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ROLE OF BCL-2 IN CARCINOGENESIS

By

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ABSTRACT

The Role of Bcl-2 in Carcinogenesis

By

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Carcinogenesis has been described as a multi-step process involving three ordered temporal phases, initiation, promotion, and progression. The promotion phase has been speculated to include the selective accumulation of an initiated cell via mitogenesis, and the escape from apoptosis. Many oncogenes and tumor promoting chemicals have been shown to contribute to mitogenesis and blockage of apoptosis. In addition, these same oncogenes and tumor promoters have been shown to block intercellular signaling through gap junctions. The objective of this study was to test the hypothesis that cooperation of proliferation, apoptosis, and gap junctional intercellular communication (GJIC) can lead to the neoplastic transformation of normal cells. To accomplish this, oncogenes that influenced proliferation and apoptosis were transfected independently and together into normal epithelial cells and subsequently assayed for neoplastic potential and GJIC.

In order to examine the involvement of apoptosis in carcinogenesis and subsequent affects on GJIC WB-F344 cells, a normal rat liver epithelial cell line, were transfected with the bcl-2 gene. Additionally, to test the synergistic roles of blocking apoptosis and inducing mitogenesis, a previously established WB.v-myc cell line was also

transfected with the bcl-2 gene. All cell lines were assessed for cell proliferation, contact inhibition saturation density, anchorage independent growth (used as a surrogate marker for neoplastic transformation), invasion, and GJIC.

The results obtained from this investigation demonstrated that bcl-2 and myc, when co-expressed, were able to cooperatively induce the neoplastic transformation of normal WB-F344 cells. Those cell lines expressing myc and bcl-2 demonstrated a loss in contact inhibition, a reduction in apoptosis and a reduction in proliferation rates. These cell lines also showed varying abilities to grow in soft agar that did not correlate to bcl-2 protein expression. In order to test if there was a correlation between neoplastic ability and GJIC, scrape load dye transfer and fluorescent recovery after photobleaching were measured. The results showed a relationship between the reduction in GJIC and neoplastic ability, in that, those cell lines that demonstrated a reduction in GJIC showed an increase in neoplastic ability.

Additionally, it has been previously hypothesized that cancer is a disease of blocked differentiation (Potter, 1978). To test this cell lines were induced to differentiate using sodium butyrate. The results demonstrated that differentiation was reduced when myc or bcl-2 were expressed independently. Those cell lines that expressed both myc and bcl-2 were unable to differentiate, which suggests that blockage of differentiation is a progressive event in carcinogenesis.

This work is dedicated to my family, especially my wife, Diana, for their love and support

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Lastly, I would like to express my deepest gratitude to my friend and wife, Diana.

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LIST OF ABBREVIATIONS

ANT.....adenine nucleotide translocator

B-HLH-LZ...basic motif, a helix-loop-helix motif, and leucine zipper motif

BH.....bcl-2 homology

Ca2 +,Mg2+ -PBS.....PBS containing Ca2+ and Mg2+

CAD .....carbamoyl-phosphate synthase/aspartate carbamoyl transferase/dihyroorotase guanosine triphosphatase (GTPase)

CREB.....cyclic-AMP response element binding protein

EGF.....epidermal growth factor

eIF-2α and eIF-4E.....eukaryotic initiation factor

EMS.....E-box Myc Sequence

Erk1 and Erk2.....extracellular signal regulated kinases

FACS.....fluorescent activated cell sorting

FBS.....fetal bovine serum

FRAP.....fluorescent recovery after photobleaching

G418.....geneticin

GJIC.....gap junctional intercellular communication

GSH.....reduced glutathione

kDa.....kilodalton

MAPK.....mitogen activated protein kinase

MEK1 and MEK2.....MAPK/ERK kinases

MEM.....minimal essential medium

MTT.....3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

myc.....myelocytomatosis

ODC.....ornithine decarboxylase

PBS.....phosphate buffered saline

PDGF.....platelet-derived growth factor

PMSF.....phenylmethylsulfonyl fluoride

PT.....permiability transition

SDS.....sodium dodecyl sulfate

SL/DT.....scrape loading dye transfer
TAT.....tyrosine aminotransferase
TFE3.....transcription factor E3
TPA.....12-tetradecanoylphorbol-13-acetate
USF.....upstream stimulatory factor
VDAC.....voltage-dependent anion channel

INTRODUCTION

Carcinogenesis has been described as a multi-step, multi-mechanism process (Pitot et al., 1981; Dragan et al., 1993). This process consists of three distinct temporal phases: initiation, promotion and progression. The initiation/promotion/progression model of carcinogenesis is an *in vivo* model that cannot be fully demonstrated *in vitro* due to the artificial nature of cell culture. However, *in vitro* models may be used to demonstrate how biochemical mechanisms could contribute to the initiation/promotion/progression model of carcinogenesis and subsequent neoplastic transformation. Initiation is characterized by an irreversible event, genetic or epigenetic, in a single cell that has the potential to proliferate, i.e. a stem or progenitor cell. Promotion is a reversible, interruptible process that brings about the clonal expansion of the initiated cell, through a chronic administration of a stimulus that is mitogenic, e.g. chemical tumor promoters. Promotion consists of those events that disrupt cellular homeostasis.

Homeostasis is described as a balance between positive and negative regulatory signals that exist to allow for the proper development and maintenance of stem/progenitor cells and their differentiated daughter cells in the tissues of multicellular organisms. Disruption of homeostasis would then involve restrictions in the response of cells to those positive and negative regulatory signals. This disruption would

involve the loss of the ability of cells to differentiate, the loss of the ability of cells to respond to cell death signals, and the loss of the cell's ability to control growth or be contact inhibited. These disturbances imply some form of communication between cells and within the cell itself. Internal, intracellular, communication is accomplished through diffusible elements restricted to the intracellular space. The cell to cell communication between cells involves either or both extracellular communication or intercellular communication. Extracellular communication can be described as regulation between distal points through hormones, growth factors, neurotransmitters, cell surface proteins, and extracellular matrices. Intercellular communication involves the direct transfer of signals between cells. One form of intercellular signaling is through specialized protein structures in the membranes of contiguous cells called gap junctions. This gap junctional intercellular communication (GJIC) represents an important means by which cells can interact directly with each other to equilibrate and exchange ions, nutrients, and other regulatory molecules 1000 daltons or less in size (Loewenstein, 1981). Many tumor cells have shown the absence of gap junction coupling (Chang et al., 1987; Fentiman et al., 1979; Corsaro and Migeon, 1977; Loewenstein and Kanno, 1966; Tosko and Ruch, 1998) and a reduced response to apoptotic stimulus. These observations led to the speculation that in solid tissues gap junctions are required for the proper transfer of apoptotic signals between cells (Trosko

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and Goodman, 1994). Uncoupling of gap junctions between cells may be a necessary event in promotion, however it is not the only step involved. Additionally, the promotion phase of carcinogenesis involves the loss of cells ability to respond to cell death signals and the loss of the cells ability to control growth. GJIC appears to be necessary event in both of these processes but it is not the sole event. In order for promotion to occur a mitogenic signal with concomitant loss of apoptosis needs to occur. Thus if an initiated cell is uncoupled from its neighbor without any specific signaling the cell will remain quiescent. However, if a mitogenic signal is present, these cells would grow in an unconstrained manner. Conversely, if an apoptotic stimulus was present in neighboring cells, then these cells would persist as their neighbors died. Thus, it would follow that, if blockage of apoptosis and induction of proliferation were sufficient for neoplasia, the induction of these states might be expected to down regulate GJIC, when expressed in a normal gap junction competent cell.

The research in this study was undertaken to investigate if bcl-2, an anti-apoptosis gene, plays a role in neoplastic transformation process and could block gap junctional intercellular communication when co-expressed with a proliferative oncogene like myc. To accomplish this, the human bcl-2 oncogene was introduced into a normal rat liver epithelial cell line, WB-F344, and in an already established v-myc expressing WB-F344 cell line. These cells were tested for their ability to proliferate, to

contact inhibit, to express neoplastic characteristics (as measured by anchorage independent growth in soft agarose, and invasion) and to modulate gap junctional intercellular communication. The expression of the bcl-2 protein was measured using western blot analysis with an antibody specific for human bcl-2.

Initiation/Promotion/Progression Model of Carcinogenesis

Multistage carcinogenesis was first demonstrated by repeated chemical treatments of mouse skin patches (Berenblum, 1941). It was found that some chemicals were able to cause tumors with repeated treatments. These were called complete carcinogens. Other chemicals could only induce tumors following treatment with an initiator, these chemicals were termed tumor promoters. It is now known that initiators and complete carcinogens are able to induce DNA lesions. In addition to causing DNA lesions initiators and complete carcinogens also provide growth (mitogenic) signals that allow for the expansion and accumulation of initiated cells. Therefore, it is believed that tumor promoters are only able to produce the mitogenic signals associated with complete carcinogens.

Figure 16. Western blot analysis of connexin 43 expression. Bands at 43 kDa represent unphosphorylated (P_0) and phosphorylated(P_1 and P_2) connexin 43 protein.

Pitot and co-workers (1981) refined this concept and characterized cancer as being three distinct stages: 1) initiation; 2) promotion; and 3) progression (Figure 1). The first step of this process is the alteration of a "normal" cell into an "initiated" cell, which can be specifically selected and clonally amplified given the proper conditions (Nowell 1976, Fialkow 1979). This phase is characterized by a fixed error occurring on a genetic level. Changes at a genetic level would include mutations to the genetic code, altering protein function or changes in gene expression that alters cellular expression in such a way that the cells are able to have some selective advantage over neighboring cells.

The promotion phase is most notably characterized by the clonal expansion of the initiated cell or cells; however, this involves a couple of cooperative mechanisms. The first is the clonal amplification of the initiated cells by mitogenesis and the second is the blockage of cell death, which, under normal circumstances, would eliminate the initiated cells. The important distinction of promotion is its reversibility, in which removal of the mitogenic stimulus could arrest the development of focal lesions, and reapplication of the mitogenic stimulus causes the resumption of growth of the focal lesion. The mitogenic stimuli could be non-cytotoxic chemicals like Phenobarbital, removal of which induces regression of tumors in the multi-stage rat liver carcinogenesis model, or even hormones like testosterone, removal of which induces regression of prostate tumors in rats and humans.

Figure 1. The initiation/promotion/progression model of carcinogenesis.

Progression
(Malignant conversion of an initiated cell)
Promotion
(Reversible mitogenic signals, blockage of apoptosis, inhibition of GJIC)
(Fixed, irreversible error in genetic material)
Initiation
Initiated Cell
Normal Cell

Figure 1

Additionally the mitogenic stimuli could be cytotoxic agents that induce concomitant hyperplasia through necrotic cell death and induction of mitogens associated with an inflammatory response (Aldaz et al., 1985; Casale et al., 1997).

The blockage of cell death, specifically apoptosis, which is programmed cell suicide and does not elicit an inflammatory response, is most notably associated with the B-cell Lymphoma gene, Bcl-2 (Tsujimoto et al., 1985). This gene allows for the accumulation of cells that would normally progress to death thus leading to the lymphoma. However, chemical tumor promoters, like 12-tetradecanoylphorbol-13-acetate (TPA) not only produce mitogenic signals, but also, they are able to block apoptosis (Magnelli et al., 1995; Rodriguez and Lopez, 1989). In this manner promotion results from mitogenesis and blockage of apoptosis leading to the accumulation of initiated cells which would have normally been excluded.

Progression is characterized by the significant acquisition of alterations in an initiated cell such that it becomes promoter independent, invasive into the surround tissues and, lastly, metastatic into distal tissues.

The "Stem Cell" Theory of Carcinogenesis

The initiation/promotion/progression model of carcinogenesis does not speculate as to the origin of the cell that is initially targeted for carcinogenesis. "The stem cell theory" posits that the target cells are the presumptive stem cells. These cells are characterized by their ability to undergo asymmetrical cell division, during which, one daughter cell retains parental "stem-cell" like characteristics and the other daughter cell is committed to a differentiation pathway.

In order to understand this concept, it is necessary to describe the hierarchical nature of multi-cellular organisms (Brody, 1973). This is the idea that the whole is greater that the sum of its parts. The concept involves atoms forming molecules, which form proteins, which are organized into specialized organelles, which make up cells, which are then organized to form tissues. These tissues then form the organs, whose functions integrate to form the systems, whose features cannot be found in any of the individual components.

Maintenance of the hierarchy of the multi-cellular organism involves positive and negative regulation of molecular, biochemical, cellular and physiological information (i.e., "homeostasis") and requires three processes of communication: 1) intracellular, those mechanisms which contribute to the signaling within an individual cell; 2) intercellular communication, those mechanisms which contribute to the signaling between neighboring cells; and 3) extracellular, which contribute to signaling between distal cells, tissues and organs. When

these communication systems functions normally, homeostasis or balance is achieved. In the case of higher multi-cellular organisms, like human beings, the hierarchy starts with the original diploid cell, the fertilized egg, that gives rise to the trillions of cells that make-up the human being. This original cell is denoted as being totipotent, for it has the capacity to form all the cells in the organism. This totipotent cell gives rise to what many term as the "pluri-potent stem cell". The "pluri-potent stem cell" is restricted to only produce cells of a specific lineage under normal conditions. This restriction may be confined to cell types of specific tissue or organ. These pluri-potent stem cells give rise to daughter cells, which are themselves committed to only produce a specific cell type within a specific tissue or organ. This daughter cell, or progenitor cell, can follow a specific differentiation pathway to form cells of specialized function. Thus, in any given tissue or organ, there are great numbers of functionally specialized cells but only a few progenitor cells and even fewer "stem cells".

Nakano and coworkers (1985), provided experimental evidence for the putative stem cells as being the targets for neoplastic transformation by demonstrating that a sub-population of less differentiated cells were more susceptible to chemical induction of neoplastic transformation. These same cells were also characterized as being contact inhibition insensitive. In other words, these cells continued to grow even when other cells in the population were growth inhibited at the same cell

density. Partially differentiated cells demonstrate contact inhibition sensitivity in that they reach a specific cell density that predetermines the size of the tissue. "Stem cells" are contact inhibition insensitive because they need to be able to replenish those cells that are lost through the normal process of programmed cell death, apoptosis, which eliminates terminally differentiated cells that are either no longer function, have provided their functional requirement, or are damaged (Levine et al., 1965). Interestingly, similar to stem cells, most tumors are contact inhibition insensitive (Abercrombie, 1979; Borek and Sachs, 1966).

Additional evidence for the putative role of stem cells in cancer comes from experiments in the isolation of "stem cells" in culture. Chang and coworkers were among the first to isolate, culture, and characterize human epithelial stem-cells *in vitro* (Chang et al., 1987; Kao et al., 1995). These human breast pluri-potent stem cells were not only contact inhibition-insensitive, but they also lacked functional GJIC, resulting from a lack of connexin gene expression. These GJIC deficient cells have been shown to be more susceptible to neoplastic transformation (Sun et al., 1999). Significantly, most cancer cells also demonstrate a lack of functional GJIC (Yamasaki, 1990; Loewenstein, 1981). These results of loss of contact inhibition and lack of functional GJIC demonstrate striking similarities and suggest that cancer cells may owe their origin to stem cells.

Gap Junctional intercellular communication

GJIC is established between cells by the formation of gap junctions. Gap junctions are intercellular channels that allow for the free passage of small molecular weight molecules (less than 1 kDa)(Beyer et al., 1990). A gap junction is comprised of plaques of connected connexons, one in each adjoining membrane (Figure 2). A connexon, the structural unit of the gap junction is a cylinder with a hydrophobic channel formed by the association of six individual protein subunits, which are called connexins. Gap junctions are formed when connexons of two closely-opposed cells align end-to-end forming intercellular channels which are responsible for GJIC.

Two descriptions of GJIC are 1) homologous communication with cells of same origin; and 2) heterologous communication with cells of different origin (Hotz-Wagenblatt and Shalloway, 1993). Very early data demonstrated that several tumor cell lines displayed reduced GJIC, but this was not proven to be a universal characteristic (Yamasaki, 1990; Loewenstein, 1981). More importantly, it was later demonstrated that, while some tumors showed homologous communication they did not display heterologous communication (Loewenstein, 1979; Yamasaki, 1990). For example, *in vitro* transformed BALB/c 3T3 cells showed no alterations in GJIC (Enomoto and Yamasaki, 1984). However, these cells

Figure 2. Graphical representation of gap junction formation between two neighboring cells. The gap junction is represented as a pairing of connexons in opposing plasma membranes. These paired connexons form the gap junctions and subsequent channel. The connexon is comprised of six individual connexin proteins.

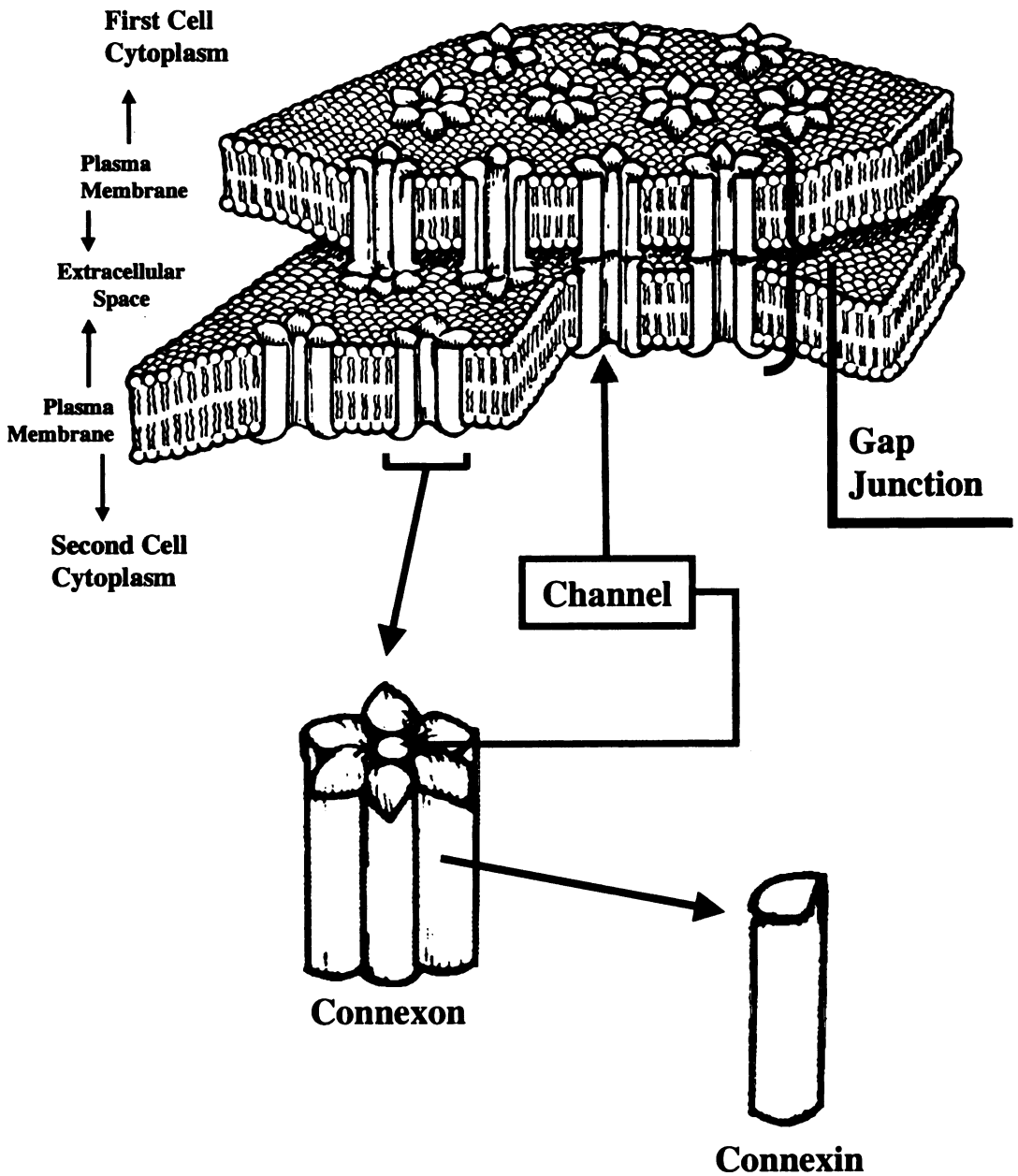

First Cell
Cytoplasm
Plasma
Membrane
Extracellular
Space
Plasma
Membrane
Second Cell
Cytoplasm
Gap
Junction
Channel
Connexon
Connexin

Figure 2

were unable to establish heterologous GJIC with their surrounding neighbors. These results were repeatable regardless of transformation conditions, i.e. chemical carcinogens, UV irradiation, or oncogenes (Yamasaki et al., 1987). Importantly, these results were repeated *in vivo* using the multistage rat liver carcinogenesis model (Krutovskikh et al., 1991). Preneoplastic foci in this model showed a significant lack of GJIC with the surrounding parenchymal cells.

The most striking evidence for the role of GJIC in promotion may come from research into chemical tumor promoters. Chemical tumor promoters were the first tools used to demonstrate multi-phase carcinogenesis (Berenblum, 1941). Many chemicals, like the phorbol ester, TPA, can promote initiated cells. TPA-induced cell proliferation and tumor promotion have been shown to be mediated through the activation of PKC (protein kinase C) (Yuspa, 1994; Dlugosz, 1994). This leads to the subsequent activation of MAPK cascades, one of which is the ras-raf-MAPK pathway (described in detail later in this report), which eventually causes the transcriptional induction of the growth related transcription factors fos and jun (Seger and Krebs; 1995).

An additional, and equally important, mechanism by which these chemicals act is by reducing/blocking gap junction-mediated intercellular communication (Yotti et al., 1979; Murray and Fitzgerald, 1979). This discovery has since led to the use of GJIC inhibition as a predictive indicator for the detection of tumor promoting activity of

environmental agents (Swierenga and Yamasaki, 1992; Budunova and Williams, 1994). More recently GJIC has been used as a predictor for carcinogenic activity (Rivedal et al., 2000; Rosenkranz et al., 1999). Interestingly, using a structure-activity relationship system as a predictive model, the blockage of GJIC has a higher correlation to carcinogenicity than did mutagenicity, meaning blockage of GJIC was a better predictor of a chemicals ability to act as a carcinogen than its ability to form mutations (Rosenkranz et al., 1999).

To further demonstrate the importance of GJIC in carcinogenesis, it has been shown that, in addition to their other properties many oncogenes also inhibit GJIC (Yamasaki, 1990; Trosko et al., 1990; de-Feijter et al., 1990; de-Feijter et al., 1996). Consistent with these findings, the evidence that tumor suppressor genes and anti-carcinogens act in an opposing manner by upregulating GJIC (de-Feijter-Rupp et al., 1998; Zhang et al., 1992; Mehta et al., 1989; Ruch et al., 1989).

