this study showed that c-myc mRNA levels were reduced in Colo320HSR cells by 60% ± 2 and 52% ± 6 (mean ± SEM) at 12 and 24 h post HN2 treatment, respectively. c-Myc message levels were reduced in HT-29 cells by only 42% ± 2 and 26% ± 2 at 12 and 24 h, respectively, while BE cells were essentially unaffected, showing reductions of 4% ± 4 and 14% ± 4 at 12 and 24 h post HN2 treatment, respectively (Figure 5). These results showed that c-myc mRNA levels were reduced in human tumor cell lines following exposure to HN2 and were
Figure 3. The effect of HN2 on the expression of c-myc mRNA in human colon carcinoma cell lines.

Human colon carcinoma cells were exposed to HN2 for 1 h at concentrations that resulted in a 1 or 3 log inhibition of clonagenicity as determined by colony formation assays. Total RNA was isolated from cells 0, 6, 12, or 24 h following drug removal and c-myc mRNA levels were determined by S1 nuclease protection analysis using a 5' 32P-end labeled c-myc exon 2 probe which resulted in a 186 bp S1 protection. Controls were included at each time point to account for any serum induced changes in c-myc mRNA levels that might have occurred as a result of refeeding following drug removal. (A) Colo320HSR cells were treated with 2 and 6 μM concentrations of HN2 and 15 μg of total RNA were used for S1 analysis. (B) HT-29 cells were exposed to 4 and 10 μM concentrations of HN2 and 25 μg of total RNA were used for S1 analysis. (C) 15-28 μg of total RNA from BE cells were used for S1 analysis and the concentrations of HN2 used were 10 and 30 μM. Protected c-myc fragments were quantitated using a Betagen Betascope 603 blot analyzer and sized by comparison to end labeled DNA size markers (HinfI digested pBR322 plasmid) which were co-electrophoresed with samples (left most lane). Probe excess was verified by including a 1:100 dilution of undigested, labeled probe (second lane from the left, panel A). Panels were representative of 3 independent experiments.
Figure 4. Graphical representation of the effect of 3-ME treatment on human colon carcinoma cell lines, showing the decrease in cellular clonogenicity and increase in cellular apoptosis after treatment. The concentration of 3-ME used in treated cells is 10 mM. The mean survival rate is calculated from the number of colonies in treated samples compared to controls.

A. Colo320HSR

B. HT-29

C. BE
Figure 4. Graphical representation of the previous figure showing the effect of HN2 on c-myc mRNA expression in human colon carcinoma cell lines.

The concentration of HN2 used in these studies produced either a 1 log (90%) (A) or 3 log (99.9%) (B) inhibition of cellular clonagenicity. c-myc mRNA expression levels in treated cells were presented as the percent change relative to each time point control. The data from three independent experiments were plotted as the mean ± standard error of the mean (SEM). The points without error bars were those points in which the error bars fell within the symbol.
Figure 5. The effect of an equi-molar concentration of HN2 on c-myc mRNA expression in human colon carcinoma cell lines.

Human colon carcinoma cells were exposed to a 6 μM concentration of HN2 for 1 h and total RNA was isolated at 12 and 24 h time points. c-myc mRNA levels were assessed by an S1 nuclease protection assay using a c-myc exon 2 probe and total RNA from Colo320HSR (15 μg), HT-29 (40 μg), and BE (25 μg) cells. mRNA levels were quantitated with a Betascope 603 blot analyzer and expressed as percent of control. Three independent experiments were performed and the data expressed as the mean ± SEM.
consistent with the possibility that reductions in c-myc mRNA levels were responsible for the inhibition of clonogenicity following HN2 treatment.

THE EFFECT OF NITROGEN MUSTARD ON C-MYC PROTEIN LEVELS IS NOT CONSISTENT WITH A ROLE FOR C-MYC IN THE INHIBITION OF Clonogenicity IN HUMAN COLON CARCINOMA CELL LINES

Since the cellular effects of c-myc are not mediated at the mRNA level, the previous studies were extended to evaluate the effects of HN2 on c-myc protein levels. c-Myc protein has a very short half life (15-30 min) (365), therefore, the effect of HN2 on c-myc protein levels would be expected to mimic those seen at the mRNA level, further supporting the working hypothesis.

Flow cytometric analysis of fixed cells stained with sulforhodamine 101 (SR101) showed that total cellular protein levels increased 2-3 fold in all three cell lines at 48 h post HN2 treatment (data not shown). These results required that specific proteins in drug treated cells be compared on a cell equivalent basis. Whole cell proteins were isolated from Colo320HSR, HT-29, and BE cells at various times following exposure to a 6, 10, or 30 μM concentration of HN2, respectively. The effect of HN2 on c-myc protein levels was assessed by western blot analysis (Figure 6A, B, and C). Following HN2 treatment, c-myc protein levels in Colo320HSR cells remained unchanged through 24 h despite the 60%
decrease in mRNA levels at the 12 and 24 h time points. c-Myc protein levels were reduced (mean ± SEM) by 52% ± 6 and 69% ± 2 at 48 and 72 h, respectively (Figure 7). Contrary to the decline in c-myc mRNA, the level of c-myc protein in both HT-29 and BE cells increased after nitrogen mustard treatment. Levels of c-myc protein began to increase in HT-29 cells 24 h following drug exposure and were maximally induced by approximately 2.5 fold at 48 h post treatment. c-Myc protein levels in BE cells were induced immediately by HN2 and showed a maximal 4 fold induction by 72 h post treatment. Protein levels of c-myc in HT-29 and BE cells exposed to HN2 remained above control values through 96 h (Figure 7). The data on c-myc protein levels were contrary to those expected and do not support a role for the down-regulation of c-myc in the inhibition of clonogenicity by HN2.

NITROGEN MUSTARD INHIBITS THE Clonogenicity OF HUMAN COLON CARCINOMA CELL LINES BY REDUCING VIABILITY

The third prediction of the working hypothesis was that the inhibition of clonogenicity by HN2 would be via a mechanism involving growth arrest, not cell death. The rationale for this prediction was based upon the known role for c-myc in cellular proliferation and differentiation. Proliferating cells contain high levels of c-myc, while quiescent cells have undetectable levels of c-myc, remain
Figure 6. Changes in c-myc protein levels in human colon carcinoma cell lines following exposure to equi-cytotoxic concentrations of nitrogen mustard.

Cells were exposed for 1 h to a concentration of HN2 that inhibited clonagenicity by 99.9%. Whole cell protein extracts were isolated at various times following drug removal, subjected to SDS-PAGE (10% gel) and western blot analysis using a rabbit polyclonal pan myc primary Ab (5 µg/ml). p64 c-myc was sized by comparison to protein molecular weight standards. Controls (C) were run at each time point to reduce variability resulting from uneven protein transfer across the membrane. The transfer efficiency was monitored by coomassie staining of post-transferred gels. (A) The effect of 6 µM HN2 on c-myc protein levels in Colo320HSR cells. 5 X 10^5 cell equivalents were used for western analysis. (B) The effect of 10 µM HN2 on c-myc protein levels in HT-29 human colon carcinoma cells. Unattached cells in drug treated flasks at the later time points were decanted, pelleted, and added back to the population of attached cells for analysis. 2.5 X 10^5 cell equivalents were used for western analysis. (C) Changes in c-myc protein levels in the BE cell line (2.5 X 10^5 cell equivalents) following exposure to 30 µM HN2. Unattached cells in drug treated flasks at the later time points were decanted, pelleted, and added back to the population of attached cells for analysis.
Figure 7. Protein level changes following exposure to PHSM in Colo320HSR, HT-29, and BE cell lines.doi:10.1177/00012037010150040601

Colo320HSR

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Figure 7. Time course analysis of the changes in c-myc protein levels in human colon carcinoma cell lines following exposure to nitrogen mustard.

A graphic representation of the previous figure showing the time course changes in c-myc protein in the Colo320HSR, HT-29, and BE cell lines following exposure to equi-cytotoxic concentrations of HN2 (6, 10, and 30 µM, respectively). Protein levels were quantitated by densitometric analysis of autoradiographs and presented as the percent change relative to time point controls. Data points represented the mean ± SEM of at least three independent experiments. Error bars fell within the symbol on some data points.
viable, and are capable of reentry into the cell cycle upon c-myc induction (351). Most differentiated cells contain undetectable levels of c-myc and also retain viability (351). Therefore, if the loss of clonogenicity induced by mustard treatment were related to the reductions in c-myc, then the cells should remain viable, since there is no evidence suggesting that the down-regulation of c-myc expression is incompatible with cellular viability.

Cellular viability of nitrogen mustard treated cells was determined using a flow cytometric assay that assessed both membrane integrity and cellular esterase activity (Figure 8A). Colo320HSR, HT-29, and BE cells were treated with 6, 10, or 30 µM HN2, respectively, and viability was measured every 24 h for seven days following drug removal. All three cell lines lost viability over the seven day time course. Colo320HSR cells rapidly lost viability (50% by approximately 38 h) while the loss of viability in both HT-29 and BE cells was substantially slower (50% by approximately 96 h) (Figure 8B). Colo320HSR and HT-29 cells were 1% and 5% viable, respectively, by the seventh day. The pattern of viability of BE cells following mustard treatment was identical to HT-29 cells out to 96 h, at which time no further loss of viability was detected in BE cells out to the seventh day (Figure 8B).

The loss of cellular viability, along with the effects observed at the protein level in all three cell lines forced
Figure 8. Time course assessment of the cellular viability of human colon carcinoma cell lines following treatment with nitrogen mustard.

Cellular viability was assessed in the Colo320HSR, HT-29, and BE cell lines at various times following a 1 h exposure to concentrations of HN2 that inhibited clonagenicity by 99.9%. Viability was determined using a flow cytometric assay that differentiated viable cells from nonviable cells based upon membrane integrity and cellular esterase function using the fluorescent dyes propidium iodide and fluorescein diacetate, respectively. (A) A scatter plot [log fluorescein (FDA) fluorescence versus log propidium iodide (PI) fluorescence] of a representative sample with each dot being equivalent to one cell. The population of viable cells was located in the lower right quadrant while the nonviable cells were located in the three other quadrants with the major portion of the nonviable cell population being located in the upper left quadrant. (B) Time course analysis of viability in the Colo320HSR, HT-29, and BE cell lines following exposure to 6, 10, and 30 µM HN2, respectively. Viability was expressed as the percent of control. Control populations of Colo320HSR, HT-29, and BE cells were 86.2%, 81.5%, and 87.3% viable. Ten thousand cells were analyzed per sample and the data expressed as the mean ± standard deviation (SD) of three independent experiments. Data points were present where the error bars fell within the symbol. For the attached cell lines HT-29 and BE, unattached drug treated cells at the later time points were decanted, pelleted, and added back to the population of attached cells for analysis.
the rejection of the original hypothesis. These studies did not conclusively rule out the partial involvement of cellular growth arrest in the inhibition of clonogenicity in the BE cell line following HN2 exposure since cellular viability was not measured out past 7 days. Therefore, based upon these results, I conclude that the inhibition of clonogenicity by HN2 in the Colo320HSR, HT-29, and BE (to a major extent) human colon carcinoma cell lines resulted from the loss of cellular viability and was not mediated by a decrease in c-myc protein expression.

Two distinct mechanisms of death are utilized by cells exposed to chemotherapeutic agents, necrosis and apoptosis. In general, cells that undergo an apoptotic cell death in response to anti-neoplastic agents are more sensitive to these drugs (88). Several gene products involved in the transformation of cells have also been shown to directly or indirectly regulate the induction of apoptosis in either a positive or negative fashion (85). The c-myc oncogene is such a gene. The constitutive ectopic expression of c-myc protein in rodent fibroblasts or murine IL-3-dependent myeloid cells has been shown to enhance the induction of apoptosis in a dose-dependent manner in cells that have been presented with a replication block (growth/survival factor deprivation, DNA damage induced by chemotherapeutic agents, etc.), thus demonstrating a role for the deregulated overexpression of c-myc in the regulation of apoptosis.
The expression of c-myc is deregulated by various mechanisms in the majority of human tumor cells (370), including the Colo320HSR (gene amplification), HT-29, and BE cell lines. Nitrogen mustard damages cellular macromolecules, including DNA, and arrests cells in the G2 phase of the cell cycle (2,456). The anti-clonogenic effect of HN2 on Colo320HSR, HT-29, and BE cells was mediated at the level of cell death and did not appear to involve the modulation of the proliferative capacity of these cells by c-myc. Based upon the previous conclusions and these observations, it seemed more logical that if c-myc were to have a role in chemotherapeutic agent-induced cytotoxicity, it would be exerted through its ability to regulate the induction of apoptosis. Therefore, the following hypothesis was investigated: Human tumor cell lines which overexpress c-myc will undergo an enhanced apoptotic cell death following exposure to chemotherapeutic agents that cause DNA damage.

