Novel Biological Approaches for Detecting Oncogenic Cooperation with Bcl-2

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science Graduate Department of Immunology University of Toronto

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ABSTRACT

Seventy to ninety percent of follicular lymphomas possess a characteristic t(14;18) translocation resulting in deregulated expression of the proto-oncogene Bcl-2. Overexpression of Bcl-2 alone is not sufficient to cause full cellular transformation. According to the “two-hit” hypothesis, cellular transformation in lymphoma occurs when, at least, one other genetic alteration cooperates with deregulation of Bcl-2. c-myc has been suggested to fulfill this role.

Using a non-transformed, overexpressing Bcl-2 only, and a transformed, overexpressing Bcl-2, c-myc and possessing mutant p53, cell line we developed various assays in which we can detect oncogenic cooperation with Bcl-2. When we used a transformed cell line that only overexpresses Bcl-2 and c-myc we were not able to detect oncogenic cooperation. Our results suggest that co-overexpression of Bcl-2 and c-myc is not sufficient to cause full cellular transformation and that additional cooperative genetic lesions must be acquired.
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LIST OF ABBREVIATIONS

aa         amino acid(s)
AIF        apoptosis inducing factor
Apaf-1     apoptosis protease activating factor-1
ATP        adenosine triphosphate
BCS        bovine calf serum
BH         Bcl-2 homology
bp         base pair(s)
BSA        bovine serum albumin
C-terminal carboxy terminal
Ca²⁺       calcium
cdc        cyclin dependent kinase
cDNA       complementary DNA
CGH        comparative genomic hybridization
DCC        Deleted in Colorectal Cancer gene
Dₜ        diversity gene segment, heavy chain
DLCL       diffuse large cell lymphoma
DNA        deoxyribonucleic acid
dNTP       deoxynucleoside triphosphate
EBNA       Epstein-Barr virus nuclear antigen
EBV        Epstein-Barr virus
ER         endoplasmic reticum
FACS       fluorescence-activated cell sorter
FISH       fluorescence in situ hybridization
FITC       fluorescein isothiocyanate
FL         follicular lymphoma
GFP        green fluorescent protein
Ig  immunoglobulin
IL  interleukin
ILGF insulin-like growth factor
JH  joining gene segment, heavy chain
kb  kilobase
kDa kiloDalton
κ  kappa
mbr major breakpoint region
MCAT-1 mouse cation amino acid transporter
mcr minor cluster region
mRNA messenger RNA
MSV Moloney murine sarcoma virus
N-terminal amino terminal
nd not done
NHL Non-Hodgkin's lymphoma
λ  lambda
LB Luria broth
LCL lymphoblastoid cell line
LMP latent membrane protein
PARP poly(ADPribose)polymerase
PBL peripheral blood lymphocyte
PBS phosphate-buffered saline
PCR polymerase chain reaction
PE R-phycoerythrin
PHA phytohemagglutinin
LIST OF ABBREVIATIONS continued

PI  propidium iodide
PT  permeability transition
PTPC permeability transition pore complex
RAG recombinase-activating gene
Rb  retinoblastoma
RNA ribonucleic acid
RNase ribonuclease
RSS recombination signal sequence
Ser serine
UV ultraviolet
vcr variable cluster region
V_{\text{H}} variable gene segment, heavy chain
CHAPTER 1: INTRODUCTION
1.1. Introductory overview and rationale

Seventy to ninety percent of follicular lymphomas and approximately 30% of diffuse B-cell lymphoma possess a characteristic t(14;18) translocation which results in deregulated expression of the proto-oncogene Bcl-2. Although overexpression of Bcl-2 is known to contribute to the pathogenesis of human B-cell lymphoma, it alone is not sufficient to cause cellular transformation. According to the "two-hit" hypothesis, cellular transformation in lymphoma occurs when, at least, one other genetic alteration cooperates with deregulation of Bcl-2.

Elucidation of the molecular mechanisms underlying lymphomagenesis may ultimately extend therapeutic options and include those based upon the specific genes involved. Therefore, we have set out to develop a system in which we can detect oncogenic cooperation with Bcl-2 in a human mature B-cell lymphoblastoid cell line in hopes of discovering novel genes capable of cooperation with Bcl-2.

1.2. Non-Hodgkin's lymphoma

Lymphoma is a solid tumour of the lymph node. Non-Hodgkin's lymphoma (NHL) is a general term referring to the gamut of malignancies which occur in peripheral lymphoid cells, the majority of which arise from germinal follicular centre cells (Hoffbrand and Pettit, 1993). Therefore, NHLs are separate from lymphoid malignancies of precursor lymphoid cells which include acute lymphoblastic leukaemias or from tumours of terminally
differentiated cell of the lymphoid lineage, for example, multiple myeloma (Gaidano and Carbone, 1996). Approximately 50,000 new cases of NHL are diagnosed each year in North America, ranging from highly proliferative and rapidly lethal diseases to some of the most indolent and well-tolerated malignancies found in humans (Hoffbrand and Pettit, 1993; Aisenberg, 1995). Close to 40% of NHL are defined as low grade and include malignancies such as follicular lymphoma (FL) which occurs as an asymptomatic and widely disseminated disease characterized by spontaneous regressions in up to 23% of cases (Horning and Rosenberg, 1984). Over 50% of low grade malignancy cases transform into high grade, are aggressive in their progression and lead to lethal disease [e.g. diffuse large cell lymphoma (DLCL)] with survival rates of 5 to 10 years (Connors, 1989).