The Role of Oncogenes

We have previously discussed how promotion involves not only mitogenesis and GJIC, but have also speculated to the role of blockage of apoptosis. Therefore, it is not surprising that these are demonstrated processes affected by many of the known oncogenes. Oncogenes are genes that induce cellular and morphological alterations leading to neoplastic transformation. Oncogenes were first discovered as the

element in transforming viruses responsible for causing tumors (Rous, 1910; Rous, 1911). Viral oncogenesis hypothesis implied that all vertebrate cells contain genes, called proto-oncogenes, analogous to the genes found in the transforming viruses (Huebner and Todaro, 1969; Todaro and Huebner, 1972; Marshall, 1986). The proto-oncogenes have been evolutionarily conserved in all metazoan organisms (Shilo and Weinberg, 1981). This conservation of proto-oncogenes underlines their great importance in normal cellular function. Proto-oncogenes can become activated by one or more of four mechanisms, namely; 1) inappropriate expression during stages of cell proliferation or differentiation, 2) gene amplification, 3) chromosome translocations, and 4) mutations. Radiation, chemical mutagens, error-prone DNA replication or even viruses can induce mutations. Once activated oncogenes are thought to contribute directly to mitogenesis.

One way to classify oncogenes is to group them based on the cellular localization of their gene products. By this manner, two major classes were established: 1) nuclear and 2) cytoplamsic/membrane bound oncogenes (Weinberg, 1985). Oncogenes could therefore produce a mitogenic signal by upregulating growth related genes in two ways: 1) directly initiating DNA transcription, and 2) initiating signal cascade mechanisms which eventually lead to initiation of DNA transcription.

The myc oncogene is an example of mitogenesis produced by direct initiation of DNA transcription. Myc is a nuclear protein that shares

many structural characteristics with known transcription factors, mainly a protein structure that contains a basic motif, a helix-loop-helix motif, and leucine zipper motif (B-HLH-LZ). This B-HLH-LZ structure demonstrated high homology with the transcription factors USF (upstream stimulatory factor), and TFE3 (transcription factor E3) (Gregor et al., 1990; Bechmann et al., 1990). The ability of myc to function as a transcription factor was confirmed using both *in vitro* yeast and mammalian cell systems (Amati et al., 1992; Kretzner et al. 1992). The role of myc in cell proliferation was demonstrated in 3 ways: 1) myc expression patterns in normal cells, 2) deregulated myc expression effects on normal cells, and 3) genes specifically targeted by myc for transcriptional activation.

The expression pattern of the normal proto-oncogene provides valuable evidence as to the role that an activated gene has in proliferation. Normal Myc expression patterns showed that there was a rapid increase in myc mRNA following mitogenic treatment (Cochran et al., 1983; Cochran et al., 1984; Kelly et al., 1983; Greenberg and Ziff, 1984; Greenberg et al., 1985; Lau and Nathans, 1987). Myc expression alone was then demonstrated to be sufficient to induce G0 to G1 cell cycle transition (Coppola and Cole, 1986; Eilers et al., 1991). Conversely, microinjection of antisense oligonucleotides to myc mRNA blocked progression beyond G1 (Coffey et al., 1988; Heikkila et al., 1987;

Holt et al., 1988, Loke et al., 1988). These results suggested that the role of myc in proliferation was to drive the transition through G1 from G0.

The overexpression of myc produced cells displaying shortened G1, and increased growth rates (Karn et al., 1989; Palmieri et al., 1983; Langdon et al., 1988). Myc overexpression was even shown to be capable of partially transforming rat fibroblasts (Kohl and Ruley, 1987; Mougneau et al., 1984). However, complete malignant transformation required two oncogenes (Land et al., 1983; Lugo and Witte, 1989).

Myc, when activated, recognizes and binds the DNA sequence CA(C/T)GTG, which is termed the E-box Myc Sequence (EMS) (Blackwell et al., 1993; Blackwood and Eisenmann, 1991; Prendergast et al., 1991; Solomon et al., 1993). The genes that are specifically targeted by myc-EMS binding and subsequent transcription can be classified into two categories: 1) cell growth, and 2) cell-cycle progression. Those genes that have been associated with cell growth are ODC (ornithine decarboxylase), which is responsible for polyamine biosysthesis necessary for S phase of the cell cycle, eIF-2α and eIF-4E (eukaryotic initiation factor), which is in turn required for transcription, and CAD (carbamoyl-phosphate synthase/aspartate carbamoyl transferase/dihyroorotase), which is responsible for pyrimidine biosynthesis (Bello-Fernandez et al., 1993; Wagner et al., 1993; Rosenwald et al., 1993; Jones et al., 1996; Miltenberger et al., 1995).

The genes directly controlled by myc that have been associated with the cell cycle are CDC25A, which removes inhibitory phosphates from CDK2, $p27^{kip1}$, which is a CDK inhibitor and is negatively regulated by myc, and the transcription factor c/EBP-a, which is responsible for cell cycle arrest and is also negatively regulated by myc (Galaktionov et al., 1996; Steiner et al., 1995; Freytag and Geddes, 1992). Myc acts to remove inhibitory signals allowing the cell cycle to proceed.

Cytoplasmic and membrane associated oncogenes function in mitogenesis through the induction of signal transduction cascades which activate the transcription of growth related genes. The best known example of this is the ras oncogene. Ras is a guanosine triphosphatase (GTPase) which has a GDP-bound inactive form and a GTP-bound active form. In a similar fashion to myc, ras function in proliferation was demonstrated by examining normal and over-expression of ras protein in normal cells. The normal function of ras was found to be associated with cytokine receptor kinases like the EGF (epidermal growth factor) and PDGF (platelet-derived growth factor) receptors (Molloy et al., 1989; Ellis et al., 1990; Kaplan et al., 1990; Satoh et al., 1990).

Activation of receptor kinases, by ligand binding leads to the activation of ras (for a detailed review see Campbell et al, 1998). Briefly, ras functions by complexing with the Raf gene product, after which raf is subsequently activated. Raf then phosphorylates MEK1 and MEK2 (MAPK/ERK kinases). MEKs phosphorylate tandem threonine and

tyrosine residues of the MAPKs (mitogen activated protein kinase) Erk1 and Erk2 (extracellular signal regulated kinases). These MAPKs can then directly induce the gene expression by phophorylating transcription factors, like Elk1 or CREB (cyclic-AMP response element binding protein), that can then induce growth related transcription factor genes like fos and jun (Marais et al., 1993; Treisman, 1996; Ginty et al., 1994). The MAPKs can also activate other kinases like $p90^{RSK}$ a serine/threonine kinase which can also induce fos and jun gene expression (Blenis, 1993). Oncogenic ras mutations lock the ras protein in its active GTP-bound form keeping the cascade always induced (Satoh et al., 1992; Boguski and McCormick, 1993; McCormick, 1994). Mutations in ras genes are some of the most frequent mutations observed in human cancers (Barbacid, 1987; Hunter, 1997).

In addition to their proliferative function, oncogenes, like *ras* and the putative oncogene, bcl-2, can reduce or even block apoptosis. The bcl-2 gene's classification as an oncogene is based on its function as an apoptosis suppressor (Hockenbery et al., 1990). However, several oncogenes like Myc and the tumor suppressor gene, p53, can increase apoptosis and promote cell cycle progression. The opposing functions of the Myc oncogene in its stimulation of both proliferation and apoptosis may explain its inability to induce signigicant neoplastic transformation (Hayashi et al. 1998), in that, as the cellular population increases so to does the apoptosis. A precarious disruption between the birth and death

of cells is formed. Conversly, those chemical tumor promoters, like TPA, and oncogenes, like *ras*, that reduce/block apoptosis and GJIC, alone are not sufficient for complete neoplastic transformation of normal cells. However, when these chemical promoters or oncogenes act in cooperation with deregulated *myc* expression, the cells become neoplastically transformed (Land et al., 1983; Storer et al., 1988; Dotto et al., 1985; Hsiao et al., 1984; Yancopoulos et al., 1985) presumably through the additional blockage of apoptosis and GJIC.

The Role of Apoptosis in Carcinogenesis

Apoptosis, commonly referred to as programmed cell death, has been speculated to be a normal pathway to eliminate initiated or pre-malignant cells thus preventing carcinogenesis (Schulte-Hermann et al., 1995). Apoptosis and necrosis were described by pathologists as two morphologically distinct process by which cells die (Kerr et al., 1972). Necrosis is a passive osmosis-driven process, which is characterized by cellular swelling. The cellular swelling observed in necrosis is proceeded by the loss of ion-pumping activity, and distention of the endoplasmic reticulum. The nucleus swells and becomes electron transparent with small-scattered masses of chromatin. During later stages, the mitochondria swell and the plasma membrane ruptures (Haukins et al., 1972).

In stark contrast to necrosis, apoptosis is an active energy requiring process, which is characterized by cellular shrinkage. The early stages of apoptosis occur in the nucleus where chromatin condenses into one or multiple masses lining the nuclear membrane (Wyllie, 1981). As apoptosis proceeds, the nucleus splits into separate round bodies, the nuclear envelope becomes uneven, the cisternae become dilated, and the nuclear pores migrate to areas adjacent to uncondensed chromatin (Arends et al., 1990). Later stages of apoptosis are manifested by a volume loss in the cytoplasm. The cell shrinks and begins blebbing outward, forming blebs around the cell. This event is proposed to be the primary cause of shrinkage (Kerr et al., 1987). As the cell further shrinks, it splits into small round apoptotic bodies that are usually phagocytosed by adjacent cells. Inward blebbing is also known to occur (Clarke, 1991). In these instances the cell membrane is endocytosed to form clear vesicles that are indicative of lysosomal activation.

This initial description led to the observations that apoptosis was the primary event that led to regression of not only non-neoplastic tissues (Wyllie et al, 1973; Kerr and Searle, 1973) and preneoplastic foci in hepatocytes (Bursch et al., 1984), but also for the hormone-dependent regression of mammary (Gullino, 1980), pancreatic (Szende et al., 1989) and prostate (Kerr and Searle, 1973) cancer. Interestingly, Kerr and Searle (1972) also attributed the slow growth rate of basal cell carcinomas, which contain numerous mitotic markers, to apoptosis.

Animal studies demonstrated that food restriction eliminated 20-30% of the normal liver cells through apoptosis. More significant is the fact that in these same animals, putative preneoplastic lesions preferentially underwent apoptosis, thereby reducing their numbers by 85% and showing the selective role that apoptosis plays in initiated cell exclusion (Grasl-Kraupp, 1994). However, not until the isolation of the B-cell Lymphoma gene 2, Bcl-2 (Cleary et al., 1986), that directly affected this pathway, was apoptosis accepted as having a significant role in cancer.

Bcl-2 was discovered as a result of a translocation between chromosomes 14 and 18 present in most follicular lymphomas (Tsujimoto et al., 1984; Bakhshi et al., 1985). Vaux and colleagues (1988) demonstrated that bcl-2 could not only block the cell death that was associated with cytokine withdrawal, but its over-expression could also block the cell death associated with the over-expression of the myc oncogene. Bcl-2's effect on cell death and prolonged cell survival was confirmed with transgeneic mice, where bcl-2 over-expression expanded the B-lymphoid compartment 4 to 5 fold (McDonnell et al., 1989). However, unlike other genes that were reported to be involved in carcinogenesis, bcl-2 did not affect the proliferation rates, it only delayed or blocked cell death (Strasser et al., 1991; Sentman et al., 1991).

Additional findings reinforced the importance of apoptosis in cancer progression by demonstrating that chemotherapeutic anti-cancer agents like cis-platin, and etoposide, which were previously thought to

kill cells by causing irreversible metabolic damage, actually functioned as inducers of apoptosis (Searle et al., 1975; Kaufmann, 1989; Eastman, 1990). Consistent with these finding were the observations that many tumors possessed the ability to escape apoptotic stimulus (Hoffman and Liebermann, 1994). In fact, using bcl-2 as the paradigm for anti-apoptotic genes, it was shown that its over-expression was sufficient to confer a resistance to chemotherapeutic drugs (Miyashita and Reed, 1992). Therefore, bcl-2 over-expression in cancer has been associated with poor prognosis as seen in neuroblastoma (Castle et al., 1993), colon cancer (Hague et al., 1994), and especially prostate cancer (McDonnell et al., 1992).

To help visualize the role of apoptosis in cancer one can view it as a mathematical equation where A equals the accumulation of cells, P equals proliferation rate and D equals cell death,

$$A = P - D$$

Homeostasis could then be defined as,

P = D, and A = 0 (no net change in accumulation of cells)

It is also possible to demonstrate the effects of oncogenes on the accumulation of cells using this equation. Myc is known enhance proliferation, but is only very mildly turmorigenic, however it also sensitizes cells to limitations in nutrients. In this scenario as cells over-expressing Myc accumulate, they also begin to die as their energy sources become depleted. Therefore P is only slightly larger than D

$P > D$, $A = P - D$

and there is only a small accumulation of cells. Ras, which enhances proliferation and inhibits apoptosis, is significantly more tumorigenic than Myc. In the Ras scenario, P is significantly larger than D.

$P >>> D$, $A = P - D$

Here the accumulation of cells is similar to the proliferation rate. Using this equation, it is also possible to visualize how bcl-2, by blocking apoptosis could increase the accumulation of cells by blocking cell death where,

$P>>D$, $A = P - D$

Here, however, unlike Ras, there is little or no effect on proliferation and the accumulation of cells is dependent on extracellular effectors of proliferation. Therefore, if an initiated cell, over-expressing a Bcl-2 like gene, failed to undergo apoptosis, it would escape the normal cell exclusion and be in a position for clonal expansion. If myc overexpression was coupled to Bcl-2 overexpression, then the rate of proliferation would be disparagingly higher than cell death and effectively accumulation of cells would equal proliferation.

$P>>>>D$, $A = P - D$, effectively $A = P$

Bcl-2 Proto-oncogene and Carcinogenesis

The importance of apoptosis is not just limited to cancer, homologues of the bcl-2 gene have been identified in metazoans and even viruses, demonstrating the importance and conservation of apoptosis in multicellular organisms (Thompson, 1995). The bcl-2 proto-oncogene codes for a 239 amino acid protein that contains a ligand domain, a receptor domain, a pore formation domain, and a membrane insertion domain (membrane anchor) (Vaux et al., 1988). The bcl-2 protein can form homodimers and, in the cell, is localized to the mitochondrial inner and outer membranes, as well as the nuclear envelope and endoplasmic reticulum. The bcl-2 proto-oncogene is a member of a larger bcl-2 related family of genes (Figure 3). The bcl-2 family members are comprised of both *pro-survival* inhibitors and *apoptogenic* promoters of cell death. The two predominant members are bcl-2 representative of pro-survival, and bax representative of apoptogenic cell death (Gross et al., 1999). Bcl-2 and its relatives inhibit apoptosis, while bax and its relatives promote cell death.

Members of the inhibitor and promoter subfamilies typically share three of the four characteristically conserved bcl-2 homology (BH) sequence motifs, BH1, BH2 and BH3 (Muchmore et al., 1996; Sattler et al., 1997). Additionally, because of the similarities in sequence, members of both the bcl-2 and bax subfamilies can form similar protein

Figure 3. Summary of the Bcl-2 family of apoptosis modulators. Topology of the bcl-2 protein is depicted, showing regions of significant function and location of the bcl-2 homolgy (BH) domains. Additional, bcl-2 family members are shown illustrating regions of conservation of BH domains.

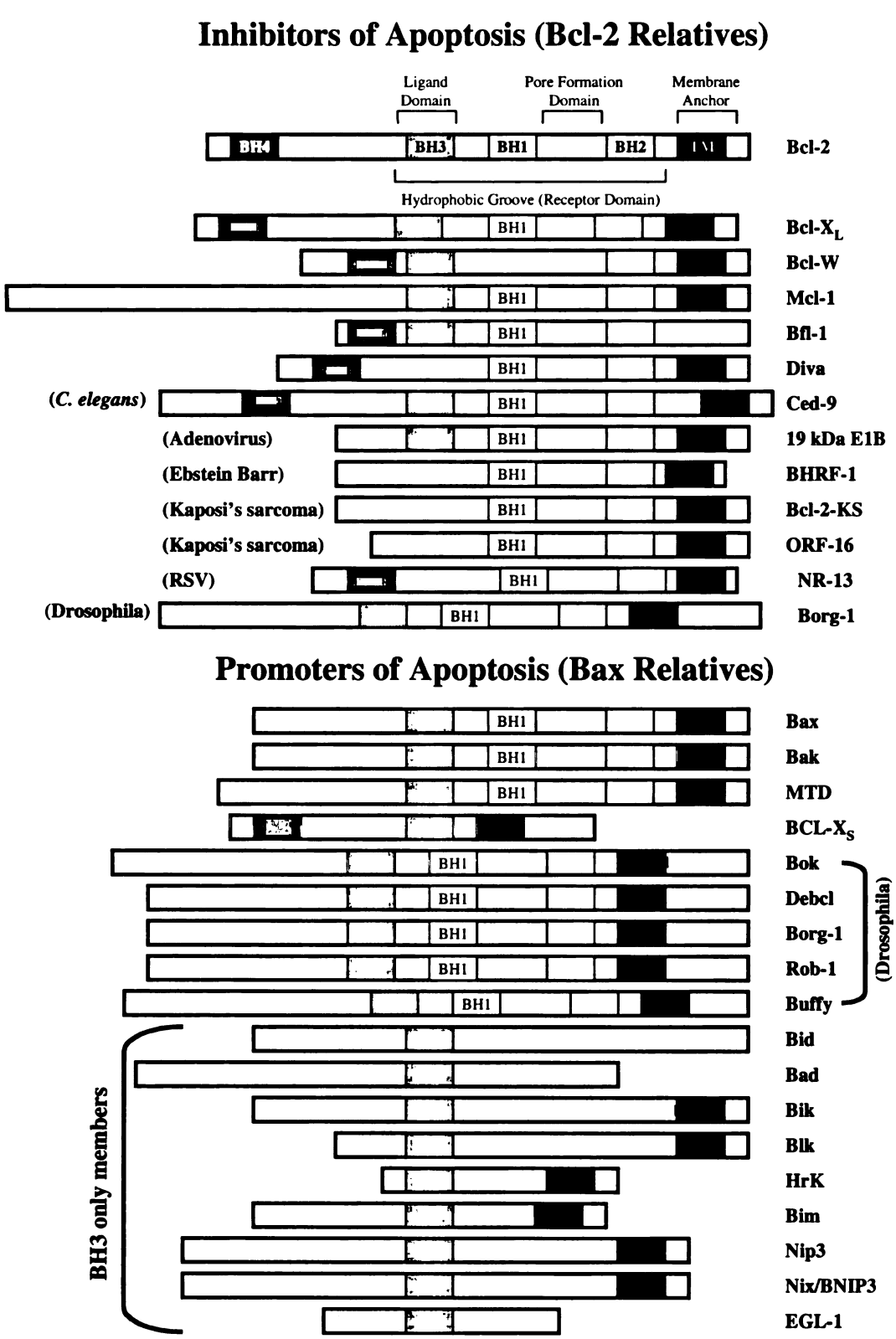

Inhibitors of Apoptosis (Bcl-2 Relatives)
Ligand Domain
Pore Formation Domain
Membrane Anchor
BH4
BH3
BH1
BH2
TM
Bcl-2
Hydrophobic Groove (Receptor Domain)
Bcl-X$_L$
Bcl-W
Mcl-1
Bfl-1
Diva
(C. elegans)
Ced-9
(Adenovirus)
19 kDa E1B
(Ebstein Barr)
BHRF-1
(Kaposi's sarcoma)
Bcl-2-KS
(Kaposi's sarcoma)
ORF-16
(RSV)
NR-13
(Drosophila)
Borg-1
Promoters of Apoptosis (Bax Relatives)
Bax
Bak
MTD
BCL-X$_S$
Bok
Debcl
Borg-1
Rob-1
Buffy
(Drosophila)
BH3 only members
Bid
Bad
Bik
Blk
HrK
Bim
Nip3
Nix/BNIP3
EGL-1

Figure 3

conformations. This similarity in protein conformation between the opposing subfamilies readily leads to heterodimerization, presumably allowing for initiation of apoptosis through the direct sequestration of bcl-2 family members' by the bax family members (Suzuki et al., 2000). This sequestration is achieved through the interaction of the ampipathic BH3 α helix of the apoptosis promoters with the hydrophobic groove, formed by the α helices in the BH3, BH1, and BH2 regions, of the apoptosis inhibitors (Sattler et al., 1997). However, there is a third subfamily, bearing little resemblance to each other or to the bcl-2 and Bax subfamilies, which contains only a BH3 domain and are promoters of apoptosis, suggesting that only the BH3 domain is necessary for the sequestration of pro-survival inhibitors (Kelekar and Thompson, 1998).

The proto-oncogenic importance of bcl-2 was demonstrated by genetic manipulation of the nematode *Caenorhabditis elegans*. Deletion of the ced-9 gene, a bcl-2 homologue, led to early embryolethality due to excessive developmental cell death (Hengartner et al., 1992). The effect of this deletion was rescued by the insertion of a human bcl-2 transgene (Vaux et al., 1992). This suggested a conservation of function between the mammalian bcl-2 and the metazoan ced-9 genes. Further studies have delineated that the bcl-2 proto-oncogene is involved in the regulation of a highly conserved cell death pathway, the caspase cascade, found in multicellular organisms ranging from metazoa to insects to mammals (reviewed in Budihardjo et al., 1999). Bcl-2 and its

homologues function as integral members of the caspase cascade, which serves as the culmination of apoptotic stimulating pathways (Figure 4). The caspases are a family of proteases that are responsible for the actual execution of apoptosis. Activation of the caspase cascade occurs with an apoptotic stimulus, ranging from radiation to DNA damage to cytotoxic chemicals, which activates apoptogenic proteins, inactivates pro-survival proteins, leading to the release of the cofactors, dATP and cytochrome C (Liu et al., 1996). These cofactors stimulate a caspase activator (initiator caspase) complex, referred to as the apoptosome, which leads to the activation of an executioner caspase and subsequent apoptosis (Hu et al., 1998; Pan et al., 1998).

How bcl-2 functions biochemically in apoptosis and the caspase cascade is still unclear. Putatively, bcl-2 can function in 3 ways; 1) direct interaction with non-bcl-2 members of the caspase cascade, 2) stabilization or perturbation of new or existing mitochondrial membrane channels, 3) other functions yet to be determined. Evidence from *C. elegans* supports a direct sequestration model of bcl-2 action, in which, ced-4, the caspase activator co-localizes with ced-9, and only when a death stimulus occurs is ced-4 displaced (Conradt and Horvitz, 1998). Since bcl-2 can rescue ced-9, it has been proposed that a similar survival mechanism exists in mammals (Hengartner and Horvitz, 1994).

The role of bcl-2 in the mitochondrial membrane is more empirical. Bcl-2 over-expression has been shown to block all disturbances

Figure 4. Diagrammatic illustration of the evolutionarily conserved caspase cascade which leads to apoptotic cell death. Typical proteins mediating five steps are depicted in boxes. Mammalian genes are listed first followed by the C. elegrans homologous gene then the Drosophila homologous gene. A nematode homologue of Caspase 3 has yet to be identified.