HN2-INDUCED LOSS OF VIABILITY IS ACCOMPANIED BY THE CONCENTRATION AND TIME-DEPENDENT APPEARANCE OF OLIGONUCLEOSOMAL DNA LADDERS ASSOCIATED WITH APOPTOTIC CELL DEATH IN COLO320HSR CELLS BUT NOT IN HT-29 OR BE CELLS.

The hypothesis predicts that the treatment of Colo320HSR, HT-29, and BE cells with HN2 would result in the rapid onset of cell death by apoptosis with little or no
necrosis observed. At the time of these studies, the presence of oligonucleosomal-sized DNA ladders was thought to be a universal biochemical hallmark of apoptosis (101). Therefore, initial experiments were performed to determine if these DNA ladders were present in Colo320HSR, HT-29, and BE cells after HN2 treatment. A time course analysis of HN2 treated cells showed that DNA ladders were present in Colo320HSR cells as early as 48 h post treatment. DNA ladders were not observed in HN2 treated HT-29 or BE cells at any of the time points (BE seven day time point not shown) (Figure 9).

Further investigation revealed that the appearance of DNA ladders in Colo320HSR cells following nitrogen mustard exposure was both time and concentration-dependent. Twenty-five and 50 µM concentrations of HN2 induced the formation of DNA ladders in Colo320HSR cells at 24 h (the earliest time point measured) while 0.5 µM (to some extent) and 1 µM concentrations of HN2 induced the formation of DNA ladders as early as 72 h post drug removal (Figure 10). A preliminary concentration-response study (10 to 100 µM HN2) in BE cells confirmed the absence of observable DNA ladders at the 96 h time point (Figure 11). These data verified the presence of apoptotic Colo320HSR cells and suggested that apoptosis was involved in the mediation of HN2-induced cell death in this cell line. These experiments were not designed to rule out the occurrence of apoptosis and should not be interpreted as
Figure 9. Nucleosome DNA laddering gels assessing the presence and time course of apoptosis in human colon carcinoma cell lines following treatment with equi-cytotoxic concentrations of HN2.

Colo320HSR, HT-29, and BE cells were treated with 6, 10, and 30 µM HN2, respectively, for 1 h. Low molecular weight genomic DNA was isolated at various times following drug removal and electrophoresed as described in the methods. Unattached cells were decanted, pelleted, and added back to the attached cells for analysis. The appearance of nucleosome-sized DNA ladders (~180 bp multimers), a classical biochemical hallmark of apoptosis, indicated the presence of apoptotic cells in drug treated populations. Actinomycin D (5 µg/ml) treated Colo320HSR cells were used as a positive control for the presence of nucleosome DNA ladders. A 1 kb DNA ladder (left most lane) was included to size nucleosome DNA fragments. Gels shown were representative of three independent experiments.
Figure 0. Gel electrophoresis of DNA from various cells at different times post-treatment. The samples were loaded at 0, 12, 24, 48, 72, and 96 hours. The gel shows the migration of DNA fragments at various positions, indicating the presence of specific DNA alterations. The bands at 1kb and 1kb-lad are marked for reference.
Figure 10. Concentration-response analysis of the time course of nitrogen mustard-induced apoptosis in Colo320HSR cells as assessed by nucleosome DNA laddering.

Colo320HSR cells were treated with various concentrations (µM) of HN2 for 1 h and low molecular weight DNA was isolated at various times following drug removal and electrophoresed through agarose gels as described in the methods. Actinomycin D (5 µg/ml) was used as a positive control for the presence of nucleosome ladders in Colo320HSR cells. The gels shown were representative of three independent experiments.
## Colo320HSR

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![Image of gel electrophoresis](image.png)

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Figure 11. Concentration-response analysis of nitrogen mustard-induced nucleosome DNA laddering in BE human colon carcinoma cells.

BE cells were treated with various μM concentrations of HN2 for 1 h and then low molecular weight DNA from attached and unattached cells was isolated 96 h following drug removal and electrophoresed through an agarose gel as described in the methods. The gel shown was representative of three independent experiments.
BE

96 h

The terminal deoxynucleotidyl transferase assay (TDTA) is a sensitive, qualitative in situ assay that detects fragmented DNA. This assay was used to verify the DNA laddering results. The time-sterependent appearance of these results was confirmed in the 100-kb and 150-kb samples of BE cells at several concentrations of drug exposure (10, 30, and 14). These results were consistent with the DNA laddering gel studies that suggested a fragmentation process following treatment. These studies suggested that 300 bp rosettes which are 90-kbp chromatin loops (107). These steps are thought to occur in cells undergoing apoptosis. In most, but not all cell types, these chromatin loops serve as the substrate for activated endonucleases that cleave the DNA into
such in the HT-29 and BE cell lines. The number of apoptotic cells in the HT-29 and BE drug treated populations may simply have been too small to be detected by agarose gel electrophoresis.

The terminal deoxynucleotidyl transferase assay (TDTA) is a sensitive, quantitative in situ assay that detects fragmented DNA. This assay was used to verify the DNA laddering results. The time and concentration-dependent appearance of fragmented DNA following HN2 exposure was confirmed in the Colo320HSR cell line (Figures 12 and 15). These results also suggested that apoptosis was the predominant mode of death utilized by Colo320HSR cells following treatment with HN2. TDT analysis of HT-29 and BE cells at several time points following exposure to various concentrations of HN2 failed to reveal the presence of oligonucleosomal-sized DNA fragments (Figures 13 and 14). These results were consistent with those from the DNA laddering gel studies.

Recently, much work has been focused upon the process of DNA fragmentation in cells undergoing apoptosis. These studies suggested that DNA is initially fragmented into 300 kbp rosettes which are subsequently degraded into 50 kbp chromatin loops (107). These steps are thought to occur in all cells undergoing apoptosis. In most, but not all cell types, these chromatin loops serve as the substrate for activated endonucleases that cleave the DNA into
Figure 12. Concentration-response analysis of the time course of nitrogen mustard-induced DNA fragmentation in the Colo320HSR human colon carcinoma cell line.

A quantitative in situ TDT assay was used to determine the number of cells in a drug treated population possessing the fragmented DNA characteristic of apoptosis. Colo320HSR cells were exposed to various µM concentrations of HN2 for 1 h and then cells were fixed at various times following drug removal and subjected to TDT analysis as described in the methods. Data are presented as the log fluorescence of TDT staining versus the number of cells. Ten thousand cells were analyzed for each sample. TDT positive cells (cells with fragmented DNA) had a higher fluorescence than TDT negative cells, therefore the population of cells furthest to the right on the x-axis were those cells that had fragmented DNA.
Figure 13. Concentration-response analysis of the time course of nitrogen mustard-induced DNA fragmentation in the HT-29 human colon carcinoma cell line.

A quantitative in situ TDT assay was used to determine the number of cells in a drug treated population possessing the fragmented DNA characteristic of apoptosis. HT-29 cells were exposed to various µM concentrations of HN2 for 1 h and then cells were fixed at various times following drug removal and subjected to TDT analysis as described in the methods. Unattached cells at the later time points were combined with attached cells for analysis. Data are presented as the log fluorescence of TDT staining versus the number of cells. Ten thousand cells were analyzed for each sample.
Figure 14. Concentration-response analysis of the time course of nitrogen mustard-induced DNA fragmentation in the BE human colon carcinoma cell line.

A quantitative in situ TDT assay was used to determine the number of cells in a drug treated population possessing the fragmented DNA characteristic of apoptosis. BE cells were exposed to various µM concentrations of HN2 for 1 h and then cells were fixed at various times following drug removal and subjected to TDT analysis as described in the methods. Unattached cells from the later time points were combined with the attached cells for analysis. Data are presented as the log fluorescence of TDT staining versus the number of cells. Ten thousand cells were analyzed for each sample.
Figure 15. Time course analysis of drug-induced DNA fragmentation in the Colo320HSR cell line following exposure to various µM concentrations of nitrogen mustard.

Graphic representation of the Colo320HSR TDT analysis of Figure 12 showing the changes in HN2-induced DNA fragmentation over a 96 h time course. The data from 1 X 10^4 cells per point were presented as the percentage of cells in the population that were stained TDT positive and thus had fragmented DNA.
oligonucleosome-sized DNA fragments (107). Therefore, the absence of DNA ladders is not sufficient evidence to rule out apoptosis as a mechanism of cell death. On closer examination, the TDT data for both HT-29 and BE cells showed a broadening of the peaks due to an increase in fluorescence at the later time points. This broadening was more prominent in HT-29 cells (Figure 13) than in BE cells (Figure 14). Although none of these peaks showed the clear separation and increase in fluorescence as observed in Colo320HSR cells, which possessed oligonucleosomal DNA fragments, their appearance was consistent with the possibility that DNA fragmentation in HT-29 and BE cells occurred at a higher order of chromatin organization (300 and 50 kbp). These results suggested that some apoptotic cells were present in populations of HT-29 and BE cells treated with HN2.

COLO320HSR, HT-29, AND BE CELLS UTILIZE APOPTOTIC AND NECROTIC MODES OF CELL DEATH TO VARYING DEGREES FOLLOWING EXPOSURE TO HN2

The morphological analysis of Colo320HSR, HT-29, and BE cells following HN2 exposure confirmed the presence of apoptotic cells in all three cell lines (Figure 16A, B, and C). Quantitation of viable, apoptotic, and necrotic cells showed that the induction of cell death in Colo320HSR cells was a rapid event and occurred exclusively via the apoptotic mechanism of cell death (Figure 17). These results
Figure 16. Photomicrographs of the morphological analysis of the time course of nitrogen mustard-induced cell death in human colon carcinoma cell lines.

Colo320HSR (A), HT-29 (B), and BE (C) cells were exposed to 6, 10, and 30 µM HN2, respectively, for 1 h and at various times following drug removal, cells were fixed, permeabilized, and stained with propidium iodide as described in the methods. Unattached HT-29 and BE cells from the later time points were added back to the population of attached cells for analysis. Nuclei were visualized by fluorescence microscopy (200X magnification). Nuclei from viable cells were small and evenly stained (control A, B, and C) while apoptotic nuclei showed the presence of condensed and fragmented chromatin (A, 96 h). Nuclei from necrotic cells were large and evenly stained (C, 96 h).
Colo320HSR

A
Figure 17. Quantitative time course of the morphological analysis of cell death in human colon carcinoma cell lines following exposure to equi-cytotoxic concentrations of nitrogen mustard.

Colo320HSR, HT-29, and BE cells were treated with 6, 10, and 30 µM HN2, respectively, for 1 h and then were fixed, permeabilized and stained with propidium iodide at various times following drug removal (see methods for details). Nuclear morphology was assessed by fluorescence microscopy at a magnification of 200X. The number of viable, apoptotic, and necrotic cells was determined by projecting the image of a photographic negative onto a grid and recording the number of cells in each category. Viable cells had a nuclear morphology characterized by a small size and evenly stained chromatin. A range of nuclear size was determined for control cells of each cell type to account for cell cycle phase-dependent changes in the size of the nucleus (generally 2-5 mm). Cells with evenly stained chromatin whose nuclei fell within this size range were considered to be viable cells. Cells with evenly stained nuclei that were larger than this size range were considered to be necrotic cells. Apoptotic cells were characterized by having nuclei that had condensed and fragmented chromatin. Any evenly stained material under 1 mm was considered to be an apoptotic body and was not scored as a cell. The data were expressed as the percentage of viable, apoptotic, and necrotic cells. Generally, 1200-3500 cells were scored for each time point.
corroborate both the viability and DNA fragmentation data which suggested that a rapid, apoptotic death was occurring in Colo320HSR cells following HN2 treatment. Morphological analysis of HT-29 cells treated with HN2 showed a loss in viability that was identical to the results seen in the FACS viability study. The non-viable fraction of HT-29 cells was equally divided between apoptotic and necrotic cells suggesting that neither mode of cell death was favored at the measured time points (Figure 17). Although apoptotic cells were present in the population of BE cells treated with HN2, the predominant mode of cell death appeared to be necrosis (Figure 17).