Each lymphoma subtype involves at least one specific chromosomal abnormality (translocation, deletion, or duplication). The most frequent abnormalities are translocations involving the proto-oncogene on one chromosome and an antigen receptor locus [immunoglobulin- (Ig) heavy or light chain loci in B-cell NHL] on the partner chromosome (Rowley et al., 1990). Previously it was thought that these translocations were due to mistakes at the time of physiologic V(D)J recombination in B lymphocytes (Bakhshi et al., 1987; Tsujimoto et al., 1985). However, recent evidence suggests that the recombinase activating genes 1 and 2 (RAG1 and RAG2) may act like transposons and therefore may be responsible for these translocations (Agrawal et al., 1998; Hiom et al., 1998; Roth and Craig, 1998). Oncogenes
implicated in lymphomagenesis include Bcl-1 (product, cyclin D, regulates mitosis), Bcl-2 (product regulates apoptosis), Bcl-3 (product inhibits the transcription factor NF-κB), Bcl-6 (product regulates cell proliferation and differentiation), and c-myc (product regulates gene transcription) (Aisenberg, 1995). It has been proposed that the initial transforming event in the development of FL is a translocation between chromosomes 14 and 18 [t(14;18)]. This translocation results in juxtaposition of the Bcl-2 protooncogene on 18q21 to the heavily transcribed Ig heavy chain J region on 14q32 (Bakhshi et al., 1985; Tsujimoto and Croce, 1986). Consequently, the Bcl-2 protein is overproduced (Tsujimoto et al., 1985a). Although Bcl-2 overexpression interferes with normal life and death of the follicular center cell and initiates the transformation process, Bcl-2 overexpression on its own is not completely transforming. Extensive evidence has shown that lymphomagenesis is a multistep process and that a secondary genetic event must occur in cooperation with Bcl-2 overexpression for the malignant phenotype to develop. Rearrangements of c-myc and Bcl-6, as well as loss of p53 (tumour suppressor gene) function, have been suggested as the additional genetic events involved in the progression from FL to DLCL in some cases of lymphoma (Yunis et al., 1987; Vaux et al. 1988; Lee et al., 1989; Farrugia et al., 1994; Aisenberg, 1995).
1.3. Human Bcl-2 gene in health and disease

1.3.a. Bcl-2: gene and protein structure

The Bcl-2 gene was initially discovered by virtue of its association with the t(14:18) translocation and elevated expression in follicular B-cell lymphomas (Tsujimoto et al., 1985; Bakhshi et al., 1985; Cleary and Sklar, 1985). The human Bcl-2 gene is located on chromosome 18q21 (Bakhshi et al., 1985; Tsujimoto et al., 1985), spans at least 230 kb and consists of three exons (Seto et al., 1988; Silverman et al., 1990). Exon I is alternatively utilized and is seemingly non-translated, possessing stop codons in all open reading frames. The open reading frame is located at the juncture of exon II and III. The Bcl-2 gene possesses two introns. The first, located between exons I and II, is 220 bp and is variably spliced. The second intron is 370 kb, correctly spliced and is located between exons II and III (Seto et al., 1988).

There are two distinct promoter regions in the Bcl-2 gene. A classic TATA (-88) plus CAAT (-106) box is found immediately 5' to the open reading frame in exon II with two distinct initiation sites (-31 and -58) before the initiation codon. An exact decanucleotide (ATGCAAAGCA) motif is present at (-119), homologous to an SV40 enhancer and the upstream enhancers of IgV regions. A totally separate GC-rich, TATA-less promoter region exists further 5' in exon I. This region contains seven (GGGCGG) motifs in either orientation and demonstrates multiple sites of transcription initiation at positions (-1394), (-1399), (-1406), (-1410) and (-1432). Therefore, multiple
different initiation sites exist both in front of the open reading frame in exon II as well as 5' to the splice in exon I with the upstream exon I sites being used most frequently. The Bcl-2 gene is transcribed into three mRNAs of different sizes; 8.5-kb, 5.5-kb, and 3.5-kb (Seto et al., 1988).