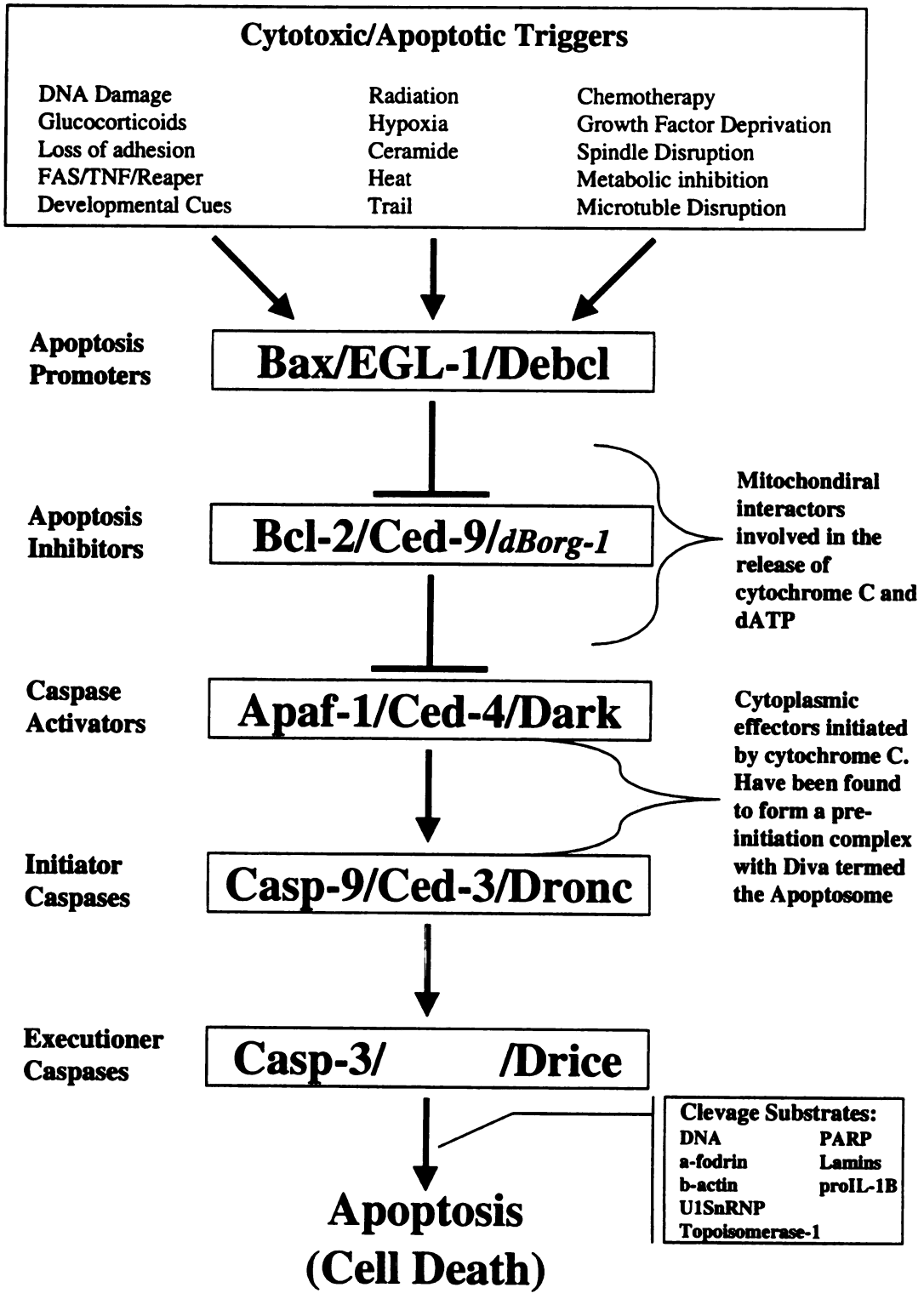

Cytotoxic/Apoptotic Triggers
DNA Damage
Glucocorticoids
Loss of adhesion
FAS/TNF/Reaper
Developmental Cues
Radiation
Hypoxia
Ceramide
Heat
Trail
Chemotherapy
Growth Factor Deprivation
Spindle Disruption
Metabolic inhibition
Microtuble Disruption
Apoptosis Promoters
Bax/EGL-1/Debcl
Apoptosis Inhibitors
Bcl-2/Ced-9/dBorg-1
Mitochondiral interactors involved in the release of cytochrome C and dATP
Caspase Activators
Apaf-1/Ced-4/Dark
Cytoplasmic effectors initiated by cytochrome C. Have been found to form a pre-initiation complex with Diva termed the Apoptosome
Initiator Caspases
Casp-9/Ced-3/Dronc
Executioner Caspases
Casp-3/ /Drice
Clevage Substrates:
DNA
a-fodrin
b-actin
U1SnRNP
Topoisomerase-1
PARP
Lamins
proIL-1B
Apoptosis
(Cell Death)

Figure 4

associated with the mitochondria that are attributed to apoptosis, such as changes in membrane permeability, pH, and overall integrity (Green and Reed, 1998). Additionally, the three dimensional crystalographic structure of the bcl-x_L gene, a splice variant of bcl-2, is similar to pore forming bacterial toxins (Muchmore et al., 1996). These findings suggested that bcl-2 might function to either: 1) form new pores in the mitochrondrial membrane, which has yet to be shown in cells or under physiological conditions, or 2) effect (either stabilize or perturb) existing channels, like the permeability transition pore (PT) comprised of a voltage-dependent anion channel (VDAC) in the outer membrane and the adenine nucleotide translocator (ANT) in the innner memebrane (Crompton, 1999). The interaction of the bcl-2 family members and mitochondrial pores has been shown using VDAC channels in liposomes (Shimizu et al., 1999). Bax was able to stabilize an open conformation that allowed for the free passage of cytochrome C, while bcl-xL perturbed the VDAC channel keeping it in a closed conformation. Additionally, bax has also been shown to interact with the ANT channel (Marzo et al., 1998).

Bcl-2s role in apoptosis may involve molecular interactions yet to be fully understood. As yet, no functions have been associated with the localization of bcl-2 to the endoplasmic reticulum (ER) or the nuclear envelope. It has been suggested that bcl-2 localization to the nuclear envelope and ER may involve intracellular Ca^{2+} stores. The major

storage site for Ca^{2+} resides between the nuclear membrane and its continuity with the ER lumen. There has been growing evidence for the role of bcl-2 in the modulation of these ER Ca^{2+} stores. He and colleagues (1997) were able to demonstrate that the depletion of the ER calcium is a possible trigger of apoptosis, and that bcl-2 overexpression inhibits ER calcium loss. In this role bcl-2 appears to be able to assist in the maintenance of the ER calcium pool, thereby blocking apoptosis at a step prior to caspase activation and independent of mitochondria. Recently, bcl-2 has been shown to actively decrease the free Ca^{2+} in the cytoplasm by upregulating the ER calcium pump (Kuo et al., 1998) and capacitative Ca^{2+} entry (Williams et al. 2000). These two systems represent the major processes by which calcium is pumped into the ER.

Less studied is the role of bcl-2 localization to the nuclear envelope. Early electron micrographs showed that bcl-2 formed highly organized patches resembling nuclear pore complexes (Krajewski et al., 1993) suggesting an active role for bcl-2. Recent evidence supports this finding in that bcl-2 was able to inhibit wild-type p53 nuclear import following DNA damage (Beham et al., 1997). This suggests that bcl-2 may act as some form of a "gatekeeper" selectively controlling protein traffic into the nucleus.

Lastly, bcl-2 localization to the mitochondria, nucleus and ER, suggests that bcl-2 may act as an oxidative stress sensor monitoring free radicals, for these compartments are postulated as the major areas of

free radical production (Thompson, 1995). Oxidative stress has been defined as "a disturbance in the prooxidant-antioxidant balance in favour of the former, leading to potential damage" (Sies, 1991). The major agent for the prooxidant state is the free radical. A free radical is a chemical species that possesses one or more unpaired electrons. These free radicals tend to be highly reactive and can readily lead to DNA damage. Many carcinogens function to induce DNA damage through free radical generation, however this has the side effect of inducing apoptosis (Clemens, 1991; Witz, 1991; Boref, 1991). Initial experiments showed that bcl-2, in addition to blocking apoptosis, was able to protect cells against hydroxyl radicals formed from gamma-radiation (Sentman et al., 1991; Strasser et al., 1991). Additionally, bcl-2 has been shown to attenuate lipid peroxidation which can lead to DNA damage (Hockenbery et al., 1993; Kane et al., 1993; Tyurina et al., 1997). Exactly how bcl-2 functions in free radical protection is not known. One mechanism by which oxidative stress and free radicals function is to cause fluxes in intracellular calcium levels, which as earlier discussed, can be blocked by bcl-2. However, and more importantly, bcl-2 has been demonstrated to upregulate the genes that act as free radical scavengers such as intracellular thiols (Bojes et al., 1997), like thioredoxin, and reduced glutathone (GSH) (Kane et al., 1993; Mirkovic et al., 1997; Voehringer et al., 1998). These proteins function in the cell as the major intracellular antioxidents.

Interestingly, oxidative stress has also been speculated to play a role in the promotion phase of carcinogenesis (Perera et al., 1987; Cerutti, 1989). Many known liver tumor promoters have been shown to induce oxidative stress (Cattley and Roberts, 2000; Roberts et al., 2000; Perera et al., 1987) at levels that do not apparently affect apoptosis. Additionally, down regulation of GJIC has also been speculated to be associated with oxidative stress signals, suggesting a possible mechanism by which tumor promoters affect gap junctions (Upham et al., 1997).

In conclusion, promotion involves mitogenesis, blockage of apoptosis, and blockage of GJIC. Classical tumor promoters can function to induce mitogenesis, block apoptosis, and down regulate GJIC, speculatively through oxidative stress. Oncogenes can function in similar manners as tumor promoters to obtain the same results.

Experimental Rationale

If one accepts the stem cell theory of carcinogenesis (Trosko and Chang, 1989), then the blockage of terminal differentiation (Potter, 1978) may be involved in the "initiation" phase of carcinogenesis, while clonal expansion (mitogenesis), inhibition of apoptosis, and down regulation of GJIC would contribute to the "promotion" phase (Shulte-Herman et al., 1995; Trosko and Goodman, 1994). Therefore, over expression of various oncogenes , cooperating with each other, might be involved in all phases of the initiation/promotion/progression model to carcinogenesis.

The cooperation of oncogenes was one of the first important molecular concepts of carcinogenesis. One of the first cooperative interactions to be reported was between the myc and ras oncogenes (Land et al., 1983; Lee et al., 1985). The myc oncogene, a transcription factor, has been associated with the cellular functions of proliferation, differentiation and apoptosis (Gandarillas, 1997; Evan et al., 1994; Koskinen and Alitalo, 1993; Evan et al., 1992; Askew et al., 1991). Disruption of any of these basic cellular functions could contribute to the multi-step, multi-mechanism process of carcinogenesis as was demonstrated by the association of myc over-expression with many types of tumors. (Cole, 1986; Kelly and Siebenlist, 1986) Similarly the ras oncogene, being a member of the G- protein family, has been shown to be involved in one of the many signal transduction pathways affecting

mitogenesis, differentiation and apoptosis (Campbell et al., 1998). Ras, as with myc, has been shown to be activated in many types of tumors (Suarez, 1989; Nishimura and Sekiya, 1987).

In addition, the demonstration that phorbol esters, such as TPA, seemed to act in a manner similar to the ras oncogene, in that they could cooperate with the myc oncogene to induce a neoplastic phenotype (Connan et al., 1985). Thus, TPA, by activating protein kinase C, triggers signal transduction pathways and can act as a modulator of mitogenesis, differentiation and block apoptosis, in affect acting as a surrogate "ras" gene (Magnelli et al., 1995; Nishizuka, 1992; Rodriguez and Lopez, 1989).

Our laboratory and others have shown that, while ras and TPA effect signal transduction, they also effect gap junction-mediated intercellular communication (GJIC) (de-Feijter et al., 1992; de-Feijter et al., 1990; Murray and Fitzgerald, 1979; Yotti et al., 1979). Gap junctions are channels that directly link the interiors of neighboring cells allowing for the free diffusion of small molecular weight molecules (Spray and Bennet, 1985). Most tumors demonstrate a reduction in GJIC activity, either between themselves (homologous GJIC) or with other cell types (heterologous GJIC) (Loewenstein, 1981). Presumably, down regulation of GJIC activity would lead to the removal of growth inhibitory signaling, thereby providing a selective advantage to the initiated or neoplastic cells. TPA was the first chemical agent shown to reduce GJIC activity

(Yotti et al., 1979; Murray and Fitzgerald, 1979). Rat liver epithelial cells stably transfected with Ha-ras demonstrate a dose-dependant reduction of GJIC with increasing levels of ras T24 protein (de-Feijter et al., 1992; de-Feijter et al., 1990; El-Fouly et al., 1989). Recently our laboratory reported that Ha-ras and v-myc could cooperate to down regulate GJIC and this down regulation correlated to the cells malignancy (Hayashi et al., 1998).

Ras and myc oncogenes have also been implicated in apoptosis (Hoffman and Liebermann, 1998; Sakai et al., 1994; Arends et al., 1993). Where myc seems to confer a susceptibility to apoptosis (Donzelli, 1999; Hoffman and Liebermann, 1998; Evan et al., 1992), ras appears to reduce the cell's response to apoptosis (Sakai et al., 1994; Arends et al., 1993). Therefore, in cells in which both myc and ras are activated, signal transduction cross-talk interacts to block differentiation, triggering cellular proliferation, resistance to apoptosis, and subsequent down regulation of GJIC. In effect, these unregulated disruptions of interacting signals bring about the appearance of the tumor.

The bcl-2 protooncogene has been one of the major genes implicated in the apoptotic process (reviewed by Korsmeyer, 1999). Early experiments in transgenic animals overexpressing bcl-2 under immunoglobulin promoter control showed increased frequency of follicular hyperplasia and B-cell survival thus suggesting the role of bcl-2

as an anti-apoptotic gene (McDonnell et al., 1989). Since this work, many have demonstrated that bcl-2 is not only involved in tumors of lymphoid origin but also in many tumors of epithelial origin (Dellas et al., 1997; Wang et al., 1998, Jiang et al., 1995; Gee et al., 1994; Hague et al., 1994; Leek et al., 1994; Lu et al., 1993;, McDonnell et al. 1992). Additionally, bcl-2 has also been demonstrated to synergistically interact with TPA to transform cells (Amstad et al., 1997). Thus, by blocking apoptosis, bcl-2 seems to be acting as a surrogate "ras" gene. However, bcl-2 itself is unable to induce proliferation or neoplastic transformation (Del-Bufalo et al., 1997; Lu et al., 1995; Nataray et al., 1994).

Many investigators have studied the co-expression of myc and bcl-2 in a variety of cell types and transgenic models (Jager et al., 1997, Fanidi et al., 1992; Strasser et al., 1990; Vaux et al., 1988). These studies were able to show that bcl-2 and myc can cooperate and increase or enhance the tumor incidence and formation. However, the cooperative assays studying myc and bcl-2 have focused on cells of lymphoid origin, fibroblasts, and targeted promoter controlled vectors, but not in other epithelial systems. The experiments described in this study were designed to test whether the bcl-2 oncogene in cooperation with v-myc could function, neoplastically, to transform the WB-F344 cell line, a normal rat liver epithelial cell line. Our laboratory has previously reported the establishment of a WB-F344 cell line stably expressing the

v-myc oncogene (Hayashi et al., 1998). This cell line demonstrates an increase in proliferation and cell saturation density but it did not form colonies in soft agar or tumors in nude mice. This cell line was used as the target for transformation by bcl-2. In this manner, this report investigated the effect that bcl-2 alone and in cooperation with v-myc would have on neoplastic transformation and GJIC.

Experimental Approach

WORKING HYPOTHESIS

Carcinogenesis has been described as a multi-step process involving three ordered temporal phases, initiation, promotion, and progression. Initiation is characterized by an irreversible genetic event in a single cell that has the potential to proliferate, i.e. the stem cell, which renders that cell sensitive to clonal expansion. The promotion phase has been speculated to include the selective accumulation of the initiate cell via mitogenesis (proliferation), and the escape from apoptosis, which would normally eliminate the initiated cell. Many oncogenes and tumor promoting chemicals have been shown to contribute to mitogenesis and blockage of apoptosis. In addition, these same oncogenes and tumor promoters have been shown to block intercellular signaling through gap junctions. Gap junctional intercellular communication has been postulated as the means by which cells communicate and transfer signals that would suppress growth and induce apoptosis. The objective of this study was to test the hypothesis that cooperation of proliferation, apoptosis, and GJIC can lead to the neoplastic transformation of normal cells. To accomplish this, oncogenes that influenced proliferation and apoptosis were transfected independently and together into normal epithelial cells and subsequently assayed for neoplastic potential and GJIC in the following manner:

SPECIFIC AIMS

1. Construction of WB cell lines that express bcl-2 and bcl-2 plus myc.

    A. Transfection of the bcl-2 plasmid along with appropriate vector controls, into rat liver epithelial cells, WB-F344, and WB.myc cell lines.

    B. Isolation of multiple clones (This will eliminate the interpretation complications associated with "insertional mutagenesis".).

2. Characterization of clonal cell lines: WB.bcl-2; WB.myc/bcl-2,

    A. Determine bcl-2 expression by western blot analysis.

    B. Test for phenotypes of neoplastic transformation, using soft agar assay for the determination of anchorage independent growth and an *in vitro* invasion assay to determine cellular motility.

    C. Test GJIC by the scrape loading dye transfer technique, and flourescent recovery after photobleaching (FRAP), in those clones expressing bcl-2.

    D. In those clones demonstrating altered GJIC, determine the level of CX43 protein.

3. Quantitate apoptosis frequency using fluorescence activated cell sorting (FACS).

4. Compare the differentiation potentical of bcl-2 and v-myc transformed clones with the parental WB-F344 cells.

MATERIALS AND METHODS

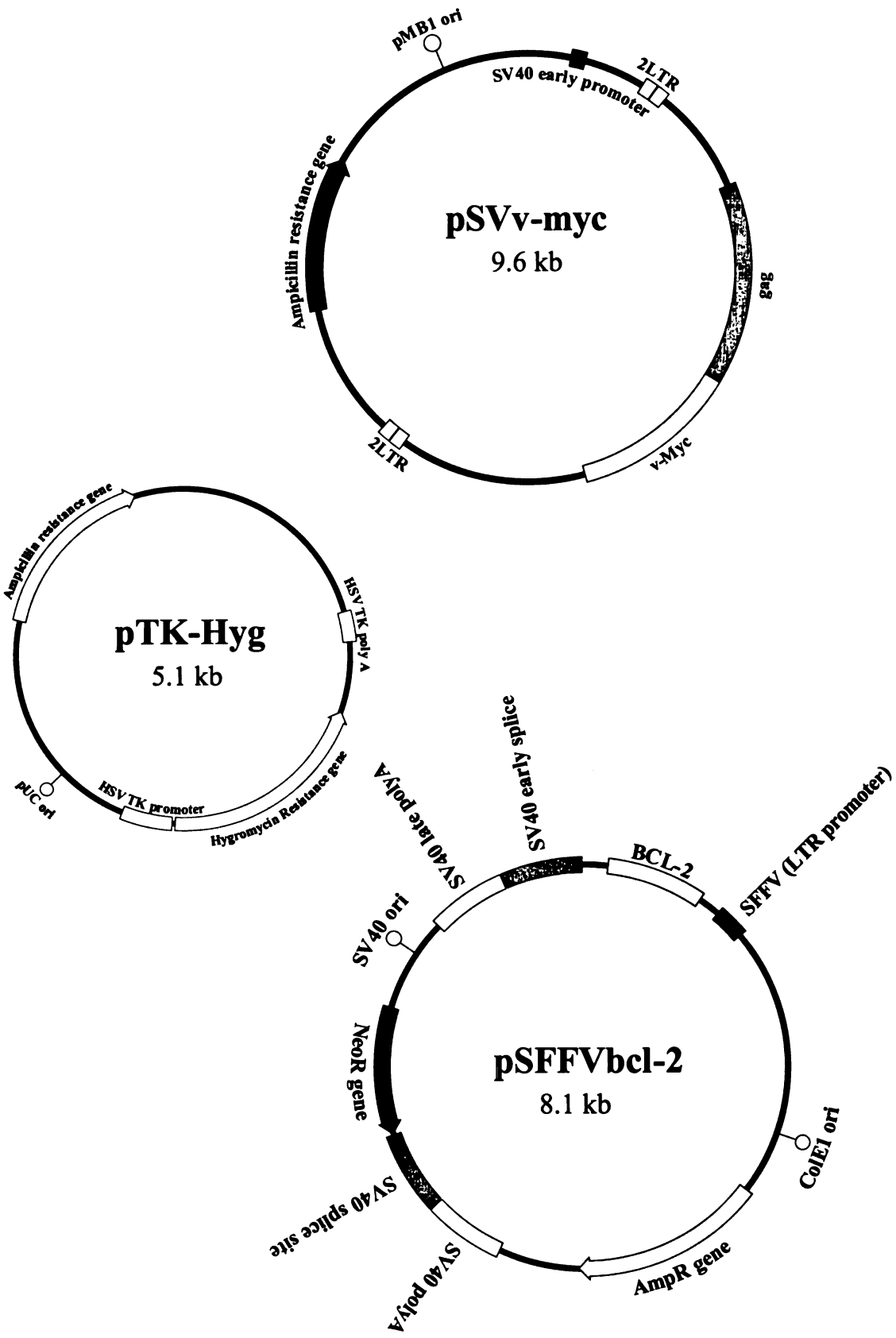
Cell Culture

WB-F344 rat liver epithelial cells, WB.myc and all subsequent bcl-2 transfectants were cultured in a modified Eagle's minimal essential medium (MEM) (Chang et al., 1981), Formula 78-5470EF, Life Technologies Inc. (Gibco/BRL, Gaithersburg, MD), supplemented with 7% fetal bovine serum (Life Technologies Inc. (Gibco/BRL, Gaithersburg, MD) and appropriate antibiotic drug for selection. All cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Transfection of bcl-2

The human Bcl-2 cDNA cloned into the pSFFV vector (kindly provided by Drs. Gabrial Nunez and Valerie Castle, University of Michigan, Ann Arbor, MI), was transfected into WB-F344 cell line and co-transfected with pTK-Hyg (Clontech Laboratories Inc., Palo Alto, CA) into the WB.myc (see Figure 5 for vector maps) cell using lipofectin (Roche Molecular Biochemicals, Indianapolis, IN). Specifically, 40 μg of Lipofectin was added to 1.5 milliliters of D-Medium (serum free) and 20 μg of DNA was added to a separate 1.5 ml of D-medum. The two samples were gently mixed and incubated at room temperature for 15 minutes. This mixture was then added to sub-confluent cultures grown in 100 mm dishes that had been rinsed twice with serum free D-medium. The plates