All three cell lines utilized the apoptotic and necrotic mechanisms of cell death to varying degrees. However, Colo320HSR cells treated with HN2 showed a rapid loss of viability, beginning as early as 24 h post treatment, that was mediated exclusively by an apoptotic mechanism of cell death. These results are consistent with a c-myc-enhanced induction of apoptosis in this cell line. The HT-29 and BE cell lines showed a gradual loss of cellular viability that was mediated by an equal combination of necrosis and apoptosis or almost exclusively by necrosis, respectively, following HN2 exposure. These results suggested that the occurrence of a c-myc-enhanced apoptosis was abrogated in HT-29 and BE cells despite an increase in c-myc protein in both cell lines following exposure to HN2. The overexpression of
c-myc in human tumor cell lines did not appear to be predictive for the enhanced onset of apoptosis following treatment with the DNA damaging agent, HN2. Therefore, I conclude that factors other than high levels of c-myc protein appeared to exist which could influence the enhancement of apoptosis in human tumor cell lines.

The fact that c-myc-enhanced apoptosis did not appear to occur in HT-29 and BE cells, despite the increase in c-myc protein levels, raised the question of whether the c-myc enhancement of apoptosis was being modulated by other gene products in these cell lines. Several gene products are known to regulate the c-myc enhancement of apoptosis and include the oncogenes c-abl, c-raf, ras, and bcl-2, and the tumor suppressor p53 (154,169,190,250,328). The Colo320HSR, HT-29, and BE cell lines did not express detectable mRNA for either c-abl, c-raf, rel, H-ras or K-ras, however, all three cell lines expressed mRNA for N-ras, with Colo320HSR cells showing the greatest level of expression followed by BE and HT-29 cells (Q. Dong, unpublished observations, 456). The regulation of both apoptosis and the c-myc enhancement of apoptosis by bcl-2 and p53 has been extensively studied. Therefore, studies were performed to rule out the involvement of these two gene products in the regulation of apoptosis in these cell lines following exposure to HN2.

The p53 tumor suppressor gene is mutated in 90% of human colon tumors (257). In some cell types, wild-type p53
protein is required for the utilization of the apoptotic pathway following exposure to various chemotherapeutic agents (173,316). In murine systems, studies have shown the following: an increase in wild-type p53 protein is sufficient for the induction of apoptosis (172); a post-translational increase in wild-type p53 protein mediates the c-myc enhancement of apoptosis (327); and the presence of mutant p53 can abrogate the enhancement of apoptosis by c-myc (328). The following hypothesis was tested: p53 protein regulates the c-myc enhancement of apoptosis in the Colo320HSR, HT-29, and BE human colon carcinoma cell lines.

CHARACTERIZATION OF THE P53 GENE AND ITS EXPRESSION IN THE COLO320HSR, HT-29, AND BE CELL LINES

The p53 tumor suppressor phenotype was characterized in the Colo320HSR, HT-29, and BE cell lines to rule out its involvement in the regulation of apoptosis following HN2 treatment. Based upon the previous conclusions and the information regarding p53, if p53 were involved in the regulation of apoptosis in these cell lines, then Colo320HSR cells would be predicted to express wild-type p53 protein, while HT-29 and BE cells would express the mutant form of the protein. The levels of p53 protein in Colo320HSR cells would also be expected to increase following exposure to HN2 while the levels of protein in HT-29 and BE cells should not change.
Figure 18. Southern, northern, and western blot analysis of p53 in the Colo320HSR, HT-29, and BE cell lines.

(A) Southern blot analysis of EcoRI and HindIII restriction endonuclease digested genomic DNA from human carcinoma cell lines. Ten µg of cut genomic DNA from each cell line was fractionated on a 0.5% agarose gel, Southern blotted and hybridized with a $^{32}$P-labeled 790 bp p53 cDNA probe. The size of each DNA fragment was shown on the left and was determined by comparison to a 1 kb DNA ladder which was included on the gel. (B) Northern blot analysis of p53 mRNA levels in human carcinoma cell lines. Total RNA (35 µg) from each cell line was fractionated on a 1% agarose gel, northern blotted, and hybridized with a $^{32}$P-labeled 790 bp p53 cDNA probe. The membrane was stripped and rehybridized with a histone 3.3 probe to assess RNA loading. Bands were sized based upon the relative positions of the 18S and 28S ribosomal RNA bands and quantitated on a Betagen Betascope 603 blot analyzer. (C) Western blot analysis of p53 protein levels in human cell lines. Whole cell protein extracts (3 X 10$^5$ cell equivalents) were fractionated on a 10% polyacrylamide gel, transferred to nitrocellulose and immunoblotted with an anti-p53 antibody as described in the methods. p53 protein levels were quantitated by densitometric analysis of autoradiographs.
Southern blot analysis of Colo320HSR, HT-29, and BE cells revealed no gross differences in p53 gene structure when compared to the HeLa cell line which is known to contain a wild-type p53 gene (325 and references within) (Figure 18A). No major differences in the expression of p53 mRNA in the three cell lines were detected when compared to HeLa cells by northern blot analysis (Figure 18B). Western blot analysis showed that p53 protein was grossly overexpressed in the Colo320HSR, HT-29, and BE cell lines when compared to the IMR-90 and HeLa cell lines, both of which contain wild-type p53 protein (Figure 18C). HL-60 cells were included in the analysis as the negative western blot control since this cell line is null for the p53 gene and does not express p53 protein. The wild-type p53 protein has a very short half life (approximately 15 min) (269) and is present in proliferating cells at low levels. The mutant forms of p53 protein tend to have longer half lives than the wild-type protein (several hours), and therefore, are overexpressed (287,288). These results suggested that the Colo320HSR, HT-29, and BE cell lines all expressed a mutant p53 protein.

**THE COLO320HSR, HT-29, AND BE CELL LINES EXPRESS A MUTANT FORM OF THE P53 PROTEIN**

The existence of mutant p53 protein in the three cell lines was confirmed by immunoprecipitation analysis. Immunoprecipitation with an antibody that recognized both the
Figure 19. Immunoprecipitation analysis of p53 protein status (wild-type versus mutant) in the Colo320HSR, HT-29, and BE human colon carcinoma cell lines.

p53 protein was immunoprecipitated from $1 \times 10^6$ cells as described in the methods using either the Ab-6 or Ab-3 monoclonal antibody. The Ab-6 antibody recognized both the wild-type and mutant forms of the p53 protein (top panel) while the Ab-3 antibody recognized only the mutant form of the p53 protein (bottom panel). Extracts from each cell line were immunoprecipitated with a specific Ab (Ab-6 or Ab-3) and an isotype control Ab ($\text{IgG}_2\alpha$ (UPC10) for Ab-6 and $\text{IgG}_1$ (MOPC21) for Ab-3). The HL-60 cell line had a deleted p53 gene and served as a negative control for the presence of p53 protein. The HeLa cell line had a wild-type p53 gene and served as the positive control for the presence of wild-type p53 protein. The data shown were representative of three independent experiments.
Colo320HSR (A), HT-29 (B), and BE (C) cells were exposed to 6, 10, and 30 \( \mu \text{M} \) HN2, respectively, for 1 h and whole cell protein extracts were isolated at various times following drug removal. Unattached HT-29 and BE cells were added back to the attached population of cells for analysis. Extracts from 5 \( \times 10^5 \) cell equivalents (Colo320HSR) or 2.5 \( \times 10^5 \) cell equivalents (HT-29 and BE) were subjected to SDS-PAGE followed by western blot analysis. p53 protein was sized by comparison to protein molecular weight standards. Controls (C) were run at each time point to reduce the variability resulting from uneven protein transfer across the membrane. The transfer efficiency was monitored by coomassie staining of post-transferred gels. The membranes used for this study were the same membranes used in the c-myc study (Figure 6). After detection of c-myc protein, the membranes were stripped of Ab as described in the methods and re-immunoblotted with the Ab-6 anti-human p53 Ab (2 \( \mu \text{g/ml} \)).
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p53
Figure 21. Time course analysis of the effect of equi-cytotoxic concentrations of nitrogen mustard on p53 protein levels in human colon carcinoma cell lines.

A graphic representation of the previous figure showing the time course changes in p53 protein levels in the Colo320HSR, HT-29, and BE cell lines following exposure to 6, 10, and 30 µM HN2, respectively. Protein levels were quantitated by densitometric analysis of autoradiographs and presented as the percent change relative to time point controls. Data points represented the mean ± SEM of at least three independent experiments.
wild-type and mutant form of the human p53 protein showed that the Colo320HSR, HT-29, BE, and HeLa cell lines all contained p53 protein, however, levels in the three colon carcinoma cell lines were greatly overexpressed relative to the HeLa cell line. Immunoprecipitation with an antibody that recognized only the mutant form of the human p53 protein showed that mutant p53 was present in the three colon carcinoma cell lines but not in the HeLa cell line (Figure 19). The HL-60 cell line is null for the p53 gene and therefore expressed no p53 protein. These results confirmed the presence of the mutant form of the p53 protein in the Colo320HSR, HT-29, and BE cell lines.

**NITROGEN MUSTARD DOES NOT AFFECT P53 PROTEIN EXPRESSION IN THE COLO320HSR, HT-29, AND BE CELL LINES**

The effect of HN2 on p53 protein expression was determined by western blot analysis. These studies showed that p53 protein levels were essentially unchanged in Colo320HSR, HT-29, or BE cells treated with HN2 (Figure 20A, B, and C). Quantitation of these data showed that p53 protein levels in treated cells were no different than controls, even at the 72 and 96 h time points (Figure 21).

The mutant status and the lack of induction of the p53 protein following HN2 treatment in the Colo320HSR cell line were inconsistent with the outcomes predicted by the working hypothesis. The fact that an enhanced induction of apoptosis
occurred in Colo320HSR cells and that apoptosis still occurred in HT-29 and BE cells, despite the presence of a mutant p53 protein in all three cell lines, suggested that the induction of apoptosis in the Colo320HSR, and possibly the HT-29 and BE cell lines, following HN2 exposure was regulated in a wild-type p53-independent manner.

Ectopic overexpression of bcl-2 protein in many cell types has been shown to block or delay the onset of apoptosis induced by a variety of physiological stimuli and chemotherapeutic agents (164,165,234-238). Bcl-2 has also been shown to be capable of blocking the c-myc enhancement of apoptosis and can cooperate with c-myc in tumorigenesis (190,192). Therefore, the following hypothesis was tested: bcl-2 regulates the c-myc enhancement of apoptosis in a subset of human colon carcinoma cell lines following HN2 exposure.

**BCL-2 PROTEIN IS NOT OVEREXPRESSED IN THE COLO320HSR, HT-29, OR BE CELL LINES**

If bcl-2 were involved in the regulation of apoptosis in these cell lines, the simplest explanation would be that bcl-2 protein is overexpressed in those cell lines which are refractory to the c-myc enhancement of apoptosis. Based upon this logic, the HT-29 and BE cell lines would be predicted to have high levels of bcl-2 protein. This prediction was tested by characterizing bcl-2 gene structure, mRNA
Figure 22. Analysis of the bcl-2 oncogene and bcl-2 mRNA and protein levels in the Colo320HSR, HT-29, and BE human colon carcinoma cell lines.