The Bcl-2 gene encodes two proteins due to an alternative splicing event. The first protein, p26-Bcl-2-α is composed of 239 amino acids while the second protein, p22-Bcl-2-β, is 205 amino acids. Sequence analysis shows that the two Bcl-2 proteins differ at their carboxyl terminus with the p26-Bcl-2-α protein possessing a very hydrophobic stretch of 23 residues at its 3' carboxyterminus. This hydrophobic region is likely responsible for the localization of the Bcl-2-α protein in the membrane. Following this lipophilic region are two highly charged basic C-terminal amino acids (His and Lys) that presumably serve to anchor the protein across the membrane (Tsujimoto and Croce, 1986; Chen-Levy et al., 1989). Mutagenesis studies have shown that the hydrophobic COOH-terminal domain (aa 219-237) allows post-translational insertion into membranes such that the bulk of the Bcl-2 protein is oriented towards the cytosol (Chen-Levy and Cleary, 1990). Previously, it was thought that membrane insertion was required for Bcl-2 to exert its function (Hockenbery et al., 1990; Tanaka et al., 1993). However, it is now known that although the COOH-terminal domain is important for membrane docking, its deletion does not abrogate the survival role of Bcl-2 (Nguyen et al., 1994; Borner et al., 1994). It is presumed that the 26 kDa form
of the Bcl-2 protein, existing as a non-glycosylated single polypeptide, is biologically more important than its 22-kDa sibling due to the fact that the Bcl-2-β protein is rarely seen in cells (Tsujimoto et al., 1987; Chen-Levy et al., 1989). In addition to the transmembrane domain, Bcl-2 also possesses a pore formation, dimerization/receptor and regulation domain (reviewed by Kroemer, 1997; Adams and Cory, 1998). Initial observations from Triton X extraction and phase separation experiments indicated that Bcl-2 had a lipophilic nature and was intimately associated with cellular membranes. Laser-scanning, confocal and electron microscopy have determined that Bcl-2 is located in the nuclear envelope, endoplasmic reticulum (ER) and the outer mitochondrial membrane (Chen-Levy et al., 1989; Monaghan et al., 1992; Krajewski et al., 1993; Akao et al., 1994).

1.3.b. Developmental expression and regulation of Bcl-2

Bcl-2 mRNA is expressed in the majority of pro-B cells and in mature naïve B cells but is temporarily down-regulated in the pre-B cell stage (Merino et al., 1994). However, the Bcl-2 pro-survival homologue Bcl-\textsubscript{xL} becomes up-regulated during the pre-B cell stage (Grillot et al., 1996). The regulation of Bcl-2 and Bcl-\textsubscript{xL} may coincide with several selection processes. The selection for functional rearrangements of IgH and IgL loci coincides with the down-regulation of Bcl-2 and up-regulation of Bcl-\textsubscript{xL}, while the opposite appears to occur with selection for recognition of self-antigen (negative selection). In addition, during positive selection of nonproliferating
centrocytes, these cells express abundant Bcl-2 mRNA transcripts but no detectable protein product. A convincing explanation is proposed to interpret this seemingly strange event. Bcl-2 transcription is initiated at an early stage of centrocyte development but immediate translation is blocked, resulting in accumulation of mRNA but little protein. If a given centrocyte fails to achieve the required amount/degree of antigen binding the translational block persists, Bcl-2 expression is prevented and the cell dies. However, signals generated from the appropriate interaction of surface Ig binding to antigen (positive selection) results in the release of the translational block. Consequently, Bcl-2 is allowed to be expressed, resulting in cellular survival and exit of the centrocytes from the germinal center (Chleq-Deschamps et al., 1993). In agreement with this theory, it was found that in vitro stimuli (which mimic in vivo selection stimuli) induce Bcl-2 and Bcl-xL up-regulation in centrocytes of splenic B cells (Liu et al., 1991; Grillot et al., 1996). A more general observation indicates that there is a striking inverse correlation between expression of Bcl-2 and lymphocyte proliferation. In B cells, staining of tonsils with antibodies specific for Bcl-2 and for the Ki-67 proliferation-associated antigen revealed an absence of Bcl-2 in the Ki-67 positive proliferative B cells of germinal centers. Conversely, high levels of Bcl-2 are seen in the quiescent recirculating IgM/IgD B cells of the mantle zone (Pezzella et al., 1990).

Bcl-2 expression is controlled by both regulation of gene transcription and post-translational modification of the protein product. Bcl-2 is induced
transcriptionally by numerous cytokines such as; Interleukin-2 (IL-2), IL-3, IL-4, IL-7, IL-10, insulin-like growth factor (ILGF) and tumour necrosis factor-α (TNF α) (Deng and Podack, 1993; Genestier et al., 1995; Jiang et al., 1996; Minshall et al., 1997; Maraskovsky et al., 1997; Akashi et al., 1997; Cohen et al., 1997). Reed et al. found that when peripheral blood lymphocytes (PBL) or purified populations of B and T cells were stimulated with the mitogen lectin phytohemagglutinin (PHA) or with IL-2, accumulation of 8.5- and 5.5-kb mRNAs for Bcl-2-α rose from undetectable to maximal levels (10-20 fold increase) within 6 to 14 hours and that the 8.5-kb and 5.5-kb mRNAs had half-lives of approximately 2.5 to 3.0 hours. They also found that stimulation of resting peripheral blood lymphocytes (PBLs) induced rapid increases in the rate of Bcl-2 transcription beginning within 1 hour and reaching peak levels approximately 6 hours later. Comparison of the kinetics of gene transcription with the time course of Bcl-2 mRNA accumulation demonstrate that Bcl-2 transcription preceded accumulation of mature mRNAs by a few hours, likely reflecting the time required for the processing of primary transcripts (Reed et al., 1987; Graninger et al., 1987).