Figure 5. Schematic diagrams of the various vectors used in this research. pSVv-myc was purchased from the American Type Culture Collection. Briefly, pSVv-myc was created by ligating an *EcoRI-Kpn1* Restriction fragment from the provial DNA into the the *EcoRI* and *Kpn1* sites of pSV2gpt (Land et al., 1983). This step effectively deletes the gpt selection and inserts the viral two long terminal repeats (LTRs). Then an entire provial fragment of the avian MC29 was ligated into the *EcoRI* site. The remaining plasmid contains the gag-myc viral genes flanked by two LTRs, and a SV40 early promoter in the opposite polarity to the direction of transcription of the viral DNA. The plasmid pTK-Hyg (Genbank accession number U40398), was purchased from Clonetech Laboratories (Palo Alto, CA) and contains a hygromycin resistance gene flanked by a herpes simplex virus TK promoter (for transcriptional initiation) and a HSV TK polyadenylylation signal. The pSFFVbcl-2 vector was kindly provided by Dr. Valerie Castle and Dr. Gabrial Nunez at the University of Michigan. Briefly, pSFFVbcl-2 was created by ligating a fragment from pFP502 containing the Friend spleen focus-forming virus 5` LTR into pBluescript (Stratagene, La Jolla, CA.). Then a fragment from pSV2cat containing the SV40 early splice region and late polyadenylylation signals was ligated into SFFV/Bluescript. For mammalian selection a fragment for pSV2neo containing the neomycin resistance gene was ligated into SFFV/SV40/Bluescript, creating pSFFVneo (Fuhlbridgge et al., 1988). Bcl-2 was then cloned into the EcoRI site of pSFFVneo. All plasmids used contained ampicillin resistance gene for selection in bacteria.


pMB1 ori
SV40 early promoter
2LTR
gag
v-Myc
2LTR
Ampicillin resistance gene
pSVv-myc
9.6 kb
Ampicillin resistance gene
HSV TK poly A
pTK-Hyg
5.1 kb
pUC ori
HSV TK promoter
Hygromycin Resistance gene
SV40 late polyA
SV40 early splice
BCL-2
SFFV (LTR promoter)
SV40 ori
NeoR gene
pSFFVbcl-2
8.1 kb
ColE1 ori
SV40 splice site
SV40 polyA
AmpR gene

were then incubated overnight at 37°C in a humidified chamber. The following day D-medium containing 7% fetal bovine serum (FBS) was added directly to the plates and incubated an additional 24 hours. Transformed cells were then split and subsequently plated with appropriate drug selection. Individual clones were then isolated using cloning rings, and subsequently recloned. Due to enhanced apoptotic properties of the WB.myc cells, co-transfection with pTK-Hyg was performed as described above with the following modifications: first, the culture was allowed to reach confluency; second, 20 μg of DNA in a ratio of 1:20 pTK-Hyg:pSFFV.bcl-2 was used to assure that selection using hyromycin B would yield bcl-2 protein positive cells. Preliminary screening of any clones was carried out using a modified dot blot assay. Putative clones were cultured in 35 mm dishes and extracted as outlined in the following section. Extracts were diluted with an equal volume of sterile distilled water, dot blotted onto Immobilon-P PVDF membrane (Millipore Corporation, Bedford, MA) and proceeded to detection as described later in the Western Blot Analysis section. Human bcl-2 protein positive clones were identified and subsequently recloned.

Protein Extraction

Total protein was extracted from confluent cell cultures grown in 25 cm^2 flasks using 20% sodium dodecyl sulfate (SDS) containing 2mM

phenylmethylsulfonyl fluoride (PMSF), 1 μM aprotinin, 1 μM leupeptin (Roche Molecular Biochemicals, Indianapolis, IN 46250), 1 μM antipain, 5 mM sodium fluoride (Fluka, Milwukee, WI), 0.1 mM sodium orthovanadate (Aldrich, Milwukee, WI). Protein extracts were subsequently sonicated three times at five-second intervals, aliquoted and stored at –20°C. The protein concentration was determined by diluting the extracts 1:5 and assayed using the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA).

Western Blot Analysis

Total proteins extracts were loaded in equal amounts (10-15 mg) into each well of a 10% SDS-polyacrylamide, in accordance with Laemmli (1970). The gels were electrophoresed at 200 volts for approximately 45 minutes, removed and equilibrated in transfer buffer and transferred to Immobilon-P PVDF membrane (Millipore Corporation, Bedford, MA). Human Bcl-2 was detected with hamster anti-human bcl-2 monoclonal antibody (Pharmingen, San Diego, CA), v-myc was detected with anti-v-myc polyclonal antibody (Caltag Laboratories, Burlingame, CA), using the supersignal ultra substrate (Pierce Chemical Co. Rockford, IL) and appropriate peroxidase labeled secondary antibody.

Measurement of Saturation Density

Multiple 60-mm dishes were seeded with $1x10^5$ cells in D-medium containing 7% FBS. Plates were trypsinized and cell counts were made 3, 4, 5, and 6 days after initial plating. The saturation density represents the maximum number of cells per cm^2 for each cell type and is presented as the average density of quadruplicate plates from three independent experiments.

Measurement of Cellular Proliferation

Cellular proliferation (growth) was assayed by staining cell cultures with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as previously described by Alley et al. (1988). A MTT stock solution was made to a final concentration of 5 mg/ml in phosphate buffered saline (PBS). Cell cultures are plated in 96 well microtiter plates at a density of $5x10^3$ cells per well. Four hours prior to assaying, 50 µl of 1:5 dilution of the MTT stock is added to each well and incubated at 37°C. Each well was then aspirated and 150 µl of dimethyl sulfoxide (DMSO) was added to each well to extract the MTT from the cells. Plates were then shaken for 1 minute to thoroughly solubilize the MTT stain. Plates were then measured for absorbance of the wells on a Titertek plate reader at 540 nm. The data reported represents quadruplicate wells from triplicate experiments.

Apoptosis

Cultured cells are trypsinized and centrifuges for 5 minutes at 1000g. Cells are then washed twice in PBS by alliquoting 500 µl of PBS and pipetting repeatedly to mix, then centrifugation for 5 minutes at 1000g. The final wash was decanted and the cells were fixed in 70% ethanol overnight at 4°C. Samples were then transferred to –20°C for long term storage. Samples were stained in propidium iodide and run on a FACS according to Fraker and co-workers (1995).

Anchorage Independent Growth

To assess anchorage independent growth, cells were grown in soft agarose as previously described (Kao et al., 1995). Briefly, an initial hard agarose layer (0.5% agarose 6013, Sigma Chemical Co.), made in D-medium containing 7% FBS, was plated in a 60 mm dish. Individual cell lines were then trypsinized, counted, and diluted to a final concentration of $1x10^4$ total cells in D-Medium containing 0.33% agarose and 7% FBS and was subsequently plated on top of the 0.5% agarose layer. Additional medium was added 3 days later and changed every 3 days for 28 days. Colonies were stained using 1 mg/ml 2-(p-iodophenyl)-3-(nitrophenyl)-5-phenyltetrazolium chloride (Sigma) in 0.9% NaCl, and

counted and is reported as the percentage of a positive control from triplicate plates assayed in quadruplicate experiments.

In Vitro Invasion Assay

The ability cells to invade through Matrigel (Collabrotive Biochemicals), a reconstituted basement membrane, was examined using a Boyden chamber *in vitro* invasion assay as previously described (Bello et al. 1997). The assay was conducted as follows: on the day prior to running the invasion assay, the cell lines were plated in T-75 flasks in 10 ml of D-medium with 7% FBS. Matrigel was diluted 1:20 with cold (4^{0}C), sterile distilled water on ice for a final concentration of 500 µg/ml. Each Nuclepore filter was coated with 25 µg Matrigel in 50 µl and left to dry overnight at room temperature under sterile conditions. Cells were rinsed with PBS 24 hours after plating and incubated with 3 ml of 1 mM EDTA for 8-10 min, dislodged by tapping and recovered by centrifugation. Pellets were re-suspended to obtain 2x10^6 cells per milliliter. The bottom chamber contained 220 µl of NIH/3T3 cell conditioned medium, which served as a chemo-attractant. For preparing conditioned medium, subconfluent NIH/3T3 cultures in 100 mm plates were fed with 7 ml of serum-free DMEM containing 50 µg/ ml ascorbic acid. Conditioned medium was collected 24 h later, centrifuged to remove cell debris and stored at -20^{0}C. A cell suspension containing

$4x10^5$ cells in 200 µl medium was added to the top chamber and allowed to remain undisturbed for 5 min before overlaying with 650 µl of medium. Cells were allowed to invade for 24 h at 37°C at which time the filters were prepared for absorbance reading. The migrated cells were fixed, stained with HEMA-3 and allowed to hydrate in distilled water. Nuclear stain was extracted by placing individual filters into individual wells of a 24-well plate containing 300 µl of 0.1 N HCl for 15 min. 200 µl from each well was then placed in a 96 well plate and the absorbance at 620 nm was measured using a Titertek microplate reader. In addition to evaluating the number of migrated cells on the filter, cells that had migrated to the bottom chamber were also counted. Medium in the bottom chambers was mixed by pipetting five times and 100 µl were taken from each of the three chambers, pooled into cuvettes containing 10 ml Isoton, mixed by inversion and counted on a Coulter ZM cell counter. Three replicate invasion chambers were prepared per treatment. All experiments were done in triplicate.

Cell-Cell Communication Assays

Two methods developed in our laboratory were used to assess gap junction-mediated intercellular communication: (a) scrape-loading dye transfer (SL/DT) (El-Fouly et al., 1987); and (b) fluorescence recovery after photobleaching (FRAP) (Wade et al., 1986). The scrape-loading dye

transfer assay utilizes confluent cultures grown in 35-mm dishes. The cultures are then rinsed three times with PBS containing Ca2+ and Mg2+ (Ca2+,Mg2+ -PBS). 1.5 milliliters of PBS containing 0.05% Lucifer yellow CH (Molecular Probes Inc., Eugene, OR) were added, and several scrapes (cuts) were made on the monolayer using a surgical scalpel. The cultures were incubated for 3 minutes at room temperature in the dye solution, and then rinsed three times with Ca2+, Mg2+-PBS (to remove any background fluorescence). The cultures were then fixed with 1 milliliter of 4% formalin and visualized using an Ultima laser cytometer (Meridian Instruments, Lansing, MI).

The scrape-loading dye transfer assay results were confirmed using the FRAP assay. Briefly, cultures grown in 35-mm dishes were rinsed 3 times with Ca2+, Mg2+-PBS and then incubated with Ca2+, Mg2+-PBS containing 5,6-carboxyfluorescein diacetate (7mg/ml, Molecular Probes Inc., Eugene, OR) at 37°C in a humidified incubator for 15 minutes. The cells were then rinsed several times with Ca2+, Mg2+-PBS and analyzed using the Ultima laser cytometer. Cells were randomly selected under a microscope (four cells were selected per field plus one unbleached control, five fields per scan), and photobleached with an argon laser beam. The transfer of fluorescence was then monitored in 4-minute intervals for a total of 12 minutes. The intensity of recovered fluorescence of the individually bleached cells is then quantitated and

rates of dye transfer can be measured as the percentage of an unbleached control cell (one per field).

Quadruplicate dishes were run per treatment group and the mean percent cells dye couples was calculated. Statistical analysis using the Kruskal-Wallis test followed by Dunnett's post hoc test was used to compare the control to treatment groups and all subsequent pairwise comparisons.

Glutathione Determination

Cells were lysed with two milliliters of 0.05 normal perchloric asid and the lysate was filtered through a 0.22 micron filter. The lysate was fractionated using an 150 mm X 4.6 mm adsorbophere C18 5 μ MF-Plus HPLC column (Alltech Associates, Deerfield, Ill.) and a mobile phase that consisted of 50 mM sodium phosphate, 0.05 mM 1-octanesulfonic acid, and 2% acetonitrile at a pH of 2.70 and a flow rate of 1.0 ml/minute. The HPLC system used was a 580 solvent delivery module from ESA (Chelmsford, MA). The glutathione was detected with a Coulochem Model 5200 electrochemical detector (ESA, Chelmsford, MA) with applied potentials of +400, +900, and +950 mV set for the screening, analytical, and guard cell electrodes, respectively.

Tyrosine Aminotransferase Assay

Cells were cultured for 6 days in D-MEM medium containing 7% FBS and 3.75 mM sodium butyrate. All cultures were supplemented with $1x10^{-7}$ M dexamethasone for the last 24 hours as previously described (Coleman et al., 1994). Cells were harvested by trypsinization and counted prior to lysis in a buffer containing 140 mM KCL, 125 mM KPO_4 (pH 7.6), 1 mM EDTA, and 1% NP-40. Insoluble material was removed by centrifugation, and protein concentrations were determined by the Biorad DC protein assay (Bio-Rad Laboratories, Hercules, CA). Tyrosine aminotransferase activities were determined using the method of Granner and Tomkins (1970), as modified from Diamondstone (1966). Enzymatic activities were calculated using the molar extinction coefficient for p-hydroxyphenylpyruvate formed per minute per $1x10^5$ cells.

RESULTS

I. Synergistic effects of Bcl-2 and V-myc in the expression of neoplastic phenotype of Rat Liver Epithelial cells

Isolation and selection of bcl-2 and myc/bcl-2 transfectants

The initial strategy to investigate the interaction between v-myc and bcl-2 in neoplastic transformation and GJIC was to generate clonal cell lines over-expressing human-bcl-2 alone and co-expressing human bcl-2 and v-myc by transfecting previously established normal WB-F344 and WB.v-myc, cell lines respectively. This methodology required the cloning of the human bcl-2 gene into a different vector, namely $pSV2_{dhfr}$. This approach was necessary because the established WB.v-myc cell line already possessed geneticin (G418) resistance. This same mammalian selection marker was also contained within the pSFFV.bcl-2 vector (provided by Dr Valerie Caslte and Gabriel Nunez). Hence, if this vector had been transfected into WB.v-myc, it would not be possible to distinguish between the untransfected and transfected colonies. Accordingly, the bcl-2 insert was excised from the pSFFV vector using the restriction enzyme EcoRI and ligated into the $pSV2_{dhfr}$ vector. Upon examination of the amplified plasmid, the insert had incorporated in the anti-sense position. Subsequent recloning was attempted without success. Therefore, a different approach was used to study the interaction between v-myc and bcl-2 oncogenes by co-transfecting WB.v-

myc with pSFFV.bcl-2 simultaneously with an additional plasmid that would contain a suitable selection marker. Since this method required no additional cloning transfections were performed on the normal WB-F344 cells in conjuction with the transfections being performed on WB.v-myc. WB-F344 cells were transfected with either pSFFV vector control or pSFFV.bcl-2 vector by lipofectin treatment and subsequently followed by selection in (G418) for 14 days. Single colonies were isolated, and tested for bcl-2 expression using Western dot blot screening and an anti-bcl-2 antibody. All bcl-2 positive clones were subsequently re-cloned and stored in liquid nitrogen for preservation.

Several initial attempts to co-transfect WB.v-myc were made using the pSFFV.bcl-2 vector and the pKT203 vector, which contained a hygromycin resistance gene as a mammalian selection marker. These attempts failed to yield any hygromycin resistant cells. Due to the success in obtaining WB.bcl-2 clonal cell lines, any errors that may have been associated with pSFFV.bcl-2 were ignored and a different vector with hygromycin resistance was sought. PTK-Hyg, purchased from Clontech Laboratories (Palo Alto, CA), was used for its specific optimization in co-transfection studies. Again, several attempts failed to transfect WB.v-myc cells. In order to determine the possible cause(s) for these failures, close scrutiny to the culture conditions was observed during the co-transfections with pTK-Hyg. WB.v-myc cells were monitored for the initial 24 hours after serum-free lipofectin treatment

and microscopic evaluation was performed every hour noting any alterations in the culture appearance. Under these conditions WB.v-myc cells appeared to be undergoing a great deal of cell death attributed to apoptosis, based on cellular morphological appearance. Additionally, the cells were not able to recover when medium containing serum was added to the culture.

Thus, a different strategy was employed to co-transfect the cells, namely the cells were allowed to reach saturation density to increase the actual number of cells and the transfection time was reduced to twelve hours to limit the induction of apoptosis caused by the serum-free conditions. This transfection protocol proved to be successful. A finally transfection was performed where WB.v-myc cells were co-transfected with pSFFV.bcl-2 and pTK-Hyg at a ratio of 20:1 to assure the selection of bcl-2 positive cells. Transfection of WB.v-myc was followed by selection in G418 (400 μg/ml) plus hygromycin B (50 μg/ml) for 14 days. All bcl-2 protein positive clones identified, using Western dot blot screening, were subsequently re-cloned, expanded and frozen at low passage number in liquid nitrogen. Vector control, four WB.bcl-2 and six WB.myc/bcl-2 clones were selected for further studies. Cultures between passages fifteen and twenty-five were consistantly used in experiementation and not used past one month in culture.

Characterization of Transfectants

The morphological appearances of the selected clones were viewed using a Nikon Diaphot phase contrast microscope (Figure 6). The WB-F344, WB.v-myc, and WB.bcl-2 cells demonstrated growth in uniform monolayers of polygonal cells. The Wb.myc/bcl-2 clonal cell lines demonstrated different morphologies: uniform polygonal (WB.MB3), spindle-shaped (WB.MB19), and multinucleated (WB.MB39).

Cultures were then allowed to reach maximal cell density, assaying for the cells ability to be contact inhibited. Neoplastic cells lose their ability to be contact inhibited and are subsequently able to grow in an unrestrained manner. All cell lines chosen for this study demonstrated contact inhibition but varied greatly in their maximal saturation densities (Table 1). WB-F344, WB.neo (vector control), and WB.bcl-2 cell lines all demonstrated similar contact inhibition saturation densities of approximately one hundred thousand cells per square centimeter. WB.v-myc attained a saturation density approximately 1.4 fold greater than that seen in the normal, vector control and bcl-2 expressing cell lines. The myc/bcl-2 expressing clones demonstrated saturation densities in a range from approximately 3 to 5 fold greater than WB-F344 and 2 to 3.5 fold greater than WB.v-myc.

Figure 6. Morphological features of the WB-F344 cell line, before and after transfection with bcl-2 and myc/bcl2. a. WB-F344 b.WB.v-myc c. WB.bcl-202 (bcl-2) d. WB.mb03 (myc/bcl-2) e. WB.mb19 (myc/bcl-2) f. WB.mb39 (myc/bcl-2). All panels are of equal magnification: X 200

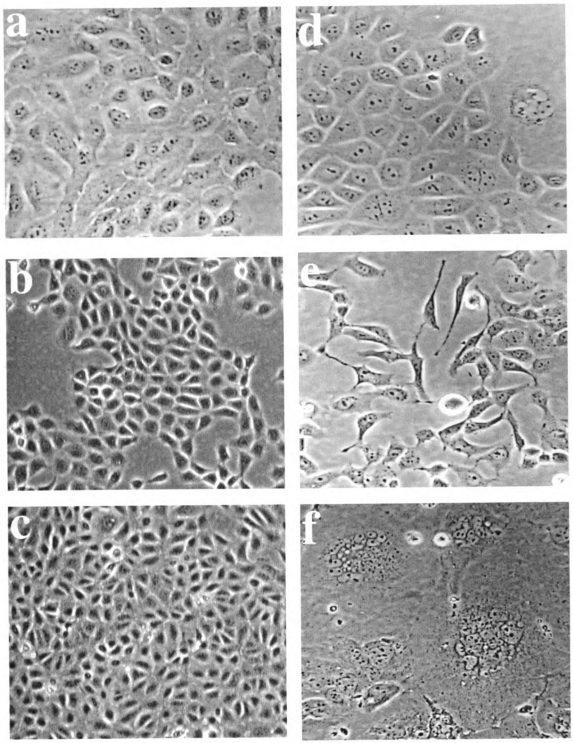


Figure 6

Table 1. Maximum Saturation Density

	Cell line	Cell Density (x $10^5/cm^2$)
	WB-F344	0.955 ± 0.053
	WB.v-myc	1.379 ± 0.053
Bcl-2 alone	WB.bcl-201	1.008 ± 0.159
	WB.bcl-202	0.950 ± 0.055
	WB.bcl-203	1.061 ± 0.212
	WB.bcl-204	1.008 ± 0.103
v-Myc and Bcl-2	WB.MB3	2.759 ± 0.159
	WB.MB6	3.130 ± 0.135
	WB.MB19	4.722 ± 0.106
	WB.MB28	2.492 ± 0.043
	WB.MB39	4.456 ± 0.096
	WB.MB50	4.234 ± 0.280

Proliferation studies were conducted next using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Mosmann, 1983; Wiedermann et al., 1990). This is a non-radioactive colorimetric assay based on the reduction of the MTT tetrazolium salt to a colored formazan. This reduction can only occur in metabolically active viable cells. Thus, by plotting a time course of cell growth from an initial seeding it is possible to calculate the proliferation rate (doubling time) for the absorbance directly correlates with cell number. Figure 7 is a representation of the proliferation analysis using MTT. It shows the growth curves for WB-F344, WB.bcl-201, WB.v-myc, WB.MB3, and WB.MB19. From these curves, doubling times were calculated, using two separate areas (four time points) during log phase growth to determine the growth rate and are shown in Table 2. WB.v-myc demonstrated the fastest growth rate of fourteen-hour doubling time, while the next was the normal WB-F344 cells had a doubling time of twenty-one hours. Interestingly the WB.bcl-2 and WB.myc/bcl-2 clones showed the slowest doubling rates of approximately 24 hours and 33 hours, respectively.

Figure 7. Incorporation of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to assess proliferation. Graph represents aborbance at 540 nm versus hours in culture. $1x10^4$ cells were initially plated in each well of a 96 well plate.