(A) Southern blot analysis of EcoRI and BamHI restriction endonuclease digested genomic DNA from the Colo320HSR, HT-29, and BE cell lines. Ten µg of cut genomic DNA from each cell line was fractionated on a 0.5% agarose gel, Southern blotted, and hybridized with a 32P-labeled 447 bp bcl-2 cDNA probe. DNA fragments were sized by comparison to a 1 kb DNA ladder as indicated to the left of the photograph. (B) Reverse transcription-PCR analysis of bcl-2 mRNA expression in human colon carcinoma cell lines. One µg of total RNA from each cell line was reverse transcribed as described in the methods. One fourth of the RT reaction was used for PCR amplification of O6 methyl guanine DNA methyl transferase (MGMT) cDNA (730 bp) which served as a positive control for the reverse transcription reaction. The Colo320HSR and BE cells were classical Mer- (Methylation Repair deficient) cell lines and did not express detectable levels of MGMT mRNA, while the HT-29 cells were classically Mer+ and showed a high level of MGMT mRNA expression. The lanes marked + or - under the MGMT section were PCR reactions using the MGMT primer pair with or without a MGMT 730 bp template added, respectively. These reactions were the negative and positive PCR controls. Three fourths of the RT product was used in a PCR amplification reaction using a bcl-2 primer pair (see methods) which amplified a 447 bp exon 1/exon 2 cDNA fragment. The lane marked - under the bcl-2 section was the negative PCR control using the bcl-2 primer pair with no template added. The HL-60 cell line was included as a positive control for the presence of bcl-2 mRNA. A 123 bp DNA ladder was included in the left most lane to size the PCR amplification products after they were electrophoresed through a 1.5% agarose gel. (C) Western blot analysis of bcl-2 protein levels in the Colo320HSR, HT-29, and BE cell lines. Whole cell protein extracts from the Colo320HSR, HT-29, BE (5 X 10^5 cell equivalents) and LM-EBV (3 X 10^5 cell equivalents) cell lines were fractionated on a 12.5% SDS-polyacrylamide gel, transferred to nitrocellulose and immunoblotted with an anti-bcl-2 Ab (5 µg/ml) as described in the methods. The 26 kDa bcl-2 protein was sized by comparison to protein molecular weight markers and quantitated by densitometric analysis of autoradiographs.
expression, and protein expression in the Colo320HSR, HT-29, and BE cell lines. Southern blot analysis showed no gross differences in gene structure between the three cell lines. Two restriction fragment length polymorphisms existed for the bcl-2 gene when digested with the restriction endonuclease, EcoRI. Colo320HSR cells were homozygous for one polymorphism while the HT-29 and BE cells were homozygous for the other polymorphism. The HL-60 cell line was heterozygous for these two polymorphisms (Figure 22A). Reverse transcription-PCR analysis revealed the presence of bcl-2 mRNA in the HL-60 (positive control cell line), Colo320HSR, and BE cell lines. Bcl-2 mRNA was present in HT-29 cells, but was barely detectable (Figure 22B). Immunoblot analysis showed that none of the three cell lines grossly overexpressed bcl-2 protein (Figure 22C). The LM-EBV cell line was included in this analysis because it overexpressed bcl-2 protein. Bcl-2 protein was present in both Colo320HSR and BE cells but was undetectable in HT-29 cells. These results showed that bcl-2 protein was not constitutively overexpressed in any of these three colon carcinoma cell lines.

BCL-2 PROTEIN LEVELS ARE ELEVATED IN THE COLO320HSR AND BE CELL LINES FOLLOWING EXPOSURE TO HN2

The lack of any constitutive overexpression of bcl-2 protein in these colon carcinoma cell lines prompted the search for a more complex explanation of how bcl-2 was
Figure 23. Time course analysis of the effect of nitrogen mustard on bcl-2 protein levels in the Colo320HSR and BE human colon carcinoma cell lines.

Colo320HSR (A) and BE (B) cells were exposed to 6 and 30 µM HN2, respectively, for 1 h and whole cell protein extracts were isolated at various times following drug removal. Both unattached and attached BE cells were combined for analysis. Extracts from Colo320HSR (4 X 10^5 cell equivalents) and BE (2.5 X 10^5 cell equivalents) cells were fractionated on 12.5% SDS-polyacrylamide gels, transferred to nitrocellulose and immunoblotted with an anti-bcl-2 Ab as described in the methods. Controls (C) were run at each time point to reduce the variability resulting from uneven protein transfer across the membrane. Transfer efficiency was monitored by coomassie staining of post-transferred gels. LM-EBV cell extracts were included in the left most lane to serve as a positive control for the presence of bcl-2 protein. (C) Quantitative analysis of changes in bcl-2 protein levels in Colo320HSR and BE cells following HN2 treatment. Bcl-2 protein levels were quantitated by densitometric analysis of autoradiographs and expressed as the percent change relative to controls. The data were presented as the mean ± SEM of at least three independent experiments. Data points existed where the error bars fell within the symbol.
possibly regulating apoptosis. A plausible explanation could be that HN2 induced the expression of bcl-2 protein, therefore, bcl-2 protein levels would be predicted to increase in HT-29 and BE cells after HN2 treatment. This hypothesis was tested by analyzing bcl-2 protein levels in Colo320HSR, HT-29, and BE cells at various times following HN2 exposure.

The results from these studies showed no apparent increase in bcl-2 protein levels in HT-29 cells (data not shown). Treatment of both Colo320HSR and BE cells with HN2 resulted in an increase in the level of bcl-2 protein at various times following drug removal (Figure 23A and B, respectively). Bcl-2 protein levels were increased approximately 3-4 fold in Colo320HSR cells from 12 to 72 h following drug treatment (Figure 23C). BE cells treated with HN2 showed an initial decrease (approximately 50%) in bcl-2 protein levels through 24 h. The decrease in bcl-2 protein was followed by a large induction from 48-96 h, with a maximum increase of 12-16 fold at 72 h (Figure 23C).

Endogenous bcl-2 did not appear to regulate the induction of apoptosis in the Colo320HSR and HT-29 cell lines following HN2 exposure since bcl-2 was not greatly induced and both cell lines underwent apoptosis. The large induction of bcl-2 protein in BE cells occurred from 48-96 h and previous results showed that the viability of BE cells following HN2 exposure was maintained at 50% from 96 h out to
seven days and that the level of apoptotic events were low in this cell line. Taken together, the results for BE cells were consistent with the possibility that bcl-2 was delaying the death of these cells. Other factors known to be involved in the bcl-2 system (bcl-x, bax, bad, and bag-1) were not examined in these studies. Further studies need to be performed to clarify the role of bcl-2 in BE cells.

The results from studies on p53 and bcl-2 were inconsistent with the involvement of these gene products in the abrogation of c-myc-enhanced apoptosis and suggested that other abrogating mechanisms existed in these cell lines. Therefore, members of the myc transcription factor network were investigated to determine their involvement, if any, in the regulation of c-myc-enhanced apoptosis. c-Myc belongs to a transcription factor network which includes its only known binding partner, max (414). c-Myc and max form heterodimers via their complimentary helix-loop-helix/leucine zipper (HLH/LZ) regions (414). Dimerization with max is required for the dose-dependent regulation of apoptosis which is thought to involve the specific binding of DNA and transactivation of genes (426,432). The level of c-myc/max heterodimers formed in a cell is determined by the ratio of c-myc and mad proteins and in normal cells is thought not to be limited by max protein (415). Mad heterodimerizes with max and antagonizes the transcriptional activation abilities of c-myc/max dimers by competitively binding to the same
consensus E-box motif (415).

Current serum starvation models of c-myc-enhanced apoptosis suggest that constitutive expression of c-myc leads to high levels of c-myc/max dimers which transactivate genes involved in the regulation of apoptosis (432). The apoptotic regulatory machinery set into place by c-myc expression is thought to be present prior to the block to replication imparted by serum starvation, but its activation is inhibited (432). However, data from the same and other studies have shown that the activation of a chimeric c-myc/ER protein in serum starved, growth arrested cells resulted in the rapid induction of apoptosis suggesting that the apoptotic regulatory system affected by c-myc expression can be functionally induced after the replication block is in place (327,432). The following working hypothesis was proposed to address the role of myc transcription factor network proteins in the regulation of c-myc-enhanced apoptosis: Genes, other than c-myc, which belong to the myc transcription factor network are capable of modulating c-myc-enhanced apoptosis in human tumor cell lines which overexpress c-myc protein.

Initial studies were based upon the assumption that the apoptotic regulatory machinery was already in place at the time of HN2 exposure as a result of c-myc overexpression. The c-myc enhancement of apoptosis has been shown to occur in a manner that is dependent upon the dose of c-myc (431,432). Therefore, c-myc-enhanced apoptosis would be predicted to
Figure 24. Comparison of the levels of p21/22 max protein, c-myc/max protein heterodimers, and p35 mad protein in the Colo320HSR, HT-29, and BE human colon carcinoma cell lines. (A) Coprecipitation of c-myc and max proteins from 5 × 10^6 Colo320HSR, HT-29, and BE cells using an anti-max rabbit polyclonal Ab as described in the Methods. Immunoprecipitated proteins were fractionated by SDS-PAGE and transferred to nitrocellulose. The membrane was cut in half and the top portion (≥ 45 kDa) was immunoblotted with an anti-myc polyclonal Ab while the bottom half (< 45 kDa) was immunoblotted with an anti-max polyclonal Ab. The p21/22 max bands were indicative of the total amount of immunoprecipitable max protein in each cell line. The p64 c-myc band did not reflect the total cellular level of c-myc protein but rather was indicative of the amount of c-myc which was coprecipitable with max and thus represented the level of c-myc/max heterodimers in each cell line. The figure shown is representative of three independent experiments. Two to four independent cell isolations of each cell line were immunoprecipitated in each experiment. (B) Immunoblot analysis of p35 mad protein levels in the Colo320HSR, HT-29, and BE cell lines. Whole cell lysates from 3 × 10^5 cell equivalents were fractionated on a 12.5% SDS-polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with an anti-mad rabbit polyclonal Ab as described in the methods. The mad protein was previously characterized and shown to migrate as a 35 kDa doublet. The relative positions of protein molecular weight size markers were shown to the left of the gel. Two independent protein isolations of each cell line were shown and were representative of at least four separate experiments. Antigen peptide blocking of the anti-mad Ab revealed the presence of several specific protein bands which included the 35 kDa doublet and other bands at 24 kDa, 44 kDa and 55 kDa.
occur more readily in cells that have a higher ratio of c-myc/max to mad/max heterodimers and max/max homodimers. Previous results suggest that apoptosis is enhanced in the Colo320HSR cell line which expresses high levels of c-myc protein, but not in the HT-29 or BE cell lines. Therefore, Colo320HSR cells would be expected to have the highest ratio of c-myc/max to mad/max heterodimers and max/max homodimers compared to HT-29 and BE cells.

CHARACTERIZATION OF THE MYC TRANSCRIPTION FACTOR NETWORK IN COLO320HSR, HT-29, AND BE HUMAN COLON CARCINOMA CELLS

To test this prediction, the levels of c-myc, max, mad, and c-myc/max heterodimers were compared between the Colo320HSR, HT-29, and BE cell lines. c-Myc protein levels were approximately 7 fold higher in Colo320HSR cells compared to both HT-29 and BE cells (Figure 2B and C). Max protein levels were 2-3 fold and 5-7 fold greater in BE cells relative to Colo320HSR and HT-29 cells, respectively (Figure 24A). The levels of mad protein in the HT-29 and BE cell lines were similar and approximately 3 fold greater relative to Colo320HSR cells (Figure 24B). The levels of c-myc/max dimers were 2 and 4 fold higher in Colo320HSR cells relative to BE and HT-29 cells, respectively (Figure 24A). The level of mad/max dimers were not determined in these studies.