Recently, considerable attention has been paid to the effects of post-translational phosphorylation of Bcl-2 and it has been shown that phosphorylation can either enhance or suppress activity depending on which of the numerous serine residues in the nonconserved "regulation domain" is phosphorylated. Ito et al., demonstrated via mutational studies that phosphorylation of Ser70 is required for complete Bcl-2 death suppressor
signaling activity. The loss of function S70 mutant (serine → alanine) was able to heterodimerize with Bax but could not prevent apoptosis, indicating that Bcl-2:Bax heterodimerization alone is not sufficient to abrogate apoptosis and that phosphorylation of the serine residue is also required for Bcl-2 activity (Ito et al., 1997). Bcl-2 phosphorylation by Cdc2/cyclin B1 kinase is also tightly associated with mitotic arrest (Ling et al., 1998). In contrast to these reports, Haldar et al. found that phosphorylation of Bcl-2 (by anticancer drugs that affect the integrity of microtubules) occurs in G2-M and abrogates the normal anti-apoptotic function of Bcl-2, resulting in the initiation of the apoptotic program (Haldar et al., 1997). Not much is known about the phosphorylation pathways involved in the activation/suppression of Bcl-2 but recently it was found that the JNK/SAPK class of MAP kinases phosphorylate Bcl-2 (Maundrell et al., 1997).

1.3.c. The Bcl-2 family

At least 15 mammalian members and several viral members of the Bcl-2 family have been identified and all possess at least one of four conserved motifs known as Bcl-2 homology domains (BH1 to BH4). Most pro-survival members (Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1, NR-13, BHRF1, LMW5-HL, ORF16, KS-Bcl-2, E1B-19K and CED-9) contain at least BH1 and BH2 with the homologues most similar to Bcl-2 possessing all four BH domains (Bcl-2: BH1, aa 136-155; BH2, aa 187-202; BH3, aa93-107; BH4, 10-30) (Zha et al., 1996). There are two pro-apoptotic subfamilies which differ considerably in their
homology to Bcl-2. The "Bax subfamily", which consists of Bax, Bak, and Bok, contain BH1, BH2, and BH3 and resemble Bcl-2 fairly closely. The other seven members of the "BH3 subfamily" possess only the central short BH3 domain (reviewed by Adams and Cory, 1998). Pro- and anti-apoptotic family members can heterodimerize and seemingly titrate each other's function. Mutagenesis studies demonstrated that the BH1, BH2, and BH3 domains heavily influence homo- and heterodimerization (Yin et al., 1994; Zha et al., 1996). The role of BH1, BH2, and BH3 domains in dimerization became apparent when the three-dimensional structure of Bcl-xL was determined. An elongated hydrophobic cleft is created when the α helices in the BH1, BH2, and BH3 regions come together. This cleft provides a perfect region for an amphipathic BH3 α helix to bind (Muchmore et al., 1996; Sattler et al., 1997). Deletional studies have shown that the BH4 domain, possessed only by Bcl-2, Bcl-xL and Bcl-w, is not important for homodimerization or heterodimerization with pro-apoptotic homologues. However, Bcl-2 (and Bcl-xL) lacking BH4 loses its ability to both bind to CED-4 (an effector of apoptosis in Caenorhabditis elegans) and antagonize its pro-apoptotic activity indicating that it is a crucial domain in the prevention of apoptosis (Huang et al., 1998).
FIGURE 1.1. Diagram indicating structure and functional domains of Bcl-2, Bcl-X₇, and Bax. Bcl-2 and Bcl-X₇ promote cell survival whereas Bax promotes cell death. BH1 (aa 136-155), BH2 (aa 187-202), BH3 (aa 93-107), and BH4 (aa 10-30) are conserved sequence motifs. Bax and its subfamily (not shown) resemble the Bcl-2 subfamily (Bcl-2, Bcl-X₇, and members not shown) but lacks the functional BH4 domain. α1 to α7 indicate helices identified in Bcl-X₇. The core hydrophobic helices, α5 and α6, are flanked by 5 amphipathic helices, and a flexible loop connects α1 with α2. Arrows indicate Ser and Thr residues phosphorylated in Bcl-2 (Adams and Cory, 1998).
1.3.d. Biological and biochemical functions of Bcl-2