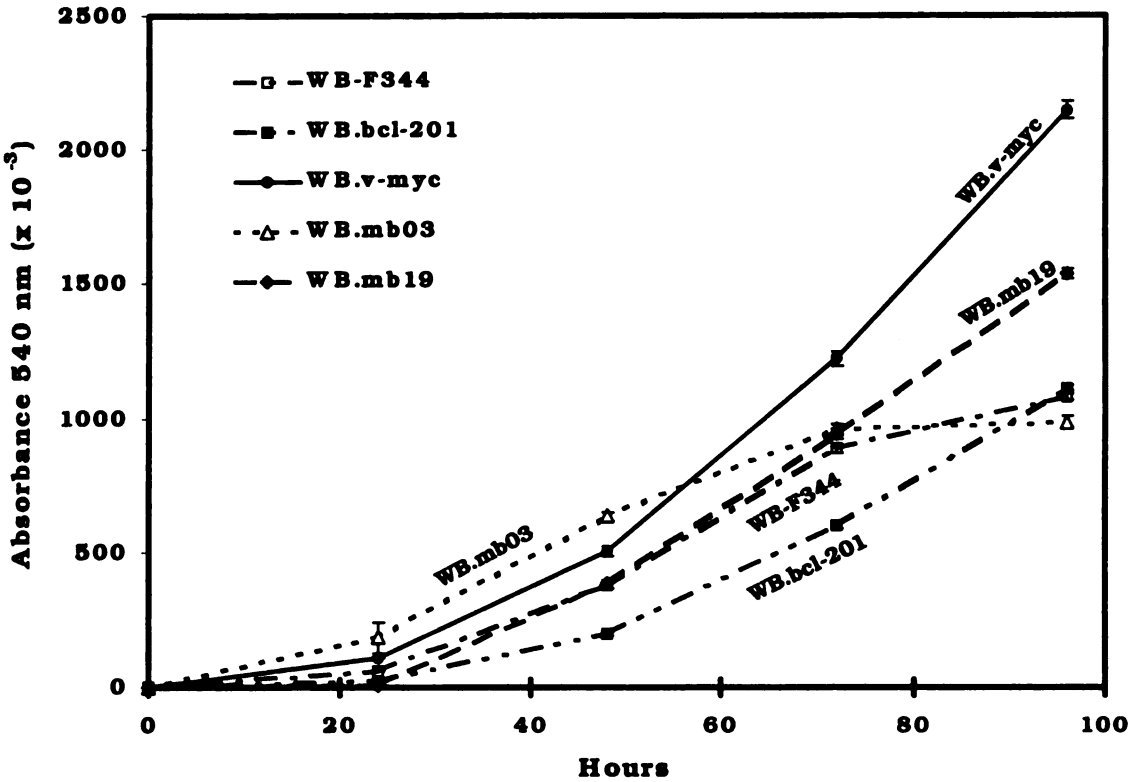

2500
2000
1500
1000
500
0
Absorbance 540 nm (x 10⁻³)
0
20
40
60
80
100
Hours
WB-F344
WB.bcl-201
WB.v-myc
WB.mb03
WB.mb19
WB.v-myc
WB.mb19
WB.mb03
WB-F344
WB.bcl-201

Figure 7

Table 2. Doubling times of transfected cell lines. Rates of doubling times as calculated from proliferation curves of MTT incorporation.

	Cell line	Hours
	WB-F344	21±0.1
	WB.v-myc	14±0.2
Bcl-2 alone	WB.bcl-201	24±0.2
	WB.bcl-202	23±0.1
	WB.bcl-203	25±0.3
	WB.bcl-204	24±0.5
v-Myc and Bcl-2	WB.MB3	34±0.6
	WB.MB6	33±0.2
	WB.MB19	32±0.3
	WB.MB28	30±0.1
	WB.MB39	31±0.4
	WB.MB50	31±0.3

Western Blot Analysis of Bcl-2

The expression of human bcl-2 was assessed using Western blot analysis with an anti-bcl-2 monoclonal antibody (Pharmingen, San Diego, CA) specific for human bcl-2 protein. Figure 8a shows high expression of a 26 kilodalton (kDa) band, corresponding to transfected human bcl-2 protein, in the WB.bcl-2 cell lines, while the control cell lines showed no expression. Figure 8b demonstrates the expression of the human bcl-2 protein in the WB.v-myc/bcl-2 cell lines but not in WB.v-myc and the tumorigenic WB.MR42 cell line which over-expresses v-myc/Ha-ras (previously described by Hayashi et al., 1998). All WB.myc/bcl-2 cell lines demonstrated similar levels of bcl-2 expression except the WB.MB28 cell line, which showed very low levels of bcl-2 expression.

The presence of v-myc in the WB.myc/bcl-2 cell lines was assessed with Western blot analysis with an anti-v-myc polyclonal antibody to verify that transfection of bcl-2 had no discernable affect on v-myc expression. Figure 9 shows the presence of a 110 kDa band that corresponds to the gap-pol-myc region of the v-myc oncogene. Its expression is consistent between the controls and transfected clones, suggesting that over-expression of bcl-2 has not effected the expression of v-myc from the parental WB.v-myc cell line.

Figure 8. Western blot analysis of human bcl-2 protien expression. WB.bcl-2 cell lines; b) WB.myc/bcl-2 cell lines. 26 kDa band represents human bcl-2 protein.

a.
WB-F344
WB.neo
WB.bcl-201
WB.bcl-202
WB.bcl-203
WB.bcl-204
26 kDa
b.
WB-F344
WB.v-myc
WB.MR42
WB.mb03
WB.mb06
WB.mb19
WB.mb28
WB.mb39
WB.mb50
26 kDa

Figure 8

Figure 9. Western blot analysis of v-myc protein expression in the WB.myc/bcl-2 cell lines. 110 kDa band corresponds to gag-myc protein expressed by full length v-myc transcript.

WB-F344
WB.neo
WB.v-myc
WB.MR42
WB.mb03
WB.mb06
WB.mb19
WB.mb39
110 kDa

Figure 9

Anchorage Independent Growth

Anchorage independent growth, commonly referred to as growth in soft agarose, has often been used as a marker for neoplastic transformation and to identify tumor cells (Stoker, 1968; Stoker et al., 1968; Hamburger and Salmon, 1977; Marshall et al., 1977). Anchorage independent growth appears to be a necessary, but not sufficient condition for tumorgenicity of cells. To assess if the bcl-2 gene was conferring neoplastic characteristics to the normal rat liver epithelial cells, we tested their ability to grow in soft agarose. Using a previously described v-myc/Ha-ras transfected tumorigenic WB-F344 cell line (designated MR-42) as a positive control (Hayashi et al., 1998), $1x10^3$ cells were plated on 60 mm dishes in triplicate. Following 28 days in culture plates were stained with 2-(p-iodophenyl)-3-(nitrophenyl)-5-phenyltetrazolium chloride, and colonies were counted. The MR-42 cell line was used as a plating (technique) positive control, which was previously demonstrated to form colonies in soft agarose with one hundred percent efficiency. Therefore all results were normalized to this positive control and colony forming efficiencies where determined as a percentage of the positive control. Figure 10 demonstrates that the control cell lines, as well as the bcl-2 only clonal cell lines, were unable to form colonies in soft agar.

Figure 10. Anchorage independent growth. Data represent colony formation in soft agarose as a percentage of a positive control WB.MR42, a known tumorigenic cell line, derived from the WB-F344 cells.

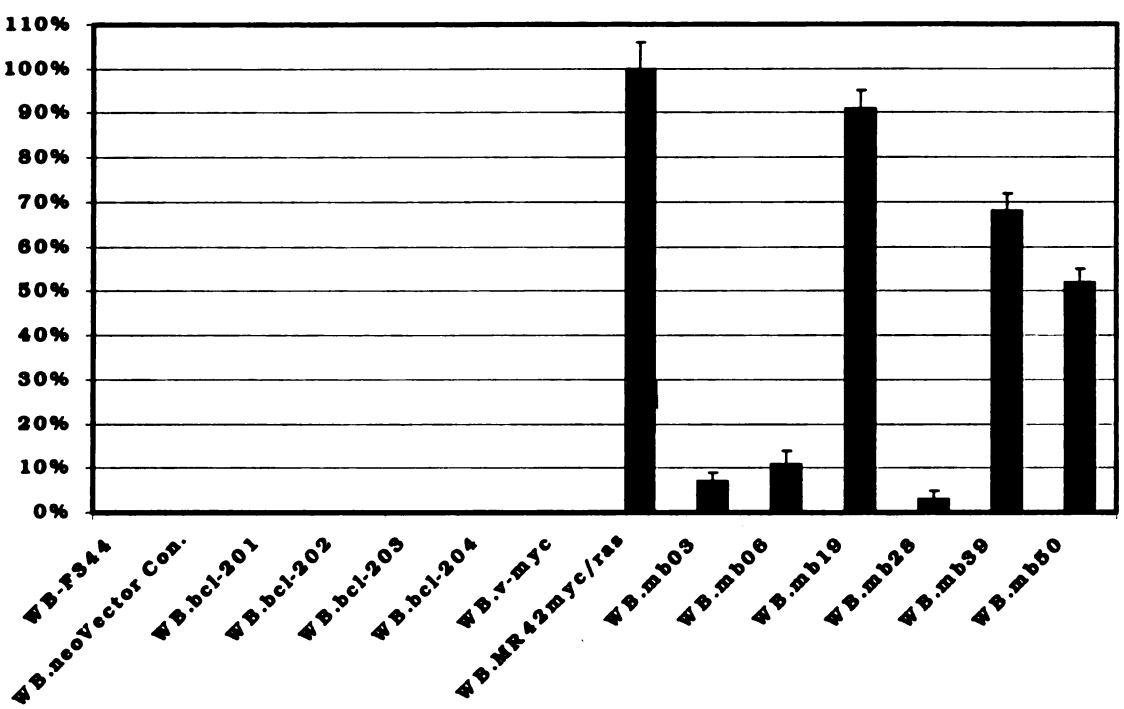

110%
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
WB-F344
WB.neoVector Con.
WB.bcl-201
WB.bcl-202
WB.bcl-203
WB.bcl-204
WB.v-myc
WB.MR42myc/ras
WB.mb03
WB.mb06
WB.mb19
WB.mb28
WB.mb39
WB.mb50

Figure 10

However, the v-myc/bcl-2 clones were able to form colonies in soft agar and they also demonstrated clonal differences in their colony forming efficiency ranging from 3 to 90 percent. When the v-myc/bcl-2 clonal cell lines colony forming efficiencies were compared to their respective bcl-2 expression level no correlation was found.

In Vitro Invasion Assay

The *in* vitro invasion assay (Albini et al., 1987) is based on the early observations of tumors both *in vivo* and *in vitro* which suggested that invasion through basement membranes represented a critical step toward progression (Liotta, 1984; Terranova et al., 1986; Mignatti et al., 1986). Basement membranes are thin extracellular structures surrounding epithelial tissue, as well as the lining of most blood and lymph vessels (Martin and Timple, 1987; Timple and Dziadek, 1986). Basement membranes are comprised of laminin, collagen type IV, fibronectin, and heparin sulfate proteoglycan (Laurie et al., 1982). The *in vitro* invasion assay uses a basement membrane reconstituted onto a filter in a Boyden chamber in which a chemoattractant, 3T3 conditioned medium, is placed below the filter and a cell suspension is placed above the filter. Two measurements are determined in this assay; 1) the number of cells that have invaded to the chemoattractant side of the filter; and 2) the number of cells that have invaded and detached. These quantities are averaged together and determine the invasive ability.

Figure 11a demonstrates that WB.MB19 had the highest amount of invasion to the other side of the membrane, while WB.MR42 had the highest amount of cells that were able to detach away from the filter (Figure 11b). However, when the data are taken together, the trend that was observed with anchorage independent growth assay was repeated in the invasion assay (Figure 11c). WB.MR42, the tumorigenic cell line had the highest average rate of invasion, followed by the WB.MB19 cell line. WB.MB03, WB.v-myc, WB.bcl-201, and WB-F344 all showed nominal invasion.

Gap Junctional Intercellular Communication (GJIC)

In order to begin to explain the differences in growth in soft agarose and invasion demonstrated by the myc/bcl-2 cell lines, the individual cell line's ability to communicate through gap junctions was assessed by the scrape loading dye transfer technique (Figure 12). The WB-F344 cell line is used as the control for communication with the dye traveling about 6-8 cell layers. When WB-F344 cells are compared to the WB.mb19 clone containing v-myc and bcl-2, there is an approximate 50% decrease in the cells ability to transfer dye (3-4 cell layers). This difference in dye transfer can be measured as an average fluorescent area for a fixed magnification, where the control WB-F344 fluorescence almost fills the total area and WB.MB19 only fills half of the area.

Figure 11. Graphical representation of invasion assay as measured by the absorbance at 620 nm. Top graph represents those cells that invaded and stayed attached to the chemoattractant side of the filter. The middle graph is a representation of those cells that detached and were free floating in the chemoattractant medium. Bottom graph represents the average of the top and middle graphs.

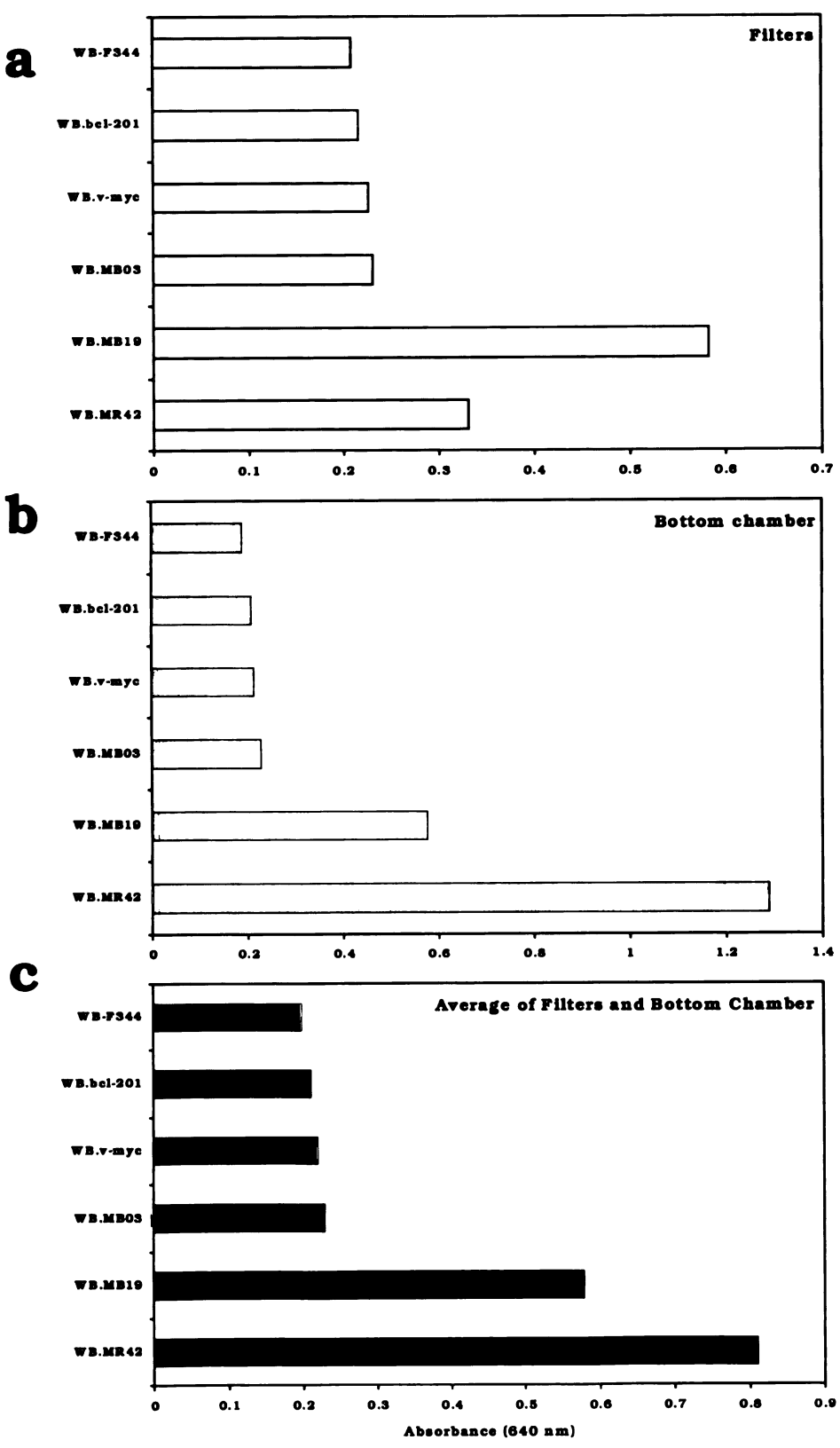

a
Filters
WB-F344
WB.bcl-201
WB.v-myc
WB.MB03
WB.MB19
WB.MR42
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
b
Bottom chamber
WB-F344
WB.bcl-201
WB.v-myc
WB.MB03
WB.MB19
WB.MR42
0
0.2
0.4
0.6
0.8
1
1.2
1.4
c
Average of Filters and Bottom Chamber
WB-F344
WB.bcl-201
WB.v-myc
WB.MB03
WB.MB19
WB.MR42
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Absorbance (640 nm)

Figure 11

Figure 12. A representation of the SL/DT assay. Note the normal WB-F344 cell line transfers the dye approximately 6-8 cells, while the myc/bcl-2 clone MB.19 is only able to transfer the dye 2-3 cells on either side of the scrape line.

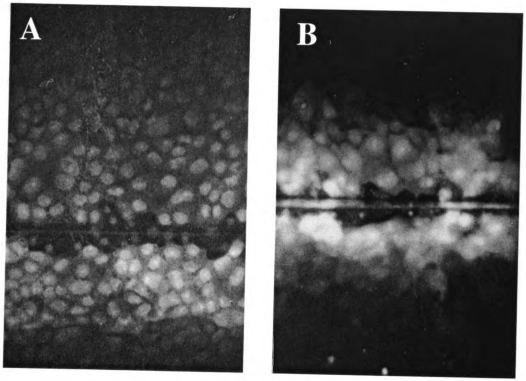


Figure 12

To reduce subjective measurements, average fluorescent area for any given SL/DT line at a fixed magnification was assessed using the Ultima laser cytometer (Figure 13). The data show that WB.F344, vector control, WB.v-myc, and bcl-2 transfect cell lines all demonstrated normal cell-cell communication. The WB.myc/bcl-2 cell lines demonstrated either normal or various reductions in cell-cell communication. These results were confirmed using the fluorescent recovery after photobleaching assay (FRAP)(Table3). This assay allows for the monitoring of individual cells in the population assessing an activity for the whole population. Figure 14 shows the GJIC activity (both SLDT and FRAP) of the various cell lines. The data represented in Figure 14 demonstrate similar findings between both the SLDT and FRAP assays, however none of the WB.bcl-2 cell lines showed any reduction in cell-cell communication. Only WB.MR42, WB.MB19, WB.MB39, and WB.MB50 showed reductions in cell-cell communication.

When AIG and GJIC, as measured by the two communication assays, are compared, they appear to be inversely correlated. Those WB.myc/bcl-2 cell lines, that showed no significant changes in GJIC, demonstrated only minimal ability to form colonies in soft agarose. However, those cell lines, that demonstrated marked reduction in cell-cell communication (WB.MB19, WB.MB39, and WB.MB50) demonstrated an enhanced or increased ability to form colonies in soft agar (Figure 15).

Figure 13. Scrape load dye transfer. Data represent average fluorescent area normalized to 100% of the control cell line WB-F344.

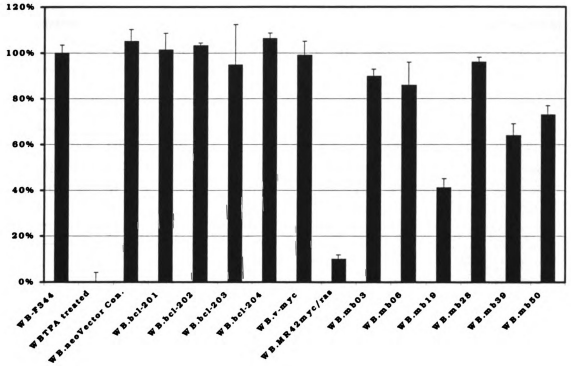

120%
100%
80%
60%
40%
20%
0%
WB-F344
WBTFA treated
WB.neoVector Con.
WB.bcl-201
WB.bcl-202
WB.bcl-203
WB.bcl-204
WB.v-myc
WB.MR42myc/ras
WB.m103
WB.m106
WB.m119
WB.m126
WB.m139
WB.m160

Figure 13

Table 3. Gap junction intercellular communication. Cell-cell communication of transfected cell lines as measured by scrape load dye transfer and fluorescent recover after photobleaching assays

	Cell line	SL/DT	% FRAP
	WB-F344	0.934± 0.035	100± 3 (404)
	WB.v-myc	0.925± 0.046	102± 5 (208)
Bcl-2 alone	WB.bcl-201	0.947± 0.068	98± 8 (192)
	WB.bcl-202	0.964± 0.011	98± 7 (160)
	WB.bcl-203	0.886± 0.163	96± 12 (172)
	WB.bcl-204	0.992± 0.022	107± 5 (184)
	WB.myc/ras (MR42)	0.093± 0.018	2± 8 (92)
v-Myc and Bcl-2	WB.mb3	0.845± 0.082	93± 6 (320)
	WB.mb6	0.803± 0.093	96± 3 (290)
	WB.mb19	0.524± 0.037	54± 5 (308)
	WB.mb28	0.897± 0.136	93± 2 (286)
	WB.mb39	0.598± 0.038	70± 6 (380)
	WB.mb50	0.782± 0.027	76± 3 (328)

( ) Denotes the number of cells assayed

Figure 14. Fluorescent recovery after photobleaching and scrape load dye transfer. Graph compares the two assays for gap junction-mediated intercellular communication.

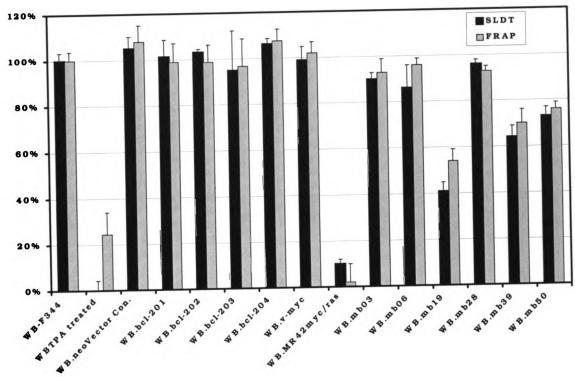

120%
100%
80%
60%
40%
20%
0%
SLDT
FRAP
WB.F344
WB.TPA treated
WB.neoVector Con.
WB.bcl-201
WB.bcl-202
WB.bcl-203
WB.bcl-204
WB.v-myc
WB.MR42myc/ras
WB.mb03
WB.mb06
WB.mb19
WB.mb28
WB.mb39
WB.mb50

Figure 14

Figure 15. Anchorage independent growth correlates to cell-cell communication. This graph represents the comparison of average cell-cell communication with AIG.