These results and others (data not shown, and Figure 25A) provided indirect evidence that max levels were limiting
in the formation of c-myc/max dimers in the Colo320HSR cell line and perhaps HT-29 cells, not c-myc, as was the case in BE cells. Therefore, the difference in c-myc/max dimer levels between the cell lines was less than would have been predicted based upon c-myc protein levels alone. The question arises about whether a 2 fold difference in c-myc/max dimer levels is sufficient to account for the differences observed in the outcome of c-myc-enhanced apoptosis in these cell lines. The observation that all available max protein was dimerized with c-myc, and that mad levels were low, was consistent with the possibility that mad/max and max/max dimers were not present in Colo320HSR cells. Max protein was in slight excess over c-myc in HT-29 cells, and in large excess over c-myc in BE cells. Mad protein levels were relatively high in both HT-29 and BE cells. These results were consistent with the possibility that mad/max dimers existed in HT-29 cells, and that both mad/max and max/max dimers existed in BE cells, and were antagonistic to the function of c-myc/max dimers. Therefore, in comparison to BE and HT-29 cells, the transcriptional activation abilities of c-myc/max dimers in Colo320HSR cells would actually be greater than the 2 or 4 fold difference predicted by dimer levels. These results were consistent with the possibility that the apoptotic machinery was in place prior to HN2 exposure and the occurrence of c-myc-enhanced apoptosis in these cell lines was determined by the
differences in the transcriptional activation abilities of c-myc/max dimers. However, the lack of direct evidence for the presence of mad/max dimers in these cell lines leaves these results open to alternative interpretations.

If the differences in the levels of functional c-myc/max dimers was not sufficient to explain why apoptosis was enhanced in Colo320HSR cells but not HT-29 or BE cells, then another possibility was that the myc network was being modulated in response to HN2 treatment. This assumption predicts that HN2 would induce changes in the expression of genes belonging to the myc network and alter the levels of c-myc/max (and possibly mad/max) dimers. Therefore, cells which undergo enhanced apoptosis would have increased levels of c-myc/max dimers following HN2 treatment, while cells which failed to enhance the induction of apoptosis would show either a decrease in c-myc/max dimers and/or an increase in mad/max (max/max) dimers. Experiments were performed to rule out the possibility that the myc network was being altered following HN2 exposure.

THE INCREASE IN C-MYC/MAX HETERODIMER LEVELS IN COLO320HSR CELLS FOLLOWING HN2 EXPOSURE OCCURS PRIOR TO THE ONSET OF APOPTOSIS AND CORRELATES WITH THE INDUCTION OF P21/22 MAX

Coimmunoprecipitation time course studies using an anti-human p21/22 max Ab showed that max protein was induced by HN2 (Figure 25A). The initial induction of p21/22 max was
Figure 25. Changes in myc transcription factor network protein levels (c-myc, max, and c-myc/max heterodimers) in Colo320HSR human colon carcinoma cells following exposure to nitrogen mustard.

(A) Coimmunoprecipitation analysis of the effect of nitrogen mustard on max protein and coprecipitable c-myc protein (c-myc/max heterodimers) levels in Colo320HSR cells. Cells were treated with 6 µM HN2 for 1 h. Whole cell protein extracts were isolated from 5 X 10^6 cells at various times following drug removal using a low stringency immunoprecipitation lysis buffer. An anti-max rabbit polyclonal Ab was used to coimmunoprecipitate c-myc and max proteins as described in the methods. Immunoprecipitated proteins were then fractionated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose. The membrane was cut in half and the top part (≥ 45 kDa) was immunoblotted with an anti-myc polyclonal Ab while the bottom half (≤ 45 kDa) was immunoblotted with the anti-max polyclonal Ab. Proteins on both halves of the membrane were detected by enhanced chemiluminescence, after which the membrane halves were realigned and exposed to autoradiographic film. The p64 c-myc protein was coprecipitated along with the p21/22 human max protein. The band below the p64 c-myc band was the rabbit IgG heavy chain from the immunoprecipitation step which was recognized by the anti-rabbit IgG secondary Ab used in the immunoblotting procedure. The specificity of the myc and max bands was ascertained by immunoprecipitating a sample with a peptide antigen-blocked anti-max Ab. Briefly, 0.1 µg of anti-max Ab was incubated with 1 µg of max peptide antigen for 1 h at room temperature and then used to coimmunoprecipitate c-myc and max as previously described.

(B) Time course analysis of the changes in c-myc, p21/22 max, and c-myc/max heterodimer levels in Colo320HSR cells following exposure to HN2. A graphical representation of the data from figure 6A (c-myc protein levels) and from the coimmunoprecipitation studies presented above. Protein levels were quantitated by densitometric analysis of autoradiographs and presented as the percent change relative to the control. Data points for coimmunoprecipitation studies represented the mean ± SEM of four independent experiments. Error bars fell within the symbol on some data points.
A

TIME (h)

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p84  
c-myc

p21/22  
max

B

PROTEIN LEVELS (% OF CONTROL)

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TIME (h)

0  24  48  72
0  12  24  36  48

max

MYC:MAX

DIMERS
approximately 7 fold by 12 h following drug removal and remained above control levels through 48 h. A maximal 22 fold induction of max protein was observed at the 24 h time point (Figure 25B). Coprecipitable levels of c-myc protein (indicative of c-myc/max dimers) also increased following nitrogen mustard treatment (Figure 25A). The levels of c-myc/max dimers (coprecipitable c-myc) increased approximately 3 fold at 18 h following drug removal and were maximally induced by approximately 5 fold at 24 h (Figure 25B). Following HN2 exposure, c-myc protein levels did not change through 24 h and then were reduced by 52% and 69% at 48 and 72 h, respectively (Figure 25B).

These results provided indirect evidence that max protein was limiting in Colo320HSR cells, not c-myc. These results also showed that the myc network was modulated by nitrogen mustard prior to the induction of apoptosis in a manner that was consistent with the possibility that the increase in c-myc/max dimers was involved in the enhancement of apoptosis in the Colo320HSR cell line.

**THE DECREASES IN C-MYC/MAX HETERODIMER LEVELS IN HT-29 AND BE CELLS FOLLOWING HN2 EXPOSURE ARE CONSISTENT WITH THE LACK OF AN ENHANCED INDUCTION OF APOPTOSIS AND OCCURS DESPITE AN INCREASE IN BOTH C-MYC AND MAX PROTEINS**

Initial studies in HT-29 cells showed that c-myc protein levels increased over a 96 h time course following
Figure 26. Changes in myc transcription factor network protein levels (c-myc, max, and c-myc/max heterodimers) in HT-29 human colon carcinoma cells following exposure to nitrogen mustard.

(A) Coimmunoprecipitation analysis of the effect of nitrogen mustard on max protein and coprecipitable c-myc protein (c-myc/max heterodimers) levels in HT-29 cells. Cells were treated with 10 \( \mu \text{M} \) HN2 for 1 h. Whole cell protein extracts were isolated from 5 \( \times \) 10^6 cells at various times following drug removal using a low stringency immunoprecipitation lysis buffer. Unattached cells from the later times points were included in the analysis. An anti-max rabbit polyclonal Ab was used to coimmunoprecipitate c-myc and max proteins as described in the methods. Immunoprecipitated proteins were then fractionated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose. The membrane was cut in half and the top part (≥ 45 kDa) was immunoblotted with an anti-myc polyclonal Ab while the bottom half (≤ 45 kDa) was immunoblotted with the anti-max polyclonal Ab. Proteins on both halves of the membrane were detected by enhanced chemiluminescence, after which the membrane halves were realigned and exposed to autoradiographic film. The p64 c-myc protein was coprecipitated along with the p21/22 human max protein. The band below the p64 c-myc band was the rabbit IgG heavy chain from the immunoprecipitation step which was recognized by the anti-rabbit IgG secondary Ab used in the immunoblotting procedure. The specificity of the c-myc and max bands was ascertained by immunoprecipitating a sample with a peptide antigen-blocked anti-max Ab. Briefly, 0.1 \( \mu \text{g} \) of anti-max Ab was incubated with 1 \( \mu \text{g} \) of max peptide antigen for 1 h at room temperature and then used to coimmunoprecipitate c-myc and max as previously described. (B) Time course analysis of the changes in c-myc, p21/22 max, and c-myc/max heterodimer levels in HT-29 cells following exposure to HN2. A graphical representation of the data from figure 6B (c-myc protein levels) and from the coimmunoprecipitation studies presented above. Protein levels were quantitated by densitometric analysis of autoradiographs and presented as the percent change relative to the control. Data points for coimmunoprecipitation studies represented the mean ± SEM of four independent experiments. Error bars fell within the symbol on some data points.
Figure 27. Changes in myc transcription factor network protein levels (c-myc, max, and c-myc/max heterodimers) in BE human colon carcinoma cells following exposure to nitrogen mustard.

(A) Coimmunoprecipitation analysis of the effect of nitrogen mustard on max protein and coprecipitable c-myc protein (c-myc/max heterodimers) levels in BE cells. Cells were treated with 30 µM HN2 for 1 h. Whole cell protein extracts were isolated from 5 X 10^6 cells at various times following drug removal using a low stringency immunoprecipitation lysis buffer. Unattached cells from the later time points were included in the analysis. An anti-max rabbit polyclonal Ab was used to coimmunoprecipitate c-myc and max proteins as described in the methods. Immunoprecipitated proteins were then fractionated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose. The membrane was cut in half and the top part (≥ 45 kDa) was immunoblotted with an anti-max polyclonal Ab while the bottom half (≤ 45 kDa) was immunoblotted with the anti-max polyclonal Ab. Proteins on both halves of the membrane were detected by enhanced chemiluminescence, after which the membrane halves were realigned and exposed to autoradiographic film. The p64 c-myc protein was coprecipitated along with the p21/22 human max protein. The band below the p64 c-myc band was the rabbit IgG heavy chain from the immunoprecipitation step which was recognized by the anti-rabbit IgG secondary Ab used in the immunoblotting procedure. The specificity of the c-myc and max bands was ascertained by immunoprecipitating a sample with a peptide antigen-blocked anti-max Ab. Briefly, 0.1 µg of anti-max Ab was incubated with 1 µg of max peptide antigen for 1 h at room temperature and then used to coimmunoprecipitate c-myc and max as previously described. (B) A shorter exposure of the p21/22 max band emphasizing the subtle changes in max levels. (C) Time course analysis of the changes in c-myc, p21/22 max, and c-myc/max heterodimer levels in BE cells following exposure to HN2. A graphical representation of the data from figure 6C (c-myc protein levels) and from the coimmunoprecipitation studies presented above. Protein levels were quantitated by densitometric analysis of autoradiographs and presented as the percent change relative to the control. Data points for coimmunoprecipitation studies represented the mean ± SEM of four independent experiments. Error bars fell within the symbol on some data points.
HN2 exposure (Figure 26B). Coimmunoprecipitation studies in HT-29 cells showed that p21/22 max was also induced by HN2 (Figure 26A). Max protein levels were induced as early as 15 h following drug removal and remained above control levels through 72 h, returning to control values by 96 h. Peak induction of max protein occurred at 24 and 48 h (almost 3 fold) (Figure 26B). c-Myc/max dimer levels never increased above control levels following HN2 exposure, despite the increase in both c-myc and max protein levels (Figure 26A). Dimer levels were decreased by approximately 44% at the 15 h time point, returned to control levels by 24 h, and then progressively declined through 96 h (Figure 26B). The decreases in c-myc/max dimer levels at the 48, 72, and 96 h time points were approximately 40%, 54%, and 75%, respectively.

c-Myc protein levels were also increased in BE cells over a 96 h time course following exposure to HN2 (Figure 27C). Max protein levels showed only a small induction (< 2 fold) in BE cells following HN2 exposure (Figure 27B and C), however max protein was previously shown to be in vast excess over c-myc protein. The level of c-myc/max dimers in HN2-treated BE cells never increased above control values (Figure 27A and C). Dimer levels were reduced approximately 21% at the 15 h time point, returned to control levels by 24 h, and then progressively declined over the remaining time course. Dimer levels were reduced by approximately 31%, 60%, and 83%
at the 48, 72, and 96 h time points, respectively (Figure 27C).

The results from the studies of HT-29 and BE cells showed that the formation of c-myc/max dimers was a complex process that was regulated by more than just the mere presence of c-myc and max proteins. The c-myc/max dimer levels in both of these cell lines were never increased above control levels despite conditions in both cell types that would have predicted an increase in dimers. The decrease in c-myc/max dimer levels in both HT-29 and BE cells occurred before the loss of viability and appearance of apoptotic events in either cell line. These results were consistent with the possibility that the decrease in c-myc/max dimers following HN2 exposure was involved in preventing the enhancement of apoptosis in the HT-29 and BE cell lines.