Using immature pre-B-cells that are dependent on IL-3 for their growth and survival, Vaux et al., were the first group to report that Bcl-2 can prolong cell survival. In this study it was noted that the stable transfer of Bcl-2 expression vectors into the IL-3 dependent cells allowed extended cellular survival in the absence of IL-3, but without concomitant cell proliferation (Vaux et al., 1988). It was known that IL-3 and several other colony-stimulating factors help maintain hemopoietic cell survival in vitro by preventing apoptosis, and Bcl-2 appeared to functionally replace IL-3 in terms of cell survival. Thus, it was reasonable to suggest that Bcl-2 was capable of blocking apoptosis (Tushinski et al., 1982; Lotem and Sachs, 1989; Williams et al., 1990). This concept was formally demonstrated by Hockenbery et al., who showed that the introduction of Bcl-2 into an IL-3 dependent cell line prevented the characteristic occurrence of apoptosis (typified by features such as nuclear membrane blebbing, volume loss, nuclear condensation, and DNA cleavage) following IL-3 deprivation (Hockenbery et al., 1990; Earnshaw, 1995). In addition to preventing or delaying cell death due to growth factor withdrawal, overexpression of Bcl-2 can prevent or reduce apoptosis induced by a broad variety of stimuli including chemotherapeutic drugs, gamma- and UV-radiation, heat shock, baculovirus infection, free-radicals, lipid peroxidation, p53 upregulation, c-myc overexpression, glutamate and calcium elevation, glucose deprivation, growth factor deprivation, neurotrophic factor withdrawal, increase in transforming growth factor-β levels, the presence of
some cytotoxic effector cells, azide, Ced 9 gene mutants in C-Elegans, prostaglandin E2 and addition of hydrogen peroxide (Sentman et al., 1991; Alnemri et al., 1992; Hockenbery et al., 1993; Miyashita and Reed, 1993; reviewed by Reed, 1994; Farlie et al., 1995; Brown and Phipps, 1996; Haldar et al., 1996; Saxena et al., 1996). Although Bcl-2 protects cells against a myriad of insults, it does not protect every cell type from every kind of apoptotic stimulus.

An interesting aspect of Bcl-2 function, which has been receiving increased attention lately, is its influence on the cell cycle. Bcl-2 involvement with cell cycle control was first suggested at by the observation that Bcl-2 expressing cells which survive cytokine withdrawal were quiescent (Vaux et al., 1988). It has also been shown that thymocyte turnover is retarded in Bcl-2 transgenic mice compared to their littermate controls. In addition, B and T cells expressing the transgene enter into the cell cycle more slowly than normal lymphocytes when stimulated with mitogens in vitro (Linette et al., 1996; Mazel et al., 1996; O'Reilly et al., 1996; O'Reilly et al., 1997). Furthermore, quiescent NIH 3T3 fibroblasts expressing either Bcl-2, Bcl-xL or their adenovirus homologue (E1B 19K protein) responded more slowly to serum stimulation than control cells (O'Reilly et al., 1996). Bcl-2 has also been observed to expedite withdrawal from the cell cycle. Constitutive Bcl-2 expression in HL60 cells had little effect on their proliferation or differentiation, but significantly accelerated their exit into G0 in response to agents inducing differentiation (Vairo et al., 1996). Therefore, under certain
conditions Bcl-2 induces quiescence rather than the cycling state. To better understand Bcl-2 function, Huang et al. carried out extensive mutational studies of the conserved N-terminal BH4 domain. Mutation of a tyrosine residue (Y28) within the BH4 domain had no effect on the ability of Bcl-2 or its closest homologues to enhance cell survival and did not prevent heterodimerization with pro-apoptotic family members (Bax, Bak, Bad and Bik). Nevertheless, upon stimulation with a cytokine or serum, starved quiescent cells expressing the mutant protein re-entered the cell cycle much faster than those expressing comparable levels of wild-type protein (Huang et al., 1997). Taken together, these results provide evidence that the anti-apoptotic function of Bcl-2 is mechanistically distinct from its inhibitory influence on the cell cycle.

Although not related to its function in lymphoma, a recent study found that Bcl-2 overexpression in a human breast cancer cell line resulted in increased metastasis. Bcl-2 overexpressing clones were found to be more invasive and migratory in response to chemotactic stimuli, with increases in secretion of 72 and 92 kDa gelatinases (which degrade the extracellular matrix) than the control transfectant clone (Del Bufalo et al., 1997).

1.3.e. Role of Bcl-2 in apoptosis

The expanding Bcl-2 family is able to register various forms of intracellular damage and determine whether the provided stimulus is positive or negative. These competing signals are combined to ascertain
whether the cell is to live or die. Anti- and pro-apoptotic family members (e.g. Bcl-2 and Bax, respectively) can heterodimerize and apparently titrate one another’s function giving rise to the notion that their relative concentrations may act as a “rheostat” for the apoptotic program (Oltvai, 1993). The mechanism by which Bcl-2 prevents apoptosis is not fully elucidated at this time, however, it has become quite clear that it functions on more than one level. The location of Bcl-2 on the cytoplasmic face of the outer mitochondrial membrane, ER, and nuclear envelope suggest a variety of functions for Bcl-2 (Monaghan et al., 1992; Krajewski et al., 1993; Akao et al., 1994).