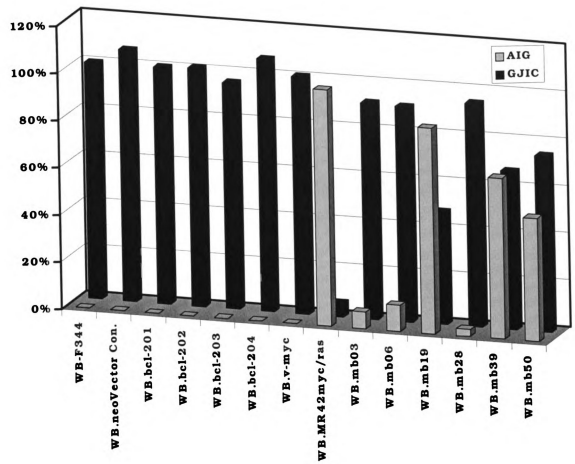

120%
100%
80%
60%
40%
20%
0%
AIG
GJIC
WB-F344
WB.neoVector Con.
WB.bcl-201
WB.bcl-202
WB.bcl-203
WB.bcl-204
WB.v-myc
WB.MR42myc/ras
WB.mb03
WB.mb06
WB.mb19
WB.mb28
WB.mb39
WB.mb50

Figure 15

Connexin 43 Expression

Due to the reduction of cell-cell communication the levels of connexin 43, the predominant connexin expressed in WB-F344 cells, was assessed using Western blot analysis. Figure 16 demonstrated that those cell lines that had normal cell-cell communication had normal connexin 43 expression, which is characterized by multiple bands at 43 kDa. These bands represent unphosphorylated and phosphorylated protein. WB.mb19 and WB.mb39, which had a 60% reduction reduced cell-cell communication, demonstrated very low expression of connexin 43 protein consistent with a reduction GJIC.

Apoptosis

To determine if bcl-2 was functioning as a pro-survival factor in the bcl-2 containing cell lines apoptosis was assayed using fluorescent activated cell sorting (FACS). Apoptosis was examined using the Becton Dickinson FACS Vantage. Cultures, which were grown in twenty five square centimeter flasks, were rinsed twice with phosphate buffered saline and serum-free medium was added to flasks. Forty-eight hours later cells were trypsinized, fixed in seventy-percent ethanol, and stored at minus 20°C. Twenty-four hours prior to analysis on FACS, cells were stained with propidium iodide. Data were analyzed using two software protocols (Figure 17). The first software was the PC-LYSYS ™ version 1.0 and the second was WinMIDI version 2.8.

WB-F344
WB.bcl-202
WB.bcl-204
WB.v-myc
WB.mb19
WB.mb39
WB.AB1
P2
P1
P0

Figure 16

Figure 17. Representation of data analyzed from FACS. Top two graphs represent data analyzed using PC-LYSYS software. Bottom four graphs represent data analyzed using WinMIDI software. Regions denoted by R represent G2/G1/Apoptotic peaks reading from right to left.

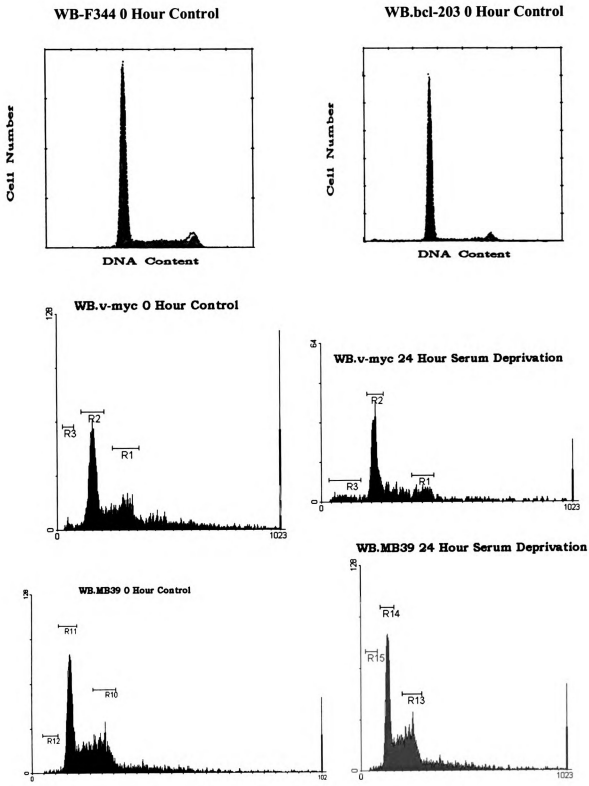

WB-F344 0 Hour Control
Cell Number
DNA Content
WB.bcl-203 0 Hour Control
Cell Number
DNA Content
WB.v-myc 0 Hour Control
128
R2
R3
R1
0
1023
WB.v-myc 24 Hour Serum Deprivation
64
R2
R3
R1
0
1023
WB.MB39 0 Hour Control
128
R11
R10
R12
0
102
WB.MB39 24 Hour Serum Deprivation
128
R14
R15
R13
0
1023

Figure 17

Both software packages allow for the identical analysis of the data, with the exception that WinMIDI was less complicated. The data demonstrate, that when expressed alone bcl-2 was able to function and block apoptosis associated with serum deprivation and caused G1 cell cycle arrest (Table 4). However, when co-expressed with myc, cell cycle arrest is not seen, even though there is an apparent block in apoptosis. In fact, it appears that there is a slight increase in cell cycle progression demonstrated by an increase the WB.myc/bcl-2 cell lines G2 population. This suggests that cell cycle progression induced by myc can be enhanced by bcl-2 possibly through the blockage of apoptosis.

Glutathione Expression

Glutathione (GSH) is a tripeptide, whose primary function is to form conjugates with reactive electrophiles rendering them inactive and represents the major soluble intracellular antioxidant. Glutathione peroxidase uses GSH to convert organic peroxides, like hydrogen peroxide, into water and alcohols. The role of GSH in cell death was first investigated by studying the action of menadione, a powerful oxidant, in the induction of necrosis (Thor et al., 1982). This work demonstrated that cell death required the depletion of intracellular GSH. Since this initial work, many researchers have shown that GSH depletion also occurs during apoptosis (Fernandez et al., 1995; Slater et al., 1995). GSH has been proposed to function as a scavenger of free oxidants in the

Table 4. Apoptosis frequency.

	Cell line	Apoptosis (Percent)		G1/G2 (Percent)	
	Serum	+	-	+	-
	WB-F344	1.3	3.9	51.7/20.6	52.9/15.0
Bcl-2 alone	WB.bcl-201	0.4	0.5	86.3/8.6	80.6/9.6
	WB.bcl-202	0.3	0.5	82.7/9.5	79.8/10.2
	WB.bcl-203	0.5	0.5	83.5/7.9	81.2/8.4
	WB.bcl-204	0.3	0.5	84.2/8.1	80.1/9.1
	WB.v-myc	1.6	8.2	43.9/24.7	49.8/14.6
v-Myc and Bcl-2	WB.MB3	0.5	0.5	30.9/31.4	39.1/23.4
	WB.MB6	0.4	0.6	28.6/29.3	40.3/28.1
	WB.MB19	0.3	0.5	31.7/30.4	40.8/26.5
	WB.MB28	0.6	0.7	32.1/31.6	41.5/22.7
	WB.MB39	0.5	0.5	29.9/30.7	38.9/24.9
	WB.MB50	0.4	0.6	31.4/31.8	41.2/25.4

cytoplasm. Macho and colleagues (1997) extended these findings by demonstrating that GSH depletion was an early event in apoptosis, taking place before much of the protein signaling. Additionally, Bcl-2 and its relatives have been shown to elevate GSH levels and protect cells against oxidant-induced apoptosis (Kane et al., 1993; Bojes et al., 1997; Mirkovic et al., 1997; Voehringer et al., 1998).

To determine if elevation of GSH was responsible for the reduction in apoptosis seen in the bcl-2 containing cell lines, GSH concentration was assessed using a HPLC technique and an electrochemical detector. Standards of GSH were injected into the injection port of an ESA solvent delivery module (ESA, Inc., Chelmsford, MA). GSH was detected using and ESA Choulochem II dual electrode electrochemical detector. Retention times were calculated from the time of injection to peak appearance on a chart recorder. Concentration was calculated by determine the area under the curve and represented as percent of the normal control WB-F344. The results demonstrate the GSH was significantly reduced in the WB.bcl-2 cell lines (Figure 18). This result was contradictory to all previous reports that showed an increase in GSH concentration in cells over-expressing bcl-2. Due to the inexplicable nature of these results, further studies were abandoned in favor of examining the bcl-2 containing cell lines differentiation potentials.

Figure 18. Glutathione Expression. Graph represents glutathione concentration normalized to protein content and expressed as a percentation of the control cell line WB-F344.

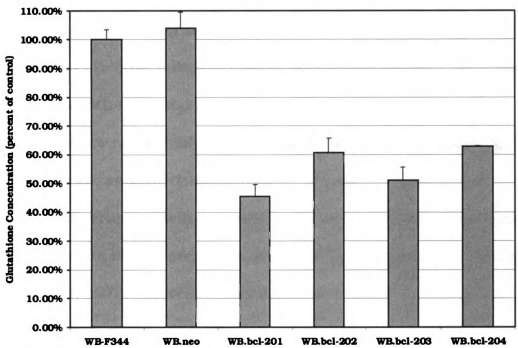

110.00%
100.00%
90.00%
80.00%
70.00%
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
Glutathione Concentration (percent of control)
WB-F344
WB.neo
WB.bcl-201
WB.bcl-202
WB.bcl-203
WB.bcl-204

Figure 18

II. Differentiation Potential of Bcl-2 and V-myc transformed Rat Liver epithelial cells

The adult liver, in mammalian species, contains many different cell types. The main cell types are hepatocytes, bile duct epithelium, stellate cells, Kupffer cells, vascular endothelium, fibroblasts and leukocytes (Desmet, 1994). The predominant cell type is the hepatocyte which comprises approximately ninety percent of the weight of the liver. Hepatocytes are large cuboidal epithelial cells that function to exchange metabolites with the blood and secrete bile into the bile duct. The hepatocytes are responsible for the metabolism of amino acids, lipids, carbohydrates, and the detoxification of xenobiotics as well as the synthesis of serum proteins. A significant, large fraction of the mature hepatocytes are binucleated, with some nuclei being tetraploid (Digernes and Blound, 1979; Medvedev, 1988). While heptocytes are capable of proliferation, they are self-limited in culture. Therefore, several *in vitro* models for hepatic progenitor cell growth and differentiation have been developed from the mouse (HBC-3: Rogler, 1997; Ott et al., 1999), the pig (PICM-19: Talbot et al., 1993), the human (AKN-1: Nussler et al., 1999), and the rat (WB-F344: Grisham, 1980). The most important of these systems is the WB-F344 cell line which was clonogenically derived from non-parenchymal cells. These cells can be cultured indefinitely and can be induced to form hepatocytes in culture. More importantly, this cell line is the only system that has been demonstrated to differentiate into

normal hepatocytes when transplanted back into rats (Colman et al., 1997; Colman et al., 1993; Grisham et al., 1993).

Initial morphological observations of the WB-F344, cells transfected with myc/bcl-2, used in this study demonstrated very different morphologies. The WB.mb39 cell line possessed cells that were very large, multinucleated and cuboidal, highly reminiscent of the description of hepatocytes. This cell line prompted the investigation into determining if bcl-2 affected the differentiation potential of WB-F344 cells. Differentiation was induced using 3.75 mM sodium butyrate (SB). Previous work demonstrating SB ability to induce differentiation in WB-F344, as well as other hepatic progenitor cells made it the ideal candidate for this study. The mechanism by which SB functions to induce differentiation is unclear. However, SB has been demonstrated to block histone deacetylases (HDAC) activity. HDAC activity results in histone hypoacetylation, chromatin condensation and transciptional repression (reviewed in Ng and Bird, 2000). Therefore by blocking HDAC activity, SB stimulates transcriptional activation and cells are released to differentiate. Using the cell lines described in the previous section, alterations in differentiation potential were investigated. The criteria used in the determination of differentiation were changes in morphology, activation of the hepatocyte specific tyrosine amino transferase enzyme, and expression of the heptocyte specific gap junction protein connexin 32.

Morphology

Cells were treated with 3.75 mM SB for six days. On the fifth day water soluble dexamethasone was added to a final concentration of $1x10^{-7}$ M. Figure 19 represents the morphology of untreated and treated WB-F344, WB.bcl-202, and WB.bcl-203 that was seen during the treatment. These cell lines all demonstrated similar induced morphological alterations of increased size, and multinucleation. WB.myc/bcl-2 and the tumorigenic WB.myc/ras cell lines all show an inability to sustain morphological alterations induced by sodium butyrate (Figure 20). These cells lines demonstrated an early response to the treatment but cells rapidly died during the prolonged exposure.

Tyrosine Aminotransferase (TAT) Assay

The morphological results suggested that the WB-F344 and WB.mcl-2 cell lines were behaving in a manner consistent with hepatocyte differentiation. To confirm that these cells were indeed differentiating into hepatocytes, TAT activity, which is specific to viable hepatocytes, was measured. SB was able to induce TAT activity in WB-F344, WB.v-myc, and WB.bcl-2 cell lines (Table 5). However the Bcl-2 cell lines showed a slightly reduced response to treatment when compared to the normal WB-F344 cell line.

Figure 19. Morphological features of the WB-F344, WB.bcl-202, and WB.bcl-203 cell lines. Images are before and after treatment with 3.75 mM sodium butyrate for 6 days and a pulse of dexamethasone ($1x10^{-7}$ M final concentation) for the last 24 hours. (Magnification: X200)

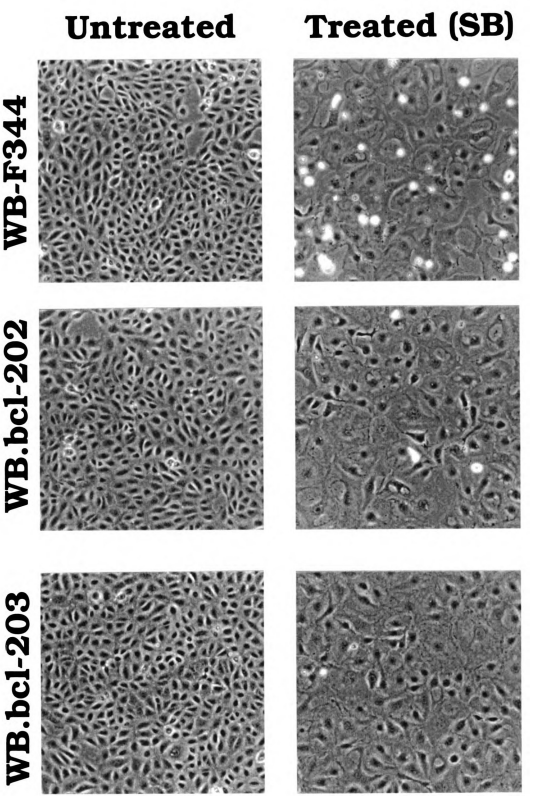


Figure 19

Figure 20. Morphological features of the myc/bcl-2 (WB.MB3 and WB.MB19) and myc/ras transfected (WB.MR42) cell lines. Images are before and after treatment with 3.75 mM sodium butyrate for 6 days and a pulse of dexamethasone ($1x10^{-7}$ M final concentation) for the last 24 hours. (Magnification: X200)

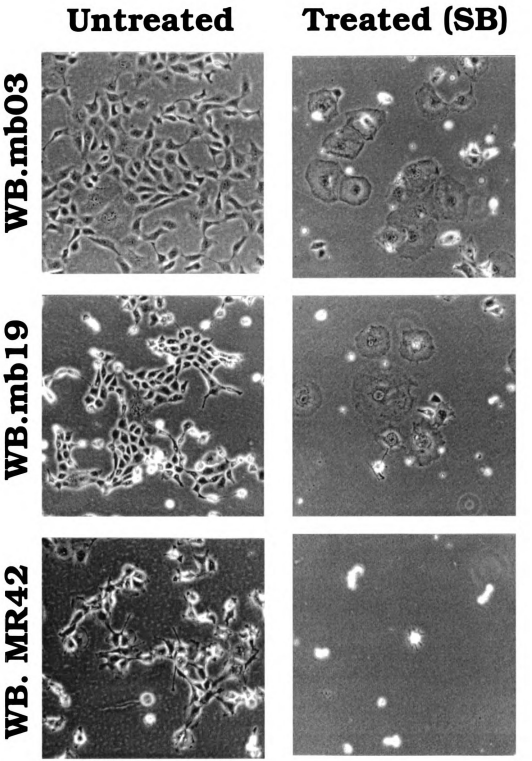


Figure 20

Table 5. Tyrosine aminotransferase activity. TAT activity of transfected cell lines before and after treatment for 6 days with 3.75 mM sodium butyrate and $1x10^{-7}$ M dexamethasone for the final twenty-four hours.

	Cell line	Basal Activity	SB Induced* Activity
	WB-F344	2.79±1.43	203.10±1.65
	WB.v-myc	1.69±1.65	73.03±2.65
Bcl-2 alone	WB.bcl-201	1.23±1.19	139.31±1.99
	WB.bcl-202	1.32±1.27	142.24±1.63
	WB.bcl-203	1.53±1.49	140.84±2.96
	WB.bcl-204	0.93±0.79	132.81±1.02
	WB.myc/ras (MR42)	0± 0.00	0 ± 0
v-Myc and Bcl-2	WB.MB3	0± 0.00	0 ± 0
	WB.MB6	0± 0.00	0 ± 0
	WB.MB19	0± 0.00	0 ± 0
	WB.MB28	0± 0.00	0 ± 0
	WB.MB39	0± 0.00	0 ± 0
	WB.MB50	0± 0.00	0 ± 0

The myc/bcl-2 and myc/ras cell lines demonstrated no induction of TAT assay, in fact they demonstrated absolutely no basal TAT activity, which was detected in the normal, v-myc and bcl-2 containing cell lines.

Connexin 32 Expression

In addition to testing for activation of a hepatocyte specific enzyme activity, expression of connexin 32 protein was used as a hepatocyte specific marker. As previously mentioned, connexin 32 is the predominant connexin protein expressed by hepatocytes. WB-F344 cells do not express connexin 32. Figure 21 shows that connexin 32 was induced in the normal WB-344, WB.bcl-2 and WB.v-myc cell lines. Induction of connexin 32 was not observed in the WB.myc/bcl-2 cell lines WB.mb3 and WB.mb19.

Figure 21. Western blot analysis of connexin 32 expression. Connexin 32 was assayed following induction by 3.75 mM sodium butyrate for 6 days and a pulse of dexamethasone ($1x10^{-7}$ M final concentation) for the last 24 hours.

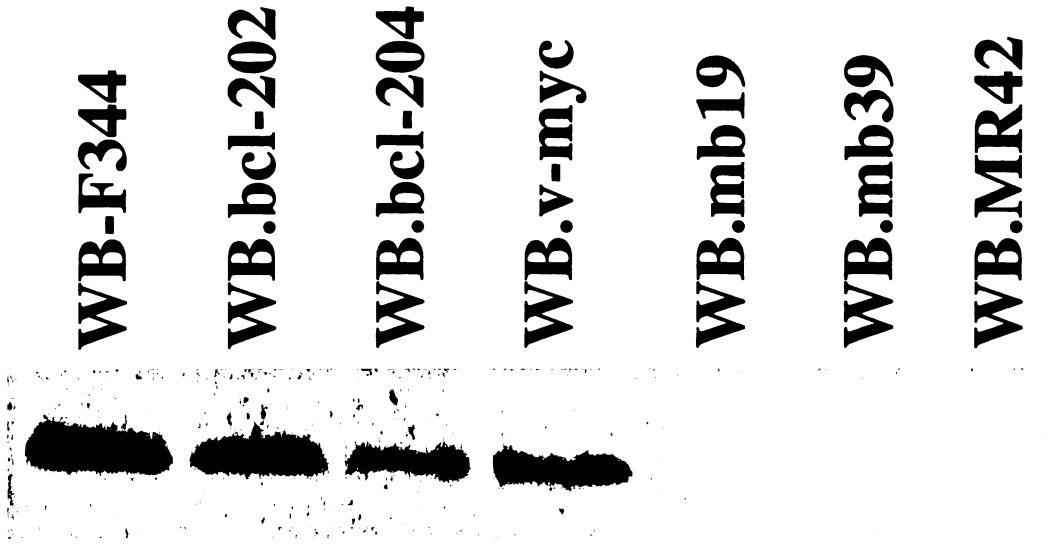


Figure 21

Discussion

In the initiation/promotion/progression model of multi-stage carcinogenesis, initiation is an irreversible genetic event that sensitizes a cell to subsequent promotion (Figure 1). Promotion is then characterized as the events that lead to the clonal expansion of the initiated cells. Progression is described as the accumulation of alterations within an initiated cell that leads to promotion-independent growth. The stem cell theory of carcinogenesis proposes that the target of initiation is the stem cell, where initiation involves the blockage of the stem cells ability to differentiate. Under normal conditions, these "initiated" cells would be contact inhibited by being coupled to the surrounding normal cells through gap junctions. Promotion would then cause the down-regulation of GJIC (leading to the loss of contact inhibition) and the induction of gene expression related to cell proliferation. Additionally, promotion would also involve the escape from apoptosis, which would normally eliminate initiated cells. Therefore, by down-regulating GJIC, stimulating proliferation, and blocking apoptosis, an initiated cell could become neoplastically transformed. The objective of this study was to determine if the oncogene, bcl-2, could act cooperatively with the myc oncogene in the neoplastic transformation of normal epithelial cells. Furthermore,

the importance of GJIC, proliferation, and apoptosis in promotion was examined.

To test the hypothesis that the inhibition of apoptosis, in conjunction with a proliferation signal and subsequent down regulation of GJIC, lead to neoplastic transformation, the bcl-2 and myc oncogenes were expressed alone and together in the WB-F344 cells, a normal rat liver epithelial cell line. The molecular biology associated with the beginning of this project was extremely labor intensive. Eventually, several WB.bcl-2 clones were established and subcloned. However, many problems occurred when transfecting the WB.v-myc cells. It was determined that WB.v-myc cells were especially sensitive to the transfection conditions. Eventually WB.myc/bcl-2 cell lines were also established.

Clones were initially screened by their human bcl-2 protein expression, using a western dot blot protocol. Subsequent characterization of subclones for bcl-2 protein expression showed a single band at 26 kDa, indicating the presence of bcl-2. This result is very interesting due to the fact that Bcl-2 has been traditionally shown to form complexes with itself and other members of the bcl-2 family, therefore, under most conditions multiple bands can be visualized. In this project, all electrophoretic conditions consistently produced similar results, a single band at 26 kDa. It is possible that the extraction methods and electrophoretic conditions precluded the observation of

protein interactions. In this system, if bcl-2 was acting as a monomer, it would call into question the mechanism by which bcl-2 is thought to function. Bcl-2's proposed functions are based on the proposition that it is the formation of complexes with other proteins and itself that allows for the sequestering of other proteins or perturbations in mitochondrial channels. The results presented in this study suggest that in this cell system, bcl-2 might be acting through alternative mechanisms than those that others have reported. Similarly, no one has actually reported findings, either *in vivo* or under physiological conditions, that bcl-2 is functioning as a multimer. What these mechanism are cannot be delineated without further investigation; however they will most likely be related to the subcellular localization of bcl-2.