The results from the myc network studies provided support for the possibility that the pretreatment status of the myc network in Colo320HSR, HT-29, and BE cells was sufficient to account for the differences observed in the c-myc enhancement of apoptosis after HN2 treatment. These results indicated that enhanced apoptosis may not be accurately predicted by the simple comparison of c-myc protein levels. The results from these studies also showed that the myc transcription factor network was modulated by exposure to nitrogen mustard, and were consistent with the possibility that the c-myc enhancement of apoptosis in human
Figure 28. The relative changes in c-myc/max heterodimer levels in the Colo320HSR, HT-29, and BE human colon carcinoma cell lines following exposure to nitrogen mustard.

A time course analysis of the relative changes in c-myc/max heterodimer levels in three human colon carcinoma cell lines following treatment with equi-cytotoxic concentrations of HN2. The data were taken from Figures 25-27 and were expressed as the percent change of control relative to HT-29 cells. The relative control levels of heterodimers in the three cell lines were determined from the results presented in Figure 24A. The data were presented as the mean ± SEM of four independent experiments. Error bars fell within the symbol on some data points. Note the break in the y-axis.
tumor cells was determined, in part, by the response of the myc transcription factor network to chemotherapeutic agent exposure. This possibility was difficult to refute when the changes in c-myc/max dimer levels in Colo320HSR, HT-29, and BE cells following HN2 exposure were graphed relative to the pretreatment dimer levels in HT-29 cells. These results demonstrated that, although pretreatment levels of c-myc/max dimers in Colo320HSR cells were 4 fold higher than in HT-29 cells, this relative difference was approximately 19-20 fold higher in Colo320HSR cells by 24 h after treatment with HN2 (Figure 28). The possibility that these HN2-induced changes in the myc transcription factor network were positively and negatively influencing the c-myc enhancement of apoptosis in these cell lines was strongly supported by the dose-dependent nature of c-myc-enhanced apoptosis. Evidence supporting both assumptions was presented, and the c-myc enhancement of apoptosis may be influenced by both factors. These results showed that max was induced in response to HN2 in a cell type-dependent manner. Taken together, these studies provided evidence supporting the hypothesis that genes, other than c-myc, which belong to the myc transcription factor network, were capable of regulating the c-myc enhancement of apoptosis in human tumor cell lines.

**ANTISENSE C-MYC STUDIES**

The modulation of c-myc protein by antisense ODNs
directed against c-myc mRNA was attempted in Colo320HSR cells to directly address the role of c-myc/max dimer increases in the enhancement of apoptosis following HN2 treatment. The goal of these studies was to prevent the HN2-induced increase in c-myc/max dimers by treatment of cells with antisense ODNs targeted against c-myc. The prevention of dimer increases would be expected to inhibit or delay the onset of apoptosis in Colo320HSR cells following HN2 exposure if these newly formed dimers were involved in the enhancement of apoptosis.

Antisense molecules were delivered to Colo320HSR cells by various methods. However, consistent reductions in c-myc protein could not be achieved. One antigene and 2 antisense ODNs (unmodified) targeted to different regions of the c-myc gene or mRNA, respectively, were used in these preliminary studies along with a phosphorothioated antisense ODN. The ODN's were delivered to either untreated cells or cells whose membranes were made permeable. ODNs delivered to non-permiabilized cells were added directly to culture media which contained serum (5%, 10%, or 15%) that was either DNase inactivated or not. Concentration-response, along with both short and long term time course studies were done with the ODNs using the various conditions previously described. Long term time course studies were done in which antisense molecules were added to the culture media every 12 or 24 h. None of these attempts at antisense modulation consistently reduced c-myc protein levels in Colo320HSR cells. Studies
involving the antisense modulation of c-myc were therefore dropped, because of the potential difficulties in interpreting the data obtained from such experiments.
CHAPTER 4
DISCUSSION

This dissertation attempts to clarify the involvement of the c-myc oncogene in bifunctional alkylating agent-induced cytotoxicity in human colon carcinoma cell lines. The initial investigations were performed prior to the discovery of the role of c-myc in the enhancement of apoptosis and were based upon the known role of c-myc in the regulation of cellular proliferation. The remaining studies dealt with the apoptotic functions of c-myc and genes which were thought to be capable of regulating this function. The studies in this dissertation were not intended to "close the book" on this area of research, an accomplishment that literally hundreds of studies have yet to achieve. The purpose of this document is simply to make a small, yet hopefully meaningful, contribution to the field and to provide further insight into how human tumor cells respond to chemotherapeutic agent exposure and ultimately survive or die.

To this end, these data show that the cytotoxic effects of the bifunctional alkylating agent, nitrogen mustard, were not mediated by specific reductions in c-myc expression in three human colon carcinoma cell lines. Therefore, these
data do not support the hypothesis that chemotherapeutic agents exert their anti-neoplastic activities by inhibiting cellular proliferation via the specific down-regulation of critical cellular oncogene expression. The results from the remaining studies are consistent with a role for c-myc in the enhancement of apoptosis in human tumor cells exposed to chemotherapeutic agents and describes a novel mechanism by which c-myc-enhanced apoptosis may be regulated in human colon carcinoma cell lines exposed to HN2.

ANTI-NEOPLASTIC ACTIVITY OF NITROGEN MUSTARD AND EFFECTS ON C-MYC EXPRESSION

The alkylating agents are clinically effective and are incorporated into many of the combination-chemotherapeutic regimens used in the fight against human cancers (1,2). Although the crosslinking of DNA appears to be important, the mechanism by which these drugs exert their anti-neoplastic activity remains to be fully elucidated (15,20). One hypothesis suggests that these drugs can interact with the DNA of activated oncogenes and inhibit their expression, thus, reversing the tumorigenic potential of the cell (12). One aim of this dissertation was to investigate this hypothesis, that nitrogen mustard exerts its anti-neoplastic activity in human tumor cell lines by inhibiting the expression of the critical cellular oncogene, c-myc.

Three human colon carcinoma cell lines were used
throughout these studies and were initially characterized with respect to c-myc gene structure and expression. The Colo320HSR cell line contains 16-33 copies of the c-myc gene, which is both amplified and translocated (457). The results from these studies confirmed the presence of a highly amplified (approximately 26-30 fold) and rearranged c-myc gene in Colo320HSR cells while HT-29 and BE cells possessed a non-amplified, non-rearranged c-myc gene (Figure 1A and B). The expression level of c-myc mRNA did not exactly correlate with the number of gene copies, nor did c-myc protein levels exactly correlate with mRNA levels when compared between cell lines. The slight discrepancy between mRNA and protein expression may be explained by the fact that protein levels were compared on a cell equivalent basis, while mRNA expression was compared based upon µg of total RNA isolated. c-Myc protein was overexpressed in all three cell lines when compared to the normal human fibroblast cell line, IMR-90 (data not shown). The overexpression of c-myc in human tumors is common and frequently occurs without the presence of an amplified or rearranged c-myc locus (458,459).

Nitrogen mustard down-regulated the expression of c-myc mRNA in all three cell lines in a concentration and time-dependent manner following drug exposure (Figure 4A and B). Studies utilizing an equi-molar concentration of HN2 showed that the level of c-myc mRNA inhibition was correlated with the degree of growth inhibition produced by the drug (Figure
5). Despite the effects seen at the mRNA level, c-myc protein expression was either unchanged through 24 h (Colo320HSR) or induced 2.5 and 4 fold in HT-29 and BE cells, respectively, over a 96 h time course (Figure 7). Finally, the anti-clonogenic effects of HN2 on these human tumor cell lines were shown to be mediated by a loss of cellular viability (Figure 8) which would not have been expected to occur if the down-regulation of c-myc expression was mediating the growth inhibition, since the reduction of c-myc expression is not cytotoxic to cells.

Several studies have shown that c-myc mRNA expression is down-regulated following exposure to various anti-cancer agents, including bifunctional alkylating agents (31,460), fluorodeoxyuridine (461), amsacrine (m-AMSA) (462), and the topoisomerase inhibitor, VM-26 (463). The mechanism by which c-myc message is down-regulated by most of these agents is unknown. However, VM-26 was shown to down-regulate c-myc via a transcriptional mechanism (463) and therefore, the other agents may affect c-myc expression by a similar mechanism. The majority of these studies have tried to link the down-regulation of c-myc to the anti-clonogenic effect of these drugs on tumor cells (31,461-463). However, all but one (461) failed to look at the drug effects at the protein level. This appears to be a critical flaw based on studies performed in this dissertation. All of these studies failed to investigate the possibility that the cells were undergoing
apoptosis following drug exposure and were not simply arrested in growth (31,461-463). Therefore, one possible explanation for the results observed in some of these studies is that the down-regulation of c-myc mRNA expression is a consequence of cell death within the population.

The effects of HN2 on c-myc mRNA expression in the Colo320HSR, HT-29, and BE cell lines appeared to be a true consequence of drug exposure since down-regulation of c-myc mRNA was maximal by 12 h post treatment, a time at which all cells were shown to be viable (Figures 4 and 8). The mechanism by which HN2 down-regulated c-myc mRNA expression is unknown. DNA-DNA interstrand crosslinks were formed in the c-myc gene of all three cell lines following exposure to HN2 (data not shown). However, no concrete evidence exists to suggest that the down-regulation of c-myc mRNA expression is a direct consequence of DNA crosslinks formed in the c-myc gene, and therefore, it is as likely to be an indirect consequence of drug exposure.

The studies on protein expression showed that c-myc protein was induced in human colon carcinoma cells following exposure to HN2. These results were unexpected in light of the effects of HN2 on c-myc mRNA expression and the short half-life of both c-myc mRNA and protein (362,365), but are not unprecedented. Sullivan and Willis showed that the mono- and bifunctional alkylating agents, dimethylsulphate (DMS), ethylmethane sulphonate (EMS), mitomycin C (MMC), bleomycin,
and gamma irradiation all induced the expression of c-myc protein in a dose and time-dependent manner in a human lymphoblastoid cell line, GM1953 (464). The bifunctional alkylating agent, MMC caused a 7 fold induction of c-myc protein 24 h following drug exposure and these levels remained elevated out to 72 h, the latest time point recorded (464). These results were similar to those produced by HN2 in the HT-29 and BE cell lines (Figure 7). Taken together, the data from these studies did not support the original hypothesis and prompted further investigations into the mechanisms by which HN2 exerted its cytotoxic effects on the Colo320HSR, HT-29, and BE human colon carcinoma cell lines.

HN2, CELL DEATH, AND C-MYC

The initial studies in this dissertation showed that the anti-neoplastic effects of HN2 on human colon carcinoma cell lines were mediated by the loss of cellular viability, and that this process did not involve the proliferative activity of c-myc. Given that HN2 caused cell death and that c-myc was overexpressed in all three of these colon tumor cell lines, and in light of the recent discoveries showing that c-myc was involved in the regulation of apoptosis (431,432), the following hypothesis was investigated: Human tumor cell lines which overexpress c-myc will undergo an enhanced apoptotic cell death following exposure to chemotherapeutic agents that cause DNA damage.
Studies were performed to detect the presence of apoptotic cells using conventional agarose gel electrophoresis and a quantitative, in situ TDT assay. These studies showed that Colo320HSR cells underwent an apoptotic cell death following HN2 exposure. Oligonucleosomal DNA fragmentation was clearly detectable in this cell line following exposure to HN2 (Figures 9, 10, and 12). Other studies have shown that Colo320HSR cells fragment their DNA following exposure to the topoisomerase II inhibitor, VM-26 and the authors suggested that the overexpression of c-myc in this cell line was involved in the induction of apoptosis (465). HT-29 and BE cells did not exhibit oligonucleosomal DNA ladders. However, the induction of apoptosis in these cell lines following exposure to HN2 could not be ruled out (Figures 9,11,13, and 14). Studies have shown that HT-29 cells do not produce nucleosomal DNA ladders following exposure to VM-26, at least out to the 48 h time point that was investigated, and the authors suggested that the lack of apoptosis seen in this cell line might be related to the lower levels of c-myc as compared to Colo320HSR cells (465). However, the results from studies in this dissertation showed that HT-29 cells did not start to lose viability until 72 h following HN2 exposure. Therefore, it is possible that fragmentation was not detected following VM-26 exposure simply because the cells were still viable.