In 1994, Newmeyer et al. developed a valuable cell-free system to study the mechanisms of apoptosis. Their results demonstrated an essential role for a dense organelle fraction highly enriched in mitochondria for apoptosis to occur in this cell-free system (Newmeyer et al., 1994). Two important aspects of mitochondrial participation in apoptosis, mitochondrial permeability transition (PT) pore opening and release of apoptogenic proteins from the mitochondria into the cytosol, have been found to involve Bcl-2 (Liu et al., 1996; Marchetti et al., 1996; Susin et al., 1996; Kluck et al., 1997; Yang et al., 1997; Marzo et al, 1998).

During apoptosis, in many different cell types induced by a variety of stimuli, the electrochemical gradient (Δψ) across the inner mitochondrial membrane becomes dissipated. Loss of Δψ has been ascribed to the opening of a large conductance inner membrane channel known as the mitochondrial
PT pore. Opening of this non-selective channel allows for an equilibration of ions within the matrix and intermembrane space of the mitochondria, thereby dissipating the electrochemical gradient and uncoupling the respiratory chain. The result of the PT pore opening are; electrons are shunted into free-radical production, mitochondrial ATP production ceases and mitochondrial volume dysregulation eventually leading to outer membrane rupture and the release of proteins from the intermembrane space into the cytosol (reviewed in Reed et al., 1998). PT has been established as a central event of the apoptotic cascade, due to the fact that when the PT is specifically inhibited (= Δψ stabilization) all major cytoplasmic and nuclear features of apoptosis fail to occur (Marchetti et al., 1996). It has been shown that Bcl-2 directly acts on the permeability transition pore complex (PTPC). Bcl-2 incorporated into membranes containing PTPC stabilizes PTPC liposomes and prevents PT pore opening, rather than increasing membrane permeability which would be a reasonable hypothesis considering Bcl-2 forms pores in lipid membranes (Schendel et al., 1997; Marzo et al., 1998). At this time it is not known whether the PT-inhibitory effect of Bcl-2 is due to interactions with PTPC constituents or rather due to the specific neutralization of Bax, a component of the PTPC (Marzo et al., 1998). However, in vitro experiments demonstrated that the channel forming ability of Bcl-2 is pH and membrane-acidity dependent. It is proposed that the opening and closing of Bcl-2 channels in vivo is modulated by pH and may be intended for transport of ion(s) or protein(s) resulting in protection of the
cell from apoptosis. The same group also suggested that Bax may transport the very same molecules but in the opposite direction (Schendel et al., 1997).

Two proteins have been found to trigger apoptosis when released from the mitochondria into the cytosol, cytochrome c and apoptosis inducing factor (AIF). Both cytochrome c and AIF are able to activate a group of intracellular cysteine proteases called caspases (Liu et al., 1996; Susin et al., 1996). Caspases are a highly conserved family of cysteine proteases with specificity for aspartic acid residues in their substrates and are the key players in the final "execution" stage of apoptosis (reviewed in Green and Reed, 1998). Bcl-2 has been shown to act directly on the mitochondria, at or near the mechanism responsible for cytochrome c release and has been shown to block it, thereby preventing apoptosis (Kluck et al., 1997; Yang et al., 1997). The precise mechanism by which Bcl-2 prevents cytochrome c release is not yet known. It is possible that Bcl-2 may block cytochrome c efflux directly via its ability to form channels in lipid membranes (Schendel et al., 1997). Another reasonable hypothesis is that Bcl-2 interacts directly with Bax. Bax is capable of forming pores in lipid membranes and has been shown to localize to the mitochondria and induce the release of cytochrome c. While Bcl-2 has been found to inhibit Bax channel-forming activity, it fails to prevent Bax-induced cytochrome c release (Antonsson et al., 1997; Rossé et al., 1998). It has also been proposed that Bcl-2 might interact with cytochrome c via the mammalian CED4 homologue Apaf-1 (apoptosis protease activating factor-1). Upon binding cytochrome c, Apaf-1 binds pro-caspase-9 which results in its
proteolytic activation (Li et al., 1997). The activation of caspase-9 in turn activates caspase-3, the downstream death protease (Zou et al., 1997). It has been found that the Bcl-2 homologue Bcl-xL physically associates with Apaf-1 and caspase-9 and inhibits Apaf-1-mediated activation of caspase-9 (Hu et al., 1998; Pan et al., 1998).

With regards to AIF, Susin et al. demonstrated that Bcl-2 does not affect the formation of AIF nor does it interfere with the action of AIF. However, Bcl-2 hyperexpressed in the outer mitochondrial membrane prevents the release of AIF from isolated mitochondria in vitro. Therefore, Bcl-2 maintains the subcellular compartmentalization of AIF (by mechanisms that are unknown), thereby preventing AIF's interaction with caspases. Without the appropriate interactions, caspases remain inactive and the apoptotic pathway does not proceed (Susin et al., 1996).