Contact inhibition functions to ensure the harmonious development of the whole tissue, affecting the way tissues organize, control their size, their morphology and synchronize their function (Jones, 1980; Lieberman and Glaser, 1981). Tumor cells have been characterized by their lack of contact inhibition. In cell culture, the loss of contact inhibition is indicated by the ability of cells to reach a higher maximum or saturation density. Transfected cells were allowed to reach their maximum density under the assumption that the more neoplastic characteristics a cell line possesses, the more cells would occupy the same space at its maximum density as compared to normal cells. It is important to note that the cells were continuously provided with fresh

medium during the course of this experiment because it has been shown that density-depend effects can be affected by decreases in available growth factors (Alberts el al, 1994). The myc/bcl-2 transfected cells were contact inhibited at a higher density than the normal WB-F344, WB.v-myc and WB.bcl-2 cell lines. Independently, neither myc nor bcl-2 was sufficient to effect contact inhibition. However, when these two oncogenes cooperate, they are able, significantly, to effect contact inhibition, indicating that their co-expression is contributing to the neoplastic transformation of WB-F344 cells. Thus, an initiated cell, lacking contact inhibition, and under the stimuli being provided by bcl-2 and myc, could escape the constraints being signaled by the surrounding cells of its tissue of origin.

Interestingly, when growth rates were examined, clones containing bcl-2 showed a reduction in proliferation. Although at the time these results were novel, they were confirmed shortly thereafter by several publications which demonstrated growth arrest in cells over expressing bcl-2 (Uhlmann et al, 1996; Vairo et al, 1996; O'Rielly et al, 1996). Similarly, the results from this project and others demonstrated that bcl-2 expression caused an accumulation of cells at G1 (Borner, 1996; Mazel et al, 1996). Bcl-2 and myc/bcl-2 cell lines equally demonstrated a reduction in proliferation, which suggest that, unlike the ras oncogene, bcl-2 does not provide myc with a cooperating mitogenic signal. Furthermore, these cell lines may prove to be very important for the

investigation into the non-apoptotic functions of bcl-2, such as the interactive role with mitogens in proliferation and cell cycle.

While bcl-2 was able to act as a pro-survival factor in both bcl-2 and myc/bcl-2 cells, the difference in apoptosis rates after serum withdrawal between the transfectants and the normal WB-F344 cells was small, exhibiting only a four-fold increase. WB.myc cells showed a higher sensitivity to serum withdrawal induced apoptosis with an eight-fold increase. These changes are admittedly small. These results are consistent with the hypothesis that the WB-F344 cells are stem cells, and their survival would be essential for they would be needed to repopulate the tissue after significant trauma. Therefore, they might be less susceptible to apoptosis. In addition, apoptosis of tissues under food restriction is a slow process lasting months, where the serum withdrawal in these experiments was only forty-eight hours.

The cell lines were also assayed for their ability to grow in an anchorage independent manner, as assayed in tissue cell culture by their propensity to form colonies in soft agarose. Anchorage-independent growth measures a cell's ability to grow in an environment lacking attachment signals. Normal cells require attachment signals to establish polarization and are therefore not able to grow in soft agarose. Tumor cells lack the need for polarization and are able to form colonies in soft agar, thus making AIG an assay for putative neoplastic transformation. Of the cell lines tested only those cells that contained myc/bcl-2 were

able to form colonies in soft agarose. Although, this suggests that all the cell lines containing myc/bcl-2 were neoplastically transformed, the degree of transformation varied greatly as expressed by large differences in their colony forming efficiencies. When the invasive ability was examined using a Boyden chamber invasion assay, they again displayed similar variability. In this context, invasion is being used a measure of the cells ability to remodel its environment and is considered a transitional neoplastic point between promotion and progression because once a cell escapes the confines of the basement membrane it is then free to metastasize. If over-expression of bcl-2 was the main factor in the neoplastic transformation of the WB.v-myc cell line, a dose correlation between bcl-2 protein expression and the cell lines colony forming efficiencies or invasive ability would expected to be seen. No such correlation between bcl-2 protein expression and colony formation or invasion was seen, suggesting that a another mechanism was occurring that was responsible for the differences observed.

Gap junction-mediated intercellular communication (GJIC) has been shown to play an important role in tumor promotion and progression. Presumably, as cells lose GJIC, they are effectively removed from intercellular signals that can regulate proliferation, differentiation, and apoptosis. When it was shown that tumor promoters, such as phorbol esters (Yotti et al, 1979, Murray and Fitzgerald, 1979), could reversibly inhibit GJIC, it was hypothesized that an "initiated" cell would

be growth suppressed by being coupled by gap junctions to surrounding normal cells. The implication is that, while the initiated cell is genotypically altered, it could still be contact inhibited by regulatory signals from the surrounding normal cells. By triggering signal transduction in the "initiated" and surrounding normal cells, growth factors, hormones and tumor promoting chemicals, would cause the down-regulation of GJIC and induce gene expression (Trosko et al, 2000). While normal cells would proliferate, differentiate and apoptose, the "initiated" cell would only proliferate, thereby increasing the number of initiated cells, i.e. clonal expansion. As long as the external exogenous chemical (tumor promoter) is applied, the clonal expansion of the initiated cell will occur.

The results in this study indicate that there might be a strong association between GJIC and promotion. Those cell lines that retained normal GJIC were restricted in their ability to form colonies in soft agar. However, as the cell lines progressively lost the ability to communicate through gap junctions, they progressively increased in their ability to form colonies in soft agarose. The results indicate that that GJIC may be contributing to the cells ability grow in soft agarose. The utilization of myc and bcl-2 was not meant to imply that all cells being initiated or neoplastically transformed represent the initiation/promotion/progression model of carcinogenesis, but rather to

show the importance of proliferation, apoptosis and GJIC during that process.

An early observation of the myc/bcl-2 cell lines was the striking dissimilar morphologies being expressed. This led to the investigation of the role that differentiation might play in multi-stage carcinogenesis. Potter (1978) proposed that cancer was a disease in which differentiation was blocked; "oncogeny as partially blocked ontogeny." Therefore, initiation may involve the blockage of a progenitor or stem cells ability to differentiate and promotion would allow that cell to clonally expand because it could not differentiate.

To test this, hypothesis the myc/bcl-2 cell lines were chemically induced to differentiate. A loss in the ability of the bcl-2 containing clones to undergo differentiation was observed. However this blockage was not consistent between the cell lines. In fact, it appeared that those cell lines that were described as being more neoplastic were less able to respond to chemical differentiation. This represents a refinement of the "oncogeny as partially blocked ontogeny" theory which suggests that blockage of differentiation is an early event before promotion. The results here could be interpreted as representing a progressive loss of the ability to differentiate as neoplasia increases. This does not exclude that blocked differentiation can be an initiation event, only that the inability to differentiate progresses with promotion.

Earlier in this report, promotion was described as an accumulation of cells using the equation A = P + D, where P was proliferation rate and D was cell death. Differentiation represents another function that cannot be additively summed with P and D, therefore this simple equation cannot be used. In addition to differentiation, another state exists in which the cells are unable to replicate, but remain viable. This is known as senescence. Cellular senescence represents another mechanism that limits clonal growth. Normal cells (excluding stem cells) have a limited proliferative life span, after which they arrest and lose the ability to divide (Hayflick, 1965). This phenomenon, termed replicative senescence has been proposed to contribute to tissue aging (Smith and Pereira-Smith, 1996). It is very important to delineate between differentiation, apoptosis and senescence. Differentiation is a functional state of cells providing specific metabolic activities to its microenvironment. Differentiating cells can begin to terminally differentiate, which results first in an irreversible loss in the ability to proliferate (senesce) and ultimately leads to apoptosis. The distinction between apoptosis and senescence is that senescent cells are still viable and remain metabolically active. They are only unable to initiate DNA replication. Interestingly, the myc and ras oncogenes, that can effect proliferation, apoptosis and differentiation, also block the cell's ability to senesce (Dean et al., 1986; Delgado et al., 1986) .

Therefore, a cell can be exhibiting one of five functions that are quiescence (homeostasis), proliferation, apoptosis, differentiation, and senescence. In order to represent the findings of this report in a broader scope, it is necessary to conceptualization these functions as unique states that a cell might reside, in which, each state is defined by a set of signaling pathways. The idea of defined signaling involved with a specific function is not new. It is well known that the signaling pathways are very different between proliferation and apoptosis even though they both involve some of the same proteins. Because some of the same proteins are involved, these states can interact. That is to say, a cell can enter other states, depending in what signaling is present. For example, replicating cells can differentiate, apoptose, senesce or quiesce. Similarly differentiated cells can proliferate, quiesce, apoptose, or senesce. However, all evidence indicates that apoptosis and senescence are irreversible. Figure 22a, depicts a directed graph demonstrating the intricate interactions of these states, in which each state is represented as a vertices. This kind of graph shows the complexity of the system. When using a directed graph such as this it becomes difficult to visualize how initiation and promotion could occur.

To simplify the complexity of these interactions a divergent/convergent model is proposed (Figure 22 b-f). In this model homeostasis (quiescence) is represented as a balance of the signaling pathways involved in the functions of proliferation, apoptosis,

differentiation, and senescence. In this model, a normal cell in homeostasis could equally respond to any signaling pathways. In tissue culture, it is difficult to demonstrate quiescence. *In vitro* culturing conditions preclude the cells from entering this state. As cells reach a density in culture that would induce contact inhibition and quiescence, prolonged maintenance at this density begins to cause genetic instabilities, thereby altering the signaling pathways. However, if a stimulus is present then a divergent path from the cells is chosen and a new state is assumed, i.e. quiescence transitioning into proliferation.

In tissue culture, this is represented as the normal passing of cells at sub-confluent condition where a majority of the culture is quiescent. When these cells are re-plated, they begin to replicate. An initiated cell in this theory would be restricted in what pathways and subsequent states that it could reside. This is demonstrated by WB.v-myc and WB.bcl-2 cells, which are precluded from senescing and apoptosing, respectively. Promotion would then be described as the blockage of divergent signals leading to fewer and fewer pathways which are available and the signals converge into a single state, for neoplastic cells that is usually proliferation. In this study, bcl-2 and myc cooperate to block differentiation and apoptosis and to reduce contact inhibition, such that the cells are able to proliferate in an unrestrained manner. That is to say, the initiated cell, when stimulated to proliferate, is inhibited to apoptose, to differentiate, and is not able to senesce. As the

tumor grows, signaling changes and some cells can proceed to other states, as is demonstrated by the heterogeneity of tumors *in vivo*. To summarize, promotion in this model is the set of signals that allows for a convergence of signals in the initiated cell, leading to the accumulation of cells. When WB-F344, WB.v-myc, and WB.bcl-2 cell lines were treated with sodium butyrate, the cumulative signaling led to differentiation. However, in the WB.myc/bcl-2 cell lines, differentiation and senescence were blocked, and proliferation could not be initiated, so the cells could only die, even though they were over-expressing bcl-2.

In this model, gap junctions would play an essential and critical role, for they would serve as a means to balance the signaling between cells. A reduction or block in GJIC would lead to an imbalance in signaling, thus leading to restrictions in what divergent pathway a cell can reside. The fact that gap junctions are so highly evolutionarily conserved suggests their importance. Bcl-2 is also conserved, however, its conservation is based on the proposal that apoptosis is a critical event to be controlled. While this is true, bcl-2 undoubtedly has other functions.

This report and published data have shown that bcl-2 can effect multiple pathways like cell growth and differentiation. As more research is conducted into bcl-2, more functions will be found. However, the demonstration that bcl-2 is involved in both differentiation and proliferation suggests that its conservation might be due to its functions

as an adaptive factor as opposed to a pro-survival factor. Bcl-2 appears to function in pathways that would allow for adaptation. This is partly represented by bcl-2's ability to facilitate the repair of DNA and inhibits p53 entry into the nucleus. Thus damaged DNA could have altered gene expression. By blocking apoptosis, slowing proliferation, bcl-2 allows for the transcriptional expression to occur. For a less organized species, this would be of profound benefit, allowing for adaptations to occur for maximization to its environment. To human beings this proves to be profoundly detrimental.

These cell lines might represent a novel model to investigate the bcl-2 functions that are distinctly associated with monomeric bcl-2 protein expression and test the functionality of bcl-2. This work lays the foundation for future studies needed to be conducted with regard to how bcl-2 is functioning on a biochemical level using these cell lines. Beginning with the determination of subcellular localization, this could be accomplished by Western blotting of isolated organelle compartments and by immunohistochemistry with anti-bcl-2 antibody to determine the localization and relative amount of bcl-2. . Additionally, future analysis of bcl-2 function and apoptosis in these cells should be considered utilizing inducers that function to produce apoptosis via different mechanisms, such as ionizing radiation or staurosporine. This would aid in the characterization of bcl-2 action, by determining exactly which apoptosis pathways were effected in this system. In these experiments,

the WB.v-myc/bcl-2 cells were only measured against themselves and found to be unable to have fully functional GJIC. Future studies will have to be conducted to test if co-culture of these cells with normal rat liver epithelial cells will behave differently and if these cells, when placed back into the rat liver, will either be suppressed or whether they can grow into tumors.

In conclusion, this study attempted to demonstrate the roles of proliferation, contact inhibition and apoptosis in promotion. Albeit, the initiation/promotion/progression model of carcinogenesis is an *in vivo* model that cannot be fully investigated in tissue cell culture. This report demonstrated that myc and bcl-2 could cooperate in the neoplastic transformation of WB-F344 cells. These myc/bcl-2 cells demonstrated characteristics consistent with a neoplastic phenotype being characterized by anchorage independent growth, blockage of apoptosis, and the loss of contact inhibition. All myc/bcl-2 cell lines grew in soft agar. This growth was independent of bcl-2 protein expression level and appeared to be related to the cell line's ability to communicate through gap junctions. This report also presented data investigating the role of differentiation in multi-step carcinogenesis and suggests that the progressive loss of differentiation is a characteristic of promotion. Lastly, this report speculated its findings into a model of carcinogenesis and suggests that bcl-2 evolutionary conservation might be related to an adaptive response rather than just apoptosis.

Figure 22. Graphical representation of proposed model for carcinogenesis. a) Direct graph demonstrating interaction of the five states of existence for a cell (H=homeostasis, A=apoptosis, D=differentiaiton, S=senescence, P=Proliferation). b-f) Signal divergence/convergence model of promotion. b) Homeostasis is represented by a balance of all signaling. c) Promotion is represented as a cumulative blockage of signaling associated with D (differentiation), S (senescence), and A (apoptosis). d) Differentiation is represented by a cumulative blockage of signaling associated with P, S, and A. e) Apoptosis is represented by a cumulative blockage of signaling associated with D, S, and P (proliferation). f) Senescence is represented by a cumulative blockage of signaling associated with P, D, and A.

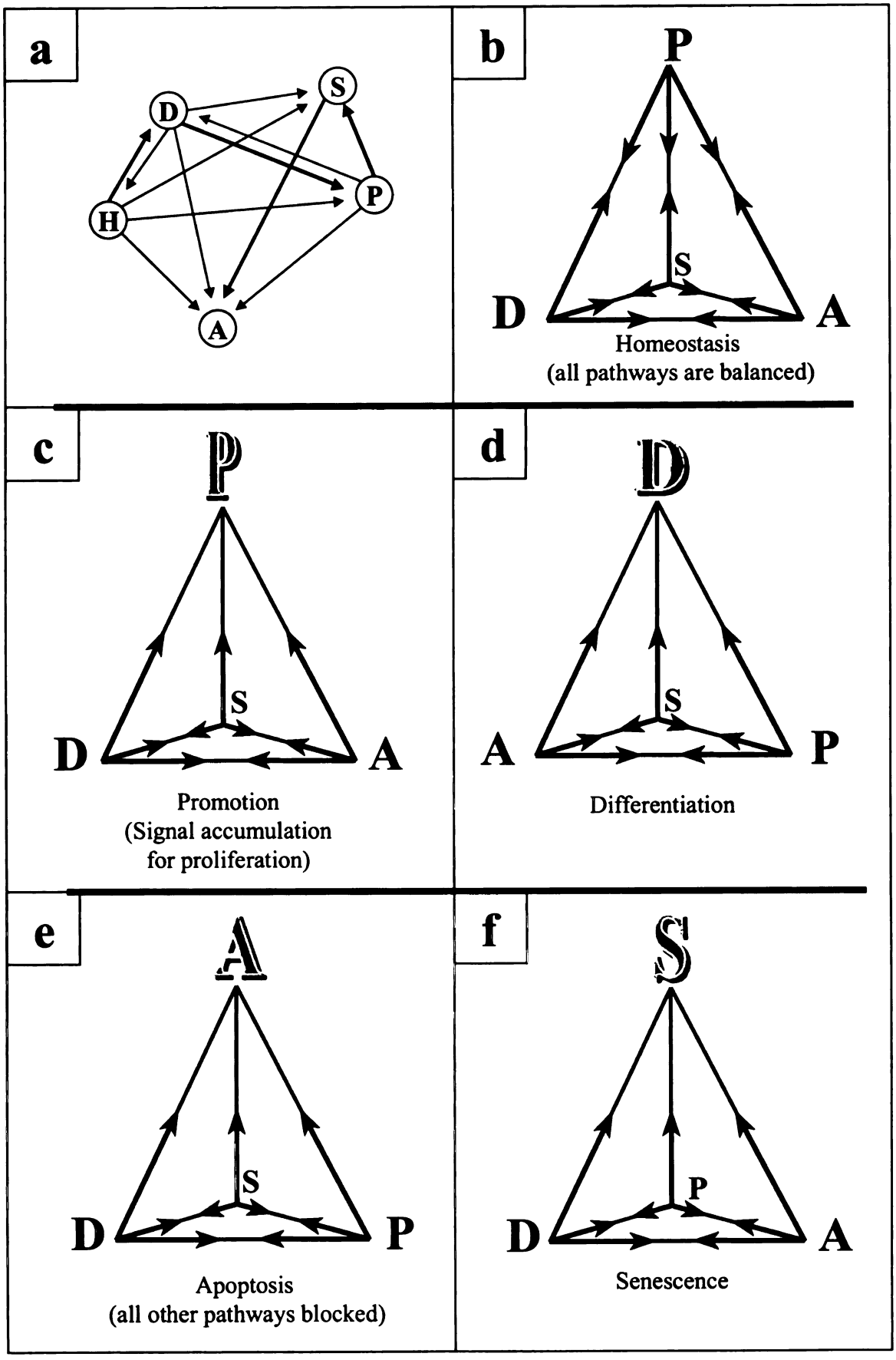

a
D
S
P
H
A
b
P
S
D
A
Homeostasis
(all pathways are balanced)
c
P
S
D
A
Promotion
(Signal accumulation
for proliferation)
d
D
S
A
P
Differentiation
e
A
S
D
P
Apoptosis
(all other pathways blocked)
f
S
P
D
A
Senescence

Figure 22

APPENDICES

APPENDIX A

PUBLICATION

Deocampo, Nestor D., Wilson, Melinda R., and Trosko, James E. (2000). Cooperation of *bcl-2* and *myc* in the neoplastic transformation of normal rat liver epithelial cells is related to the down-regulation of gap junction-mediated intercellular communication. *Carcinogenesis* 21, 1501-1506.

TITLE

Cooperation of Bcl-2 and Myc in the Neoplastic Transformation of Normal Rat Liver Epithelial Cells is Related to the Down Regulation of Gap Junction-Mediated Intercellular Communication.

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ABSTRACT

The objectives of this study were to isolate several rat liver epithelial cell clones, containing the human bcl-2 and myc/bcl-2 genes, in order to study their potential cooperative effect on neoplastic transformation and gap junction-mediated intercellular communication and to test the hypothesis that the loss of gap junction-mediated intercellular communication leads to tumorigenesis. Using anchorage independent growth as a surrogate marker for neoplastic transformation, we transfected both the normal rat liver epithelial cells, WB-F344, and a WB-F344 cell line overexpressing v-myc with human bcl-2 cDNA. Those cell lines that only expressed v-myc or human bcl-2 were unable to form colonies in soft agar. However, those cell lines that overexpressed both v-myc and human bcl-2 showed varying ability to form colonies in soft agar, which did not correlate with their human bcl-2 expression level. In order to test if there was a correlation between the cell lines' growth in soft agar and their ability to communicate through gap junctions, we performed scrape load dye transfer and fluorescent recovery after photobleaching assays. Our results show that v-myc and human bcl-2 can cooperate in the transformation of normal cells, but the degree to which the cells are transformed is dependant on the cells ability to communicate through gap junctions.

Abbreviations: AIG, anchorage independent growth; FBS, fetal bovine serum; FRAP, fluorescent recovery after photobleaching; GJIC, gap junction-mediated intercellular communication; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonyl fluoride; SL/DT, scrape load dye transfer; SDS, sodium dodecyl sulfate

INTRODUCTION

If one accepts the stem cell theory of carcinogenesis (1), then the blockage of terminal differentiation (2) might be involved in the "initiation" phase of carcinogenesis, while clonal expansion (mitogenesis), plus inhibition of apoptosis, might contribute to the "promotion" phase (3). Within this conceptual framework, over expression of various oncogenes , acting together with other genes, might be involved in either the initiation or promotion phases.

The myc oncogene, a transcription factor, has been associated with the cellular functions of proliferation, differentiation and apoptosis (4-8). Disruption of any of these basic cellular functions could contribute to the multi-step, multi-mechanism process of carcinogenesis. Over expression of the myc oncogene has been associated with many tumors (9,10).

The ras oncogene, being a member of the G- protein family, has been shown to be involved in one of the many signal transduction pathways affecting mitogenesis, differentiation and apoptosis. Ras, as with myc, has been shown to be activated in many types of tumors (11,12).

Cooperation of oncogenes is one of the first important molecular concepts of carcinogenesis. One of the first interactions to be reported was the cooperation between the myc and ras oncogenes (13). In addition, the demonstration that phorbol esters, such as 12-

tetradecanoylphorbol-13-acetate,TPA, seemed to act in a manner similar to the ras oncogene, in that it could cooperate with the myc oncogene to induce a neoplastic phenotype (14). TPA, by activating protein kinase C, triggers signal transduction pathways and can act as a modulator of mitogenesis, differentiation and apoptosis (15-17).

Ras and myc oncogenes have also been implicated in apoptosis (18-20). Where myc seems to confer a susceptibility to apoptosis (6,19,21), ras appears to reduce cellular responses to apoptosis (18,20). Therefore, in cells in which both myc and ras are activated, signal transduction crosstalk interacts to block terminal differentiation, triggering cells to proliferate and become resistant to apoptosis. In effect, these unregulated disruptions of interacting signals bring about the appearance of the tumor.

The bcl-2 protooncogene has been one of the major genes implicated in the apoptotic process (22). Early experiments in transgenic animals overexpressing bcl-2 under immunoglobulin promoter control showed increased frequency of follicular hyperplasia and B-cell survival thus suggesting the role of bcl-2 as an anti-apoptotic gene (23). Since this work many have demonstrated that bcl-2 is not only involved in tumors of lymphoid origin but also in many tumors of epithelial origin (24-31). Bcl-2 has also been demonstrated to synergistically interact with TPA to transform cells (32). Thus, by blocking apoptosis, bcl-2 seems to be acting as a surrogate "ras" gene. However, bcl-2 itself is

unable to induce proliferation or neoplastic transformation that is often associated with the overexpression of other oncogenes, like myc or ras (33-35).