Recent studies have demonstrated that cells exposed to
chemotherapeutic agents can undergo apoptosis without the appearance of nucleosomal DNA ladders (107,108), suggesting that the lack of this biochemical hallmark does not correlate with the absence of apoptotic cell death. Cells undergoing apoptosis have been shown to initially fragment their DNA into 300 kbp rosettes and 50 kbp chromatin loops which are thought to represent fragmentation at higher orders of chromatin structure (107,108). HT-29 cells exposed to 5-fluorodeoxyuridine (5-FdUrd) have been shown to lack oligonucleosomal DNA ladders, but possessed DNA fragments in the low megabase to 50 kbp range which could have represented the higher order chromatin fragmentation seen in apoptotic cells. However, the presence of apoptotic cells was not morphologically assessed in these studies (466). Studies from this dissertation provided no direct evidence that these large DNA fragments existed in HT-29 and BE cells treated with HN2. However, the results from the TDT analyses were consistent with the possibility that these cells were fragmenting their DNA at a higher order of chromatin structure, as evidenced by the broadening of the peaks at the later time points (Figures 13 and 14).

Morphological analysis of HN2-treated cells provided direct evidence that both HT-29 and BE cells underwent apoptosis following exposure to HN2 to some degree (Figure 16B and C). These studies also confirmed that Colo320HSR cells underwent an apoptotic cell death after HN2 treatment
(Figure 16A). The morphological analyses of the three cell lines quantitated the occurrence of both apoptotic and necrotic cell deaths at various time points following exposure to HN2. This approach was unique, compared to most studies of this type, which simply document the loss of cellular viability with the assumption that all cell deaths are by apoptosis.

The results from the morphology studies showed that Colo320HSR cells died exclusively by apoptosis. The results from the viability studies (Figure 8) were consistent with the results from the TDT analysis (Figure 15) which showed that, in Colo320HSR cells, cell death began as early as 24 h post HN2 exposure. DNA fragmentation was also detectable at this time point. Fragmentation and loss of viability were fairly well correlated in this cell line. The morphological analysis of Colo320HSR cells (Figure 17) corroborated both the viability and fragmentation studies. However, few apoptotic cells were morphologically detectable at the 24 h time point, and the levels detected at 48 h were less than those predicted by the viability studies. This apparent discrepancy may have been due to the differences in sensitivity between techniques. Both the viability and TDT assays were performed by FACS analysis while the morphological analysis relied on human analysis and, therefore, was subject to error. Another possibility was that apoptotic cells, which have a higher buoyant density
than normal cells, may have been mechanically restricted to various areas under the cover slip when in the presence of large numbers of viable cells and, therefore, this discrepancy may simply have been an artifact of cell spreading. Regardless, the results from these studies clearly showed that Colo320HSR cells underwent a rapid induction of apoptotic cell death following exposure to HN2 with no necrosis observed.

The morphology results corroborated the viability studies for HT-29 cells and showed that these cells utilize apoptotic and necrotic modes of cell death to similar extents (Figures 8 and 17). The results for BE cells were also consistent between the viability and morphology studies (Figures 8 and 17). The only discrepancy for BE cells was at the 96 h time point in which viability was slightly overestimated by morphological analysis. This discrepancy was probably caused by the underestimation of necrotic cells at this time point. Although G2 phase cells were clearly distinguishable from most necrotic cells, those which have just begun to lose membrane permeability may not have been swollen enough to be distinguished as necrotic by morphological criteria and, therefore, were counted as viable. The viability assay was clearly a more accurate method of determining the number of viable cells remaining since the subjectivity was removed. Also, the apoptotic events may have been underestimated at this time point for
the reasons described previously. Clearly, necrosis was the major mode of cell death in BE cells, although apoptotic events were detected to a small degree at the time points investigated.

The role of c-myc in the regulation of apoptosis is important in carcinogenesis and tumor response to chemotherapy (154,434). Many studies have shown that c-myc overexpression in a variety of cell types is capable of enhancing the induction of apoptosis induced by a variety of physiological stimuli (431,435,437,467-469) and chemotherapeutic agents (154,432,433,470,471). Malde and Collins suggested that the levels of c-myc in tumor cells may be predictive of their sensitivity to cancer therapy (471). This notion is somewhat short-sighted, however, since many oncogenes have been shown to cooperate with c-myc in the transformation of cells by inhibiting its apoptotic functions (153,154,166,169,250,251). Therefore, high levels of c-myc would not always be predictive of chemotherapeutic agent sensitivity. The studies presented in this dissertation clearly demonstrate the difficulty in trying to use c-myc protein levels alone as a predictive measure for the induction of apoptosis of human tumor cells exposed to chemotherapeutic agents.

Taken together, the results for HT-29 and BE cells suggested that c-myc was not enhancing the induction of apoptosis in these cell lines following exposure to HN2.
These results were interesting since c-myc protein levels in both of these cell lines were high compared to normal human fibroblasts and were actually induced following exposure to HN2. This observation suggested that factors other than high levels of c-myc could influence the c-myc enhancement of apoptosis in human colon carcinoma cell lines. Colo320HSR cells exposed to HN2 were shown to die in a manner that was consistent with a role for c-myc in the enhancement of apoptosis.

THE INVOLVEMENT OF P53 AND BCL-2 IN THE REGULATION OF C-MYC INDUCED APOPTOSIS

Many oncogenes have been shown to negatively regulate c-myc-induced apoptosis and these include c-abl, c-raf, ras, bcl-2 and p53 (153,154,166,169,250,251,328). The Colo320HSR, HT-29, and BE cell lines have been shown not to express mRNA for c-abl, c-raf, or k-ras. However, all three cell lines express mRNA for N-ras (Q. Dong, unpublished observations). These findings suggested that these oncogenes were not involved in the regulation of c-myc-induced apoptosis in these cell lines. Therefore, studies were performed to rule out the possible involvement of either p53 or bcl-2 in the regulation of c-myc-induced apoptosis in the three colon tumor cell lines.

Preliminary studies showed that p53 gene structure and mRNA expression were similar in all three cell lines when
compared to HeLa cells, which contained a wild-type p53 gene (Figure 18A and B). The expression of p53 protein was high in all three colon tumor cell lines compared to HeLa and IMR-90 cells (Figure 18C) and suggested the presence of mutant p53 protein in these cells, since mutant forms of p53 protein have substantially longer half-lives than their wild-type counterpart, resulting in their overexpression (287,288). The presence of mutant p53 protein in Colo320HSR, HT-29, and BE cells was confirmed by immunoprecipitation analysis with conformation-specific antibodies (Figure 19). Other studies have since confirmed that Colo320HSR cells have lost the wild-type allele and possess a single mutant p53 allele (arginine 248 to tryptophan) (472). HT-29 cells have also been shown to possess a mutant p53 gene containing an arginine to histidine replacement at codon 273 (473).

The p53 tumor suppressor gene has been shown to be a direct inducer of apoptosis (172) and its involvement in the suppression of tumor formation is strongly associated with its apoptosis-inducing functions (310). Studies in murine systems have shown that wild-type p53 is required for the induction of apoptosis by chemotherapeutic agents in many but not all cell types (173,175,316,317,320). Mutant p53 protein has been shown to confer resistance to cells exposed to many types of chemotherapeutic agents and has been shown to abrogate c-myc-induced apoptosis (175,328,332). However, the situation is much more confusing in human systems where some
studies have shown that p53 status is an important determinant of the outcome of chemotherapy (333,334) while others have shown that it is not (335-338).

All three colon tumor cell lines expressed mutant forms of p53 protein and all could undergo apoptosis to varying degrees following exposure to HN2 suggesting that p53 status may not be an important determinant in the cytotoxic response to drug exposure. Nitrogen mustard had no effect on mutant p53 protein expression in any of the three cell lines out to 96 h post treatment (Figure 21). These data were consistent with other studies which showed that the expression of mutant p53 protein was not affected in several Burkitt's lymphoma cell lines following gamma-irradiation (474). Colo320HSR cells, which appeared to undergo a c-myc-enhanced apoptosis following exposure to HN2, did not show an induction of p53 protein following drug treatment and expressed only a mutant p53 protein. These findings were contrary to those in murine systems which showed that the induction of wild-type p53 was involved in the c-myc enhancement of apoptosis and that mutant p53 protein abrogated this process (327,328). The studies on p53 showed that HN2-induced apoptosis in human colon carcinoma cell lines occurred in a wild-type p53-independent manner and suggested that p53 was not a regulator of c-myc-enhanced apoptosis in these cell lines.

Bcl-2 has been shown to cooperate with c-myc in the transformation of cells both in vitro and in vivo by
abrogating the apoptotic effects of c-myc overexpression (163,190-192). The ectopic overexpression of bcl-2 has been shown to increase the resistance of many tumor cell types to a variety of anti-neoplastic agents (164,165,234-238). Therefore, studies were performed to rule out the involvement of bcl-2 in the abrogation of c-myc-induced apoptosis in the HT-29 and BE cell lines. The Colo320HSR and BE cell lines expressed mRNA and protein for bcl-2, while the expression of bcl-2 mRNA was low and bcl-2 protein was undetectable in HT-29 cells (Figure 22B and C). The lack of bcl-2 protein expression in HT-29 cells has been previously reported (475). Bcl-2 protein did not appear to be overexpressed in Colo320HSR and BE cells since bcl-2 protein levels were well below those seen in the LM-EBV cell line (Figure 22C). This cell line overexpressed bcl-2 protein as a result of the presence of the latent membrane protein 1 (LMP-1) of the Epstein-Barr virus, which up-regulates bcl-2 expression and prevents virally infected cells from undergoing apoptosis (476).

Bcl-2 expression can be either up- or down-regulated in response to various physiological stimuli including estrogen, TGFβ1, and wild-type p53 protein (322,323,477). Studies on the modulation of bcl-2 protein expression by HN2 in this dissertation demonstrated that bcl-2 protein expression was induced in a cell type specific and time-dependent manner following HN2 exposure (Figure 23). Based upon the many
studies involving the forced, ectopic overexpression of bcl-2 (234-238), the data from this dissertation suggested that Colo320HSR and BE cells would have been protected from the apoptosis-inducing effects of HN2 exposure. However, we now know that the regulation of cell death by bcl-2 is extremely complex and probably cell type specific. Therefore, the status of bcl-2 expression alone may be insufficient to predict a protective effect (218,222,223,226-230). Colo320HSR cells died rapidly and exclusively by apoptosis despite the presence of bcl-2 and its induction following HN2 exposure. The induction of bcl-2 in BE cells was a robust and late event following drug exposure. However, the peak induction preceded the point in time at which further cell death in these cells was inhibited (Figure 8) and, therefore, was consistent with the possibility that bcl-2 was delaying the death of BE cells following HN2 exposure. Ectopic overexpression of bcl-2 has been shown to delay the death of murine FL5.12 prolymphoid progenitor cells following exposure to either HN2 or the topoisomerase I inhibitor, camptothecin (242).

Few studies have investigated the effects of chemotherapeutic agents on the modulation of bcl-2 expression. Most, if not all, of the studies involving the effects of bcl-2 on chemotherapeutic agent responses have been performed on cells engineered to ectopically overexpress bcl-2. If the results from the studies in this dissertation
are confirmed to be a real and consistent effect of anti-neoplastic agent exposure on human tumor cells, then the modulation of bcl-2 and other death repressor family genes such as bcl-x, mcl-1, A1, bax, bag-1, and bad by chemotherapeutic agents may be playing an important, as yet unexplored role in the modulation of drug resistance in human tumor cells. In the cell lines studied in this dissertation, bcl-2 did not appear to be involved in the abrogation of c-myc-enhanced apoptosis following HN2 treatment. The induction of bcl-2 at the later time points appeared to be associated with a delay in the death of BE cells. However, the low levels of constitutive expression, along with the decline in bcl-2 levels through 24 h post HN2 treatment and lack of induction until 48 h post treatment suggested that bcl-2 was not overexpressed through 48 h following drug exposure. Therefore, the involvement of bcl-2 in the abrogation of c-myc-induced apoptosis did not appear to be warranted in this cell line. This conclusion is not contradictory, since the death repressor activity of bcl-2 occurs in a manner that is independent of c-myc functions.