Although mitochondrial location and function is usually emphasized, significant portions of the Bcl-2 protein are also integrated into the membranes of the ER and nuclear envelope (Monaghan et al., 1992; Krajewski et al., 1993; Akao et al., 1994). In this regard, Bcl-2 has been reported to regulate Ca^{2+} homeostasis in the ER by preventing or delaying efflux of Ca^{2+} from this organelle resulting from some, but not all, inducers of apoptosis (Lam et al., 1994; Zörnig et al., 1995; He et al., 1997). Recently, Kuo et al. reported that overexpression of Bcl-2 in breast epithelial cells modulates the ER calcium store by upregulating calcium pump expression without affecting the release channel which results in accelerated Ca^{2+} uptake and
FIGURE 1.2. General diagram outlining Bcl-2's involvement in apoptosis, functioning at the level of the mitochondria. Apoptotic stimuli results in the release of cytochrome c and apoptosis inducing factor (AIF) from the mitochondria. Cytochrome c forms a complex with Apaf-1 and pro-caspase-9 which results in proteolytic activation of pro-caspase-9 (caspase-9). Caspase-9 activates pro-caspase-3, -6, and -7, which in turn cleave downstream substrates resulting in apoptosis. Alternatively, AIF directly activates pro-caspase-3. Caspase-3 then cleaves downstream substrates leading to apoptosis. Bcl-2 and Bcl-xl inhibits the release of cytochrome c and AIF from the mitochondria by mechanisms that are not fully understood to date.
enhanced loading (Kuo et al., 1998). Lastly, Bcl-2 has been localized to the nuclear envelope and may associate with nuclear pore complexes (Monaghan et al., 1992; Krajewski et al., 1994). Consistent with this hypothesis, it was found that, in some cell types, overexpression of Bcl-2 prevents translocation of p53 (an apoptosis-inducing protein) from the cytosol into the nucleus. This suggests that Bcl-2 might seize selected proteins as they attempt to pass through nuclear pore complexes (Ryan et al., 1994). Another suggested function of Bcl-2 at the nuclear level is sequestration of glutathione into the nucleus resulting in alteration of nuclear redox and inhibition of caspase activity (Voehringer et al., 1998).

1.3.f. Alterations of Bcl-2 in lymphoma; t(14;18)

In up to 90% of FL and 30% of DLCL cases, a specific translocation, t(14;18), occurs resulting in productive rearrangements of the Bcl-2 gene and overexpression of the Bcl-2 protein product (Yunis et al., 1987; Aisenberg et al., 1988; Ngan et al., 1988). The t(14;18) translocation involves the Ig heavy chain locus on chromosome 14q32 and the Bcl-2 gene on chromosome 18q21. During the translocation event, the tip of chromosome 18 including the coding region of Bcl-2 is joined to one of the IgH joining segments (JH) on chromosome 14. The tip of chromosome 14 including the IgH variable (VH) and diversity (DH) gene segments is coupled to the shortened reciprocal chromosome 18, downstream of the Bcl-2 gene. The breakpoints on chromosome 18 are clustered within two regions. The first is a 150 bp region
in the 3' untranslated region of Bcl-2 exon 3 and is termed the major breakpoint region (mbr). The second, called the minor cluster region (mcr), is a 500 bp region 20 kilobases downstream of Bcl-2, 3' of the mbr (Bakhshi et al., 1985; Cleary et al., 1985; Cleary et al., 1986). In FLs that are positive for t(14;18), approximately 60-80% of the breakpoints on chromosome 18 occur within the mbr and about 10-20% occur in the mcr (Cleary et al., 1986; Meijerink, 1997; Tsujimoto et al., 1987). A third breakpoint region was discovered in 1987 in one case of follicular lymphoma that was localized 1.5 kb 5' of the Bcl-2 gene and within the first exon (Tsujimoto et al., 1987). This breakpoint region, termed the variant cluster region (vcr), is rarely observed in FL but is more common in B-cell chronic lymphocytic leukemia (Merup et al., 1996). One might presume that some of these breakpoints may disrupt the function of Bcl-2, however, this is not the case, and the functional and regulatory domains are preserved (Dirks et al., 1996).