Many investigators have studied the co-expression of myc and bcl-2 in a variety of cell types and transgenic models (36-39). These studies were able to show that bcl-2 and myc can cooperate and increase or enhance the tumor incidence and formation. However, these experiments studying myc and bcl-2 cooperation have focused on cells of lymphoid origin, fibroblasts, and a whey acidic protein promoter controlled bcl-2 vector in the mouse lactating mammary gland, but not in other epithelial systems. The experiments we describe in this study were designed to test whether the bcl-2 oncogene in cooperation with v-myc could function to neoplastically transform the WB-F344 cell line, a normal rat liver epithelial cell line. We have previously reported the establishment of a WB-F344 cell line stably expressing the v-myc oncogene (40). This cell line demonstrates an increase in proliferation and cell saturation density but it did not form colonies in soft agar or tumors in nude mice. This cell line will be used as the target for transformation by bcl-2.

Our laboratory and others have shown that while ras and TPA effect signal transduction, they also affect gap junction-mediated intercellular communication (GJIC) (41-44). Gap Junctions are channels that directly link the interiors of neighboring cells allowing for the free

diffusion of small molecular weight molecules (45). Most tumors demonstrate a reduction in GJIC activity, either between themselves (homologous GJIC) or with other cell types (heterologous GJIC) (3). Presumably, down regulation of GJIC activity would lead to the removal of growth inhibitory signaling, thereby providing a selective advantage. TPA was the first agent shown to reduce GJIC activity (43,44). Rat liver epithelial cells stably transfected with Ha-ras demonstrate a dose-dependant reduction of GJIC with increasing levels of ras T24 protein (41,42,46). Recently our laboratory reported that Ha-ras and v-myc could cooperate to down regulate GJIC and this down regulation correlated to the cells malignancy (40). In this same manner, we have investigated the effect that bcl-2 alone and in cooperation with v-myc will have on GJIC and look for any correlation to neoplastic transformation.

In this report we demonstrate that bcl-2 can cooperate with v-myc to induce neoplastic transformation of a normal rat liver epithelial cell line. We also demonstrate that the extent of neoplastic transformation is dependant on the cells ability to communicate through gap junctions.

MATERIALS AND METHODS

Cell Culture

WB-F344 rat liver epithelial cells, WB.myc and all subsequent bcl-2 transfectants were cultured in D-media (Formula 78-5470EF, Life Technologies Inc.(Gibco/BRL), Gaithersburg, MD), supplemented with 7% fetal bovine serum (Life Technologies Inc.(Gibco/BRL), Gaithersburg, MD). All cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Transfection of bcl-2

The human Bcl-2 cDNA cloned into the pSFFV vector (kindly provided by Gabrial Nunez, University of Michigan, Ann Arbor, MI) was transfected into WB-F344 cell line and co-transfected with pTK-Hyg (Clontech Laboratories Inc., Palo Alto, CA) into the WB.myc cell using lipofectin (Roche Molecular Biochemicals, Indianapolis, IN). Briefly, 40 □g of Lipofectin was added to 1.5 milliliters of D-Medium (serum free) and 20 □g of DNA was added to a separate 1.5 ml of D-medium. The two samples were mixed and incubated at room temperature for 15 minutes. This mixture was then added to sub-confluent cultures grown in 100 mm dishes that had been rinsed twice with serum free D-media. The plates were then incubated overnight at 37°C in a humidified chamber. The following day D-medium containing 7% fetal bovine serum was added

directly to the plates and incubated an additional 24 hours. Transformed cells were then split and subsequently plated with appropriate drug selection. Individual clones were then isolated using cloning rings, and subsequently recloned. Due the enhanced apoptotic properties of the WB.myc cells, co-transfection with pTK-Hyg was performed as described above with the following modifications: first, the culture was allowed to reach confluency; second, 20 mg of DNA in a ratio of 1:20 pTK-Hyg:pSFFV.bcl-2 was used to assure that selection using hyromycin B would yield bcl-2 positive cells.

Protein extraction

Proteins were extracted from confluent cell culture grown in 25 cm^2 flasks using 20% sodium dodecyl sulfate (SDS) containing 2mM phenylmethylsulfonyl fluoride (PMSF), 1 □M aprotinin, 1 □M leupeptin (Roche Molecular Biochemicals, Indianapolis, IN 46250), 1 □M antipain, 5 mM sodium fluoride (Fluka, Milwukee, WI), 0.1 mM sodium orthovanadate (Aldrich, Milwukee, WI), and subsequently sonicated three times at five second intervals, aliquoted and stored at –20°C. The protein concentration was determined by diluting the extracts 1:5 and assayed using the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA).

Western Blot analysis

Proteins were loaded in equal amounts (10-15 □g) into each well of a 10% SDS-polyacrylamide, in accordance with Laemmli (47). The gels were electrophoresed at 200 mV for approximately 45 minutes, removed and equilibrated in transfer buffer and transferred to Immobilon-P PVDF membrane (Millipore Corporation, Bedford, MA). Human Bcl-2 was detected with hamster anti-human bcl-2 monoclonal antibody (Pharmingen, San Diego, CA), endogenous rat bcl-2 was detected with a polyclonal anti-bcl-2 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), v-myc was detected with anti-v-myc polyclonal antibody (Caltag Laboratories, Burlingame, CA), using the supersignal ultra substrate (Pierce Chemical Co. Rockford, IL) and appropriate peroxidase labeled secondary antibody.

Anchorage Independent Growth

To access anchorage independent growth cells were grown in soft agarose as previously described (48). Briefly, an initial hard agarose layer (0.5% agarose 6013, Simga Chemical Co)made in D-medium containing 7% FBS was plated in a 60 mm dish. Individual cell lines were then trypsinized, counted, and diluted to a final concentration of 100 total cells in D-Medium containing 0.33% agarose and 7% FBS and was subsequently plated on top of the 0.5% agarose layer. Additional medium was added 3 days later and changed every 3 days for 28 days.

Colonies were stained using 1 mg/ml 2-(p-iodophenyl)-3-(nitrophenyl)-5-phenyltetrazolium chloride (sigma) in 0.9% NaCl, and counted.

Cell-Cell Communication assays

Two methods developed in our laboratory were used to assess gap junction-mediated intercellular communication: (a) scrape-loading dye transfer (SL/DT) (49), (b) fluorescence recovery after photobleaching (FRAP) (50). The scrape-loading dye transfer assay utilizes confluent cultures grown in 35-mm dishes. The cultures are then rinsed three times with Ca2+, Mg2+- phosphate-buffered saline (PBS). 1.5 milliliters of PBS containing 0.05% Lucifer yellow CH (Molecular Probes Inc., Eugene, OR) was added, and several scrapes (cuts) were made on the monolayer using a surgical scalpel. The cultures were incubated for 3 minutes at room temperature in the dye solution, and then rinsed three times with Ca2+, Mg2+-PBS (to remove any background fluorescence). The cultures were then fixed with 1 milliliter of 4% formalin and visualized using an Ultima laser cytometer (Meridian Instruments, Lansing, MI).

The scrape-loading dye transfer assay results were confirmed using the FRAP assay. Briefly, cultures grown in 35-mm dishes were rinsed 3 times with Ca2+, Mg2+-PBS and then incubated with Ca2+, Mg2+-PBS containing 5,6-carboxyfluorescein diacetate (7mg/ml, Molecular Probes Inc., Eugene, OR) at 37°C in a humidified incubator for 15 minutes. The

cells were then rinsed several times with Ca2+, Mg2+-PBS and analyzed using the Ultima laser cytometer. Cells were randomly selected under a microscope (four cells were selected per field plus one unbleached control, five fields per scan), and photobleached with an argon laser beam. The transfer of fluorescence was then monitored in 4-minute intervals for a total of 12 minutes. The intensity of recovered fluorescence of the individually bleached cells is then quantitated and rates of dye transfer can be measured as the percentage of an unbleached control cell (one per field).

RESULTS

Characterization of Isolated Clones

To examine the interaction between v-myc and bcl-2 oncogenes in neoplastic transformation and GJIC in rat liver epithelial cells, we generated clonal cell lines, utilizing established normal WB-F344 and WB.v-myc cell lines, over-expressing human-bcl-2 alone and co-expressing human bcl-2 and v-myc. The morphological appearances under phase-contrast microscopy of the different cell lines are shown in figure 1. The WB-F344, WB.v-myc, and WB.bcl-2 cells grew in uniform monolayers of polygonal cells and exhibited contact inhibition of growth. The Wb.myc/bcl-2 cell lines demonstrated different morphologies, such as seen between the different clones [uniform polygonal, spindle-shaped, and multi-nucleated].

Western Blot Analysis of Bcl-2

The expression of human bcl-2 was assessed using western blot analysis with an anti-bcl-2 monoclonal antibody (Pharmingen, San Diego, CA) specific for human bcl-2 protein. Figure 2a shows high expression of a 26 kDa band corresponding to transfected human bcl-2 protein, while the control cell lines show no expression. We also verified the presence of v-myc using western blot analysis with an anti-v-myc polyclonal antibody. Figure 2b shows the presence of a 110 kDa band that

corresponds to the gap-pol-myc region of v-myc. Its expression is consistent between the controls and transfected clones, suggesting that over-expression of bcl-2 has not effected the expression of v-myc from the parental WB.v-myc cell line.

Anchorage Independent Growth

Anchorage independent growth, more commonly referred to as growth in soft agar, has often been used as a marker for neoplastic transformation and to identify tumor cells (51-54). While this assay does not confirm that cells are indeed neoplastically transformed, it does demonstrate that those cells that are able to grow contain a characteristic shared with all tumors. To assess if the bcl-2 gene was conferring neoplastic characteristics to the normal rat liver epithelial cells, we tested their ability to grow in soft agar. Using a previously described (40) v-myc/Ha-ras tumorigenic WB-F344 cell line (designated MR-42) as a positive control (100% colony forming efficiency), we plated replicate plates with 100 cells per plate in soft agar. Figure 3 demonstrates that the control cell lines, as well as the bcl-2 only clones, were unable to form colonies in soft agar. However, the v-myc/bcl-2 clones were able to form colonies in soft agar and they also demonstrated clonal differences in their colony forming efficiency ranging from 7 to 90 %. When we compared the v-myc/bcl-2 clones colony forming efficiencies to their respective bcl-2 expression level, we found no correlation.

Gap Junction-Mediated Intercellular Communication (GJIC)

In order to explain the differences in growth in soft agar demonstrated by the myc/bcl-2 cell lines, we assessed the individual cell lines ability to communicate through gap junctions. Figure 4a demonstrates typical results obtained from the scrape loading dye transfer technique. The WB-F344 cell line is used as the control for communication with the dye traveling about 6-8 cell layers. When WB-F344 cells are compared to the WB.mb19 clone containing v-myc and bcl-2, there is an approximate 50% decrease in the cells ability to transfer dye (3-4 cell layers). These results were confirmed using the fluorescent recovery after photobleaching assay (FRAP). Figure 4b shows the GJIC activity (both SLDT and FRAP) of the various cell lines. This assay allows for the monitoring of individual cells in the population assessing an activity for the whole population. When compared to colony forming efficiency, the two communication assays appear to be inversely correlated. Those cell lines that showed no significant changes in GJIC demonstrated a marked decrease in colony forming ability in soft agar. However those cell lines that demonstrated marked reduction in cell-cell communication demonstrated an enhanced or increased ability to form colonies in soft agar.

DISCUSSION

In this study our objective was to determine if myc and bcl-2 could act cooperatively in the induction of tumorigenicity by the inhibition of gap junction-mediated intercellular communication. To test this idea, myc/bcl-2 genes were co-expressed in WB-F344 cells, a normal rat liver epithelial cell line. The parental WB-F344 cells, myc transformed, and bcl-2 transformed cells grew in uniform monolayers of polygonal cells but did not grow in soft agar. The myc/bcl-2 transformed cells demonstrated various phenotypes and the cells were able to form colonies in soft agar. When these cell lines were compared to a cell line with 100% colony forming efficiency, they demonstrated varying abilities to form colonies is soft agar. The ranges in colony forming ability did not correlate to the cell lines respective bcl-2 expressions. We further characterized these myc/bcl-2 cell lines by examining their gap junction-mediated intercellular communication. We found that there was an inverse correlation between the cell ability to communicate and their ability to form colonies in soft agar. Those cells lines that demonstrated a reduced GJIC showed an increased ability to form colonies in soft agar.

The v-myc/bcl-2 transformed cells did indeed grow in soft agar, while the controls (WB-F344, WB.v-myc, WB.bcl-2) did not grow. The degree to which the cell lines were able to form colonies varied greatly. If over-expression of bcl-2 was the main factor in the neoplastic

transformation of the WB.v-myc cell line, we would expect to see a dose correlation between bcl-2 and the cell lines colony forming efficiencies. We did not see any correlation between bcl-2 expression and colony formation, which suggests another mechanism occurring that was responsible for the differences we were observing.

Gap junction-mediated intercellular communication (GJIC) has been shown, by our group and others, to play an important role in tumor promotion and progression. Presumably, as cells lose GJIC, they are effectively removed from intercellular signals that can regulate proliferation, differentiation, and apoptosis. In this manner an "initiated" cell could escape growth regulation.

The role of GJIC in the regulation of cell behavior (e.g., growth control, differentiation, apoptosis, and adaptive responses of terminally-differentiated cells), while still not known in detail, seems to involve the transfer of ions and small molecular weight regulatory molecules through the gap junction channels to act as either a "sink" or "source" (55). GJIC is known to synchronize electronic or metabolic responses between cells (56). Coupling of normal homologous or heterologous cells could alter the behavior of the cells. When it was shown that tumor promoters, such as phorbol esters (44), could reversibly inhibit GJIC, it was hypothesized that an "initiated" cell (a stem-like cell which has been prevented from terminally differentiating or which has been "immortalized") (57) would be growth suppressed by

being coupled by gap junctions to surrounding normal cells. The implication is that, while the initiated cell is genotypically altered by the "initiator", it still could be "partially blocked from terminally differentiating" (2) and be "contact inhibited" by regulatory signal equilibration from surrounding normal cells. Growth factors, hormones and tumor promoting chemicals, by triggering signal transduction in the "initiated" and surrounding normal cells, would (a) cause the down-regulation of GJIC (leading to the inhibition of contact inhibition) and (b) induced gene expression related to cell proliferation/differentiation (57). While the normal cells would proliferate and differentiate, the "initiated" cell would only proliferate or not die by terminal differentiation or apoptosis, thereby increasing the number of initiated cells. As long as the external exogenous chemical (tumor promoter) is applied, the clonal expansion of the initiated cell can occur. If, and when, other genetic changes within the initiated cell occur that can stably down regulate GJIC, external dependence on tumor promoters declines and the cell becomes independent of the suppressing effect of surrounding normal cells.

When we examined the myc/bcl-2 cell lines GJIC activity, we found a striking correlation between GJIC and colony formation. Those cell lines that possessed functional GJIC, formed very few colonies in soft agar. However, those cell lines that demonstrated a reduction in GJIC activity, showed dramatic increases in their ability to form colonies.

This suggests an active role for GJIC in contributing to neoplastic transformation. The use of myc and bcl-2 was not used to imply that all cells being initiated or neoplastically transformed represent the initiation/promotion/progression model of carcinogenesis, but rather to show the potential importance of GJIC during that process. The myc transformed cell still has functional GJIC and could be suppressed by surrounding normal cells. In these experiments, the WB.v-myc/bcl-2 cells were only measured against themselves and found to be unable to have fully functional GJIC. Future studies will have to be conducted to test if co-culture of these cells with normal rat liver epithelial cells will behave differently and if these cells, when placed back into the rat liver, will either be suppressed or whether they can growth into tumors.

These new clones demonstrate that bcl-2 can cooperate with myc to neoplastically transform rat liver epithelial cells. However, the degree to which the cells are neoplastically transformed is dependent on their ability to communicate through gap junctions.

Acknowledements

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Fig. 1. Phase Contrast photomicrographs of WB-F344, control and transfected cells. (A) WB-F344; (B) vector control, WB.neo; (C) WB.v-myc; (D-F) WB.myc/bcl-2 clones, WB.mb3, WB.mb19, WB.mb39 respectively.

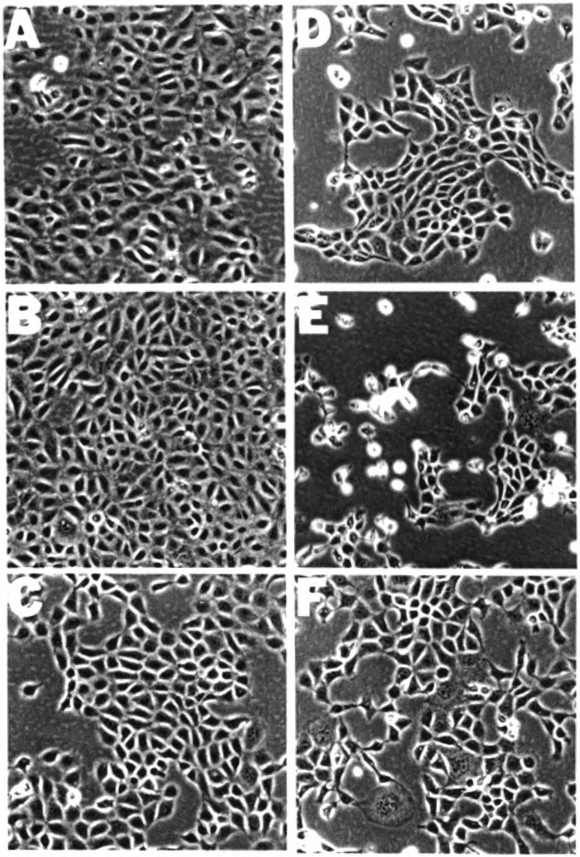
A
D
B
E
C
F

Fig. 2. Analysis of expression of human bcl-2 and v-myc proteins. (A) Detection of 26 kDa protein corresponding to human bcl-2 from extracts of normal WB-F344 and transfected cell lines. (B) Detection of a 110 kDa protein corresponding to v-myc from extracts of normal WB-F344 and transfected cell lines.

a.
WB-F344
WB.v-myc
WB.myc/ras
WB.mb3
WB.mb6
WB.mb19
WB.mb28
WB.mb39
WB.mb50
26 kDa
b.
WB-F344
WB.v-myc
WB.myc/ras
WB.mb3
WB.mb6
WB.MB19
WB.mb39
110 kDa

Fig. 3. Assessment of anchorage independent growth using growth in soft agar. The data represent the efficiency of colony formation represented as the number of colonies formed per 10^2 cells plated.

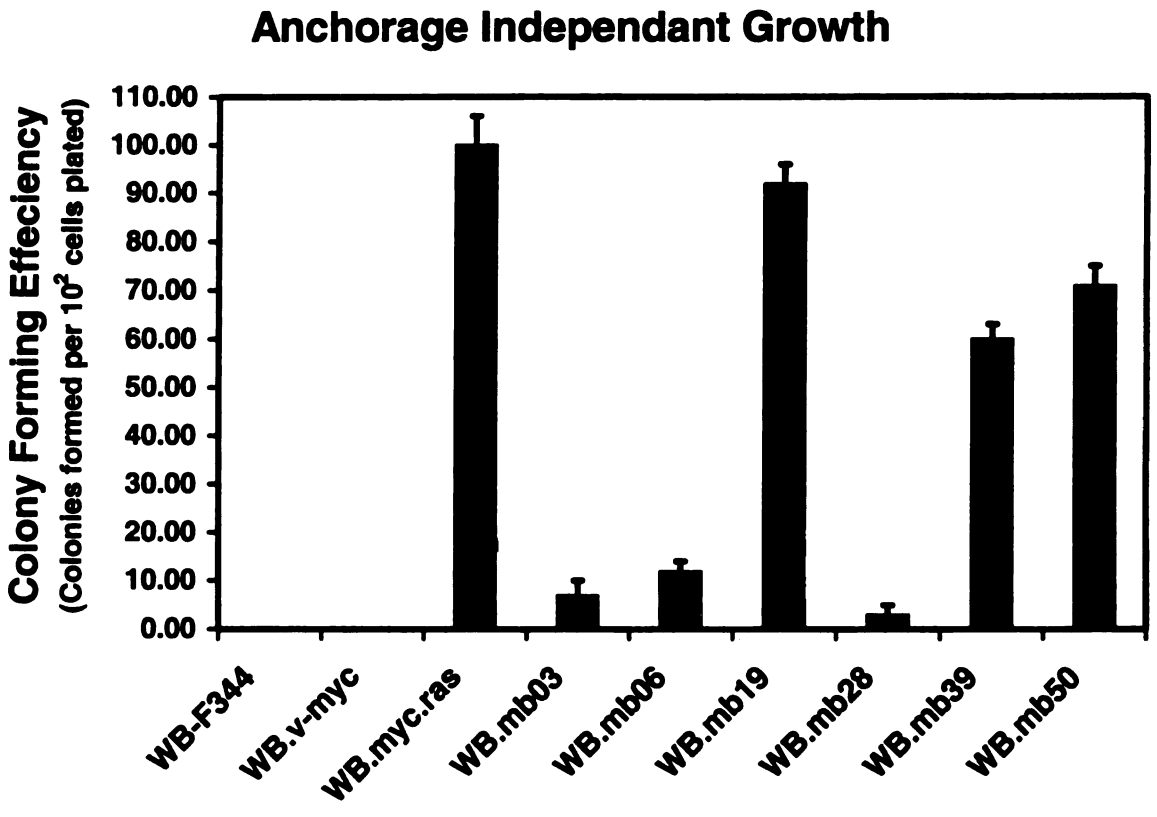
Anchorage Independant Growth
Colony Forming Effeciency
(Colonies formed per 10² cells plated)
110.00
100.00
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
WB-F344
WB.v-myc
WB.myc.ras
WB.mb03
WB.mb06
WB.mb19
WB.mb28
WB.mb39
WB.mb50

Fig. 4. Analysis of GJIC expression using the scrape loading dye transfer (SL/DT) and fluorescent recovery after-photobleaching (FRAP) techniques. (A) A representation of the SL/DT assay. Note the normal WB-F344 cell line transfers the dye approximately 6-8 cells, while the myc/bcl-2 clone, MB.19, is only able to transfer the dye 2-3 cells on either side of the scrape line. (B) Assessment of GJIC using the FRAP technique. The data represent the percentage of fluorescent recovery after a single cell is photobleached amongst a population of cells.

a
A
B
b
Gap Junction-mediated Intercellular Communication
Percent of Control
120%
100%
80%
60%
40%
20%
0%
SLDT
FRAP
WB-F344
WB.v-myc
WB.myc-ras
WB.mbc3
WB.mbc6
WB.mbc19
WB.mbc28
WB.mb39
WB.mb50

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