INVOLVEMENT OF THE MYC TRANSCRIPTION FACTOR NETWORK IN THE C-MY C ENHANCEMENT OF APOPTOSIS INDUCED BY HN2

The previous studies in this dissertation suggested that c-myc-enhanced apoptosis in HT-29 and BE cells was not abrogated by the various oncogenes known to regulate the
enhancement of apoptosis by c-myc. Therefore, the involvement of the myc transcription factor network in this process was investigated.

It has been suggested that the apoptosis-inducing functions of c-myc require its dimerization with max and occur in a manner that is dependent upon the dose of c-myc (426,432). However, this is a simplistic view of the system. In fact, the level of c-myc/max dimers is what determines the outcome, since this is the functional transactivating unit of this system (426). The amount of c-myc/max dimers is thought to be determined by the relative levels of c-myc and mad proteins, which, unlike max, are thought to be the limiting factors in this system (415). The mad/max, mxil/max, and max/max dimers all antagonize the transcriptional activity of the c-myc/max dimer (415,416). Therefore, a true assessment of the transcriptional capabilities of this system would require knowledge of the levels of all four of these dimer types at any point in time.

In these studies, the levels of c-myc, max, and mad protein, and c-myc/max dimers were measured in an attempt to characterize this system and determine its status prior to and following exposure to HN2. Mad/max dimers were not measured in these studies. The involvement of mxil in these systems was not determined. When this system was characterized, several important observations were noted. The levels of max protein varied greatly between the three
colon tumor cell lines, suggesting that tumor cells may show a wide range of max expression which could have important implications on tumor cell studies involving c-myc (Figure 24A). Indeed, c-myc/max dimer levels did not correlate with c-myc protein expression in either Colo320HSR or HT-29 cells (Figure 29, control panel). In Colo320HSR cells max protein levels were clearly limiting the formation of c-myc/max dimers which was contrary to the results from previous studies which suggested that all of the c-myc protein in cells was dimerized with max, and that max was not the limiting factor of this system in cells (426,478).

Three lines of indirect evidence all suggested that max protein levels were limiting the formation of c-myc/max dimers in Colo320HSR cells. First, the amount of immunoprecipitable max protein was never greater than the amount of co-precipitable c-myc in Colo320HSR cells as was clearly the case in both HT-29 and BE cells, showing that excess max protein was present in these two cell lines (Figure 24A). Second, the levels of immunoprecipitable c-myc were greater than the levels of max-co-precipitable c-myc implying that not all of the c-myc present in Colo320HSR cells was dimerized with max (data not shown). Finally, and most convincingly, the increase in c-myc/max dimers in Colo320HSR cells exposed to HN2 was shown to be mediated by the induction of max protein, which could have occurred only if max levels were limiting the formation of c-myc/max dimers.
Figure 29. The relative pre-treatment status and post-treatment changes in the major components of the myc transcription factor network in Colo320HSR, HT-29, and BE cell lines following exposure to HN2.

The figures in this diagram were based upon the previous data presented on the myc transcription factor network and data not shown. The relative protein and dimer levels were expressed as arbitrary units and were comparable within each cell line and between cell lines. The c-myc/max dimers for BE cells were arbitrarily set as 1 unit, and were the basis upon which everything else was compared. The 0, 12/15, 72, and 96 h time points were not shown.
RELATIVE PROTEIN LEVELS (Arbitrary Units)
These results clearly underscore the limitations of studies in tumor cells that only measure effects on c-myc protein.

If the pretreatment status of the myc transcription factor network was involved in determining the outcome of c-myc-enhanced apoptosis in the three colon tumor cell lines, then the transactivating capabilities of this system would have been predicted to be the highest in Colo320HSR cells. Although c-myc protein levels were 7 fold higher in Colo320HSR cells, the levels of c-myc/max dimers were only 2 or 4 fold greater relative to BE and HT-29 cells, respectively. Therefore, the question arises, was a 2 fold difference in c-myc/max dimers enough to account for the lack of enhanced apoptosis in BE cells? The answer to this question is, probably not. However, if the whole system is taken into account, then perhaps this difference was enough. In Colo320HSR cells, all available max protein appeared to be dimerized with c-myc (Figure 29), and it appeared that mad levels, although detectable in these cells, were not high enough to compete with c-myc for max binding. Therefore, in Colo320HSR cells, it was possible that only c-myc/max dimers existed and little or no max/max or mad/max dimers were present to antagonize the activity of these dimers. In HT-29 cells, mad levels were higher and c-myc levels were lower than in Colo320HSR cells. The fact that c-myc/max dimer levels were lower than the levels of c-myc protein even
though max protein levels were equivalent to c-myc levels suggested that mad/max or max/max dimers may have existed in these cells (Figure 29). The existence of these antagonistic dimers would have effectively lowered the transactivating activity of c-myc/max dimers in these cells. Therefore, the difference in c-myc/max dimer transactivating activity between Colo320HSR and HT-29 cells would have been much greater than the 4 fold difference predicted by c-myc/max dimer levels alone. The same logic held true for BE cells, which expressed high levels of mad, low levels of c-myc (relative to Colo320HSR cells) and an almost 5 fold excess of max protein. However, a direct demonstration of the existence of mad/max or max/max dimers in these cell lines, along with an index of the in vivo transactivation capacity of the myc network is required to validate these statements.

The final set of studies described in this dissertation were designed to investigate the effects of HN2 on the myc transcription factor network in human colon carcinoma cell lines to determine if post-treatment effects could explain the apparent lack of c-myc-enhanced apoptosis in HT-29 and BE cells. These studies were novel in that no other studies have been found describing the effect of a chemotherapeutic agent on the members of the myc transcription factor network, including c-myc/max dimers.

In the Colo320HSR cell line, HN2 induced the expression of both p21 and p22 max, which led to the approximately 5
fold induction of c-myc/max dimers by 24 h post treatment (Figure 25). The increase in c-myc/max dimers occurred without any changes in c-myc protein expression (through 24 h) and prior to the onset of apoptosis in these cells. This large increase in the "dose" of c-myc/max dimers prior to the onset of apoptosis induced by HN2 was consistent with the possibility that these newly formed dimers were contributing to the c-myc enhancement of apoptosis in Colo320HSR cells. The decrease in c-myc/max dimers at the 48 h time point was due, in part, to the decrease in c-myc protein at this time which was most likely a consequence of cell death (Figure 25B).

The effect of HN2 on the myc transcription factor network in both HT-29 and BE cells was consistent with the possibility that decreases in c-myc/max dimers were involved in the abrogation of c-myc-enhanced apoptosis in these cell lines. In HT-29 cells, both c-myc and p21/p22 max proteins were induced following exposure to HN2. However, c-myc/max dimer levels never increased above control levels and declined over the post-treatment time course despite the continued elevation of both c-myc and max protein levels (Figure 26). An almost identical situation was observed in BE cells exposed to HN2, except that the induction of max was minimal. However, max levels in this cell line were already very high relative to HT-29 cells (Figure 27).

The finding that c-myc/max dimer levels did not increase
Despite an increase in both c-myc and max protein levels in both HT-29 and BE cells was quite unexpected given that current thinking suggests that all c-myc in the cell is associated with max (426, 478). These results suggested that the c-myc and max proteins were prevented from interacting and forming dimers by alterations in their cellular localization since no known posttranslational modifications in c-myc or max proteins are known to prevent or antagonize their association (479). These findings merit further investigation.

The studies on the myc transcription factor network were consistent with a role for this system in the regulation of c-myc-enhanced apoptosis in human tumor cell lines, and showed the problems involved in trying to predict the occurrence of c-myc-induced apoptosis based upon the levels of c-myc protein alone. These studies were consistent with the dose-response relationship between c-myc/max dimers and the induction of apoptosis (432). However, these studies did not conclusively determine whether the pre-treatment status or post-treatment responses were responsible for determining whether apoptosis was enhanced in these cell lines. Convincing arguments can be made for either scenario. The argument for the pre-treatment status has already been discussed. The post-treatment changes in the myc transcription factor network following HN2 exposure strongly suggested that these changes were involved in regulating
apoptosis. However, these results, as presented, were only correlative and not causative. Regardless, the dose-response relationship between c-myc/max dimer levels and the enhancement of apoptosis was quite impressive when compared between cell lines, relative to HT-29 cells (Figure 28). These results were certainly consistent with the possibility that post-treatment changes in the myc transcription factor network were involved in the regulation of c-myc-induced apoptosis in these cell lines.

Experiments were designed to address the relative importance of pre-treatment status versus post-treatment changes in the myc transcription factor network in the regulation of c-myc-enhanced apoptosis in Colo320HSR cells exposed to HN2. These experiments relied upon the specific reductions in c-myc protein by antisense ODNs targeted against c-myc mRNA (449,454). Numerous attempts were made to modulate this system in a consistent manner, with antisense technology, but were unsuccessful. These studies were not continued since they were deemed unlikely to produce interpretable results. Therefore, the investigations into the myc transcription factor network remain correlative at best.

The involvement of deregulated c-myc expression in the regulation of apoptosis has been demonstrated in Burkitt’s lymphoma cells, Epstein-Barr virus-immortalized B cells, rat fibroblasts, murine myeloid cells, and T cells
However, the participation of c-myc is not a requirement in all apoptotic pathways in all cell types (431,435,480,481). The regulation of apoptosis by c-myc has been shown to require dimerization with max and is thought to involve the transactivation of genes (426,432). Several genes have been described as possible mediators of c-myc-induced apoptosis (327,408,444), the most convincing of which seems to be the ODC gene (444). The ODC gene is a direct transcriptional target of c-myc/max dimers (404) and the enforced overexpression of ODC has been shown to induce apoptosis upon factor withdrawal (444). To this end, it would be interesting to look at the expression of ODC and cyclin A, two known mediators of c-myc-induced apoptosis, in Colo320HSR cells following exposure to HN2, to determine whether their expression correlates with changes in c-myc/max dimer levels. Also, the bax gene has recently been shown to contain 4 E-box motifs (321) suggesting that c-myc may be capable of inducing the expression of bax. Since bax is known to be directly involved in the cell death pathway (218), it would be interesting to determine whether bax expression is induced in Colo320HSR cells following HN2 treatment.

The current thinking of how anti-neoplastic agents exert their cytotoxic effects on tumor cells has begun to change. The impetus behind this change in thinking has come from the growing body of evidence which has shown that the induction
of apoptosis is the end result of drug exposure in most, if not all, tumor cell types regardless of the chemotherapeutic agent used (156,157; 85,86 and references within). The cell can no longer be thought of as simply a dart board in which drugs are interacting with specific cellular targets, killing cells as a direct result of this drug/target interaction (90,156,161). The cell is a complex structure and the fact that drugs with so many varied mechanisms of action all evoke a similar, genetically programmed response, suggests that these drugs produce cellular responses downstream of the drug/target interaction that impinge at various nodes upon a common cell death pathway. This "stimulus-response coupling" theory suggests that the drug/target interaction is only the initial event; the stimulus, and the cellular context in which this stimulus is received is probably more important than the actual type of drug/target interaction (90,156). The probability that the apoptotic program is engaged following drug/target interaction is thought to be determined by the cellular response to the stimulus which is cell type-specific (90). This theory suggests that cells can become resistant to chemotherapeutic agents by either not receiving the stimulus (classical drug resistance) or more importantly, by not responding to it. Cells that do not respond to the stimulus have effectively uncoupled the response from the stimulus and are effectively protected from cell death. However, these cells can still undergo apoptosis in response
to other stimuli that utilize a different signal transduction pathway to impinge upon the final, common cell death pathway (173,316). Many studies support such a "stimulus-response" type of model (39,90,162,173,243,316,323,456), including the studies from this dissertation.
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