The mechanism by which the t(14;18) translocation occurs has been a matter of debate for years. The widely held belief that t(14;18) results from mistakes that occur during V(D)J recombination has much evidence to support it. For instance, occasional t(14;18)s have been described that are the result of faulty IgH rearrangements during either the D<sub>H</sub> → J<sub>H</sub> or the V<sub>H</sub> → D<sub>H</sub>J<sub>H</sub> rearrangement processes (Cotter et al., 1990; Meijerink et al., 1995). The presence of two opposite sets of Ig gene recombination signal-like (or cryptic signal) sequences in the mbr of Bcl-2 (resembling a pseudo D<sub>H</sub> gene) were reported and may participate in IgH recombination (Tsujimoto et al.,
Nevertheless, approximately half of the mbr translocations cannot be explained by the presence of these signal-like sequences. Often there is no recombination signal-like sequence or it is too far away to be useable. Another explanation was proposed by Wyatt et al., who suggested that prokaryotic recombination signal \( \chi \)-related sequences may be involved. \( \chi \)-like sequences are present in the mbr, \( V_H, D_H, \) and \( J_H \) gene segments and could possibly be targets for illegitimate recombination by the V(D)J recombinase complex (Wyatt et al., 1992). In the cases where recombination signal-like sequences or \( \chi \)-like sequences cannot be found or cannot provide a probable explanation, the "end donation" pathway has been incorporated. In this pathway, it has been suggested that a V(D)J-induced break in an antigen receptor locus might be accompanied by a random double-strand break on the partner chromosome, followed by interchromosomal joining (Bakhshi et al., 1987; Tycko and Sklar, 1990; Lewis, 1994). Like all other suggested mechanisms for t(14;18), this explanation has never been proven conclusively with the major criticism being that the source of the sporadic random breakage has never been apparent. Recent data suggests that translocations involving only one recombination signal sequence (RSS) may arise by RAG-mediated transposition. An RSS from an Ig locus could be linked to a random site on another chromosome by a one-ended transposition. In the resulting three-branched structure, the branch with the exposed 3'-hydroxyl group is chemically analogous to a nicked RSS in normal RAG-mediated cleavage and could be further processed to generate a hairpin end and one
interchromosomal junction containing the RSS. Due to the fact that this break presumably occurs within a complex that also contains the hairpin coding end from the original cleavage at the Ig locus, joining of the two hairpin ends would then generate the reciprocal chromosomal translocation. Single cleavages, such as the one proposed here, are about 20-fold less frequent than coupled cleavages, but they cannot be resolved by normal V(D)J joining because of the absence of a cleavage partner. Therefore, the ends may be unusually available for transposition. This mechanism is distinct from end donation because the breaks on both partner chromosomes are generated by RAG proteins (Agrawal et al., 1998; Hiom et al., 1998; Roth and Craig, 1998).

The consequence of the t(14;18) is that the Bcl-2 gene is neighboring the IgH enhancer. This results in continuous high expression of the Bcl-2 gene (Seto et al., 1988; Graninger et al., 1987; Tsujimoto et al., 1985). Interestingly, the number of FL cases positive for t(14;18) is considerably lower than the number of cases that express the Bcl-2 protein. Only about half of the cases expressing abundant Bcl-2 protein have detectable Bcl-2 rearrangements. It is possible that more cases do have rearrangements but they are not detected due to alternative breakpoints (Pezzella et al., 1990; Monni et al., 1997). While this explanation is likely valid for some undetected translocation it is not, however, an explanation for all of them. It is more likely that additional mechanisms exist which result in a deregulated Bcl-2 gene expression. Another mechanism suggested to be responsible for Bcl-2 overexpression is chromosomal amplification. Monni et al. reports that high-level
amplification of 18q, resulting in Bcl-2 amplification, was found more frequently than t(14;18) in DLCL and that these two mechanisms for Bcl-2 overexpression may be mutually exclusive due to the fact that they were never present in the same lymphoma sample. While this study reported findings on DLCLs only it is reasonable to assume that Bcl-2 amplification also occurs in FL (Monni et al., 1997).

1.4. Oncogenic cooperation in lymphoma

While deregulated expression of Bcl-2 (due to chromosomal translocation, mutation, or amplification) is an early and critical event in lymphomagenesis, it is not, by itself, sufficient to cause cellular transformation. Reed et al. found that transfection of Bcl-2 constructs into NIH 3T3 cells did not result in transformation of 3T3 cells in vitro but did lead to enhanced tumourigenicity (with a latency period of 4 weeks). However, cells recovered from Bcl-2 mediated tumours exhibited a transformed morphology when re-established in culture (Reed et al., 1988). Other groups have also shown that overexpression of Bcl-2 in lymphoblastoid cell lines results in growth advantages, as demonstrated by limiting dilution and soft agar assays, but does not render the cells tumourigenic (Nuñez et al., 1989; Tsujimoto, 1989). This indicates that additional secondary events probably cooperated with Bcl-2 overexpression resulting in tumour formation. PCR assays designed to detect t(14;18) have found that; 1) rare somatic translocations at the t(14;18) locus occur in people without
lymphoma or lymphoid hyperplasia, 2) the frequency of such translocations is highly variable among individuals, 3) the frequency of such translocations increase substantially with age and 4) the increase in t(14;18) frequency appears to be a result of expansion of persisting clones (Liu et al., 1994). Although this group found that the frequency of t(14;18) increases with age, as does the incidence of NHL, the incidence of Bcl-2 mutations in spleens (35%) and peripheral blood lymphocytes (55%) is extremely high compared with the lifetime risk for NHL (0.6%). These findings, in addition to the fact that normal individuals harbour t(14;18) positive lymphocytes, persistent polyclonal B-cell lymphocytosis is a t(14;18)-positive benign disorder and that there is an extremely long latency period before Bcl-2 transgenic mice develop lymphoma strongly implies that Bcl-2 overexpression alone is not sufficient to cause malignancy (McDonnell and Korsmeyer, 1991; Liu et al., 1994; Limpens et al., 1995; Delage et al., 1997).

A transgenic murine model of the human t(14;18) translocation demonstrated that overexpression of Bcl-2 initially resulted in the accumulation of small, resting, IgM/IgD-positive B lymphocytes (McDonnell et al., 1989; McDonnell et al., 1990). Not surprisingly, it was determined that the lymphocytes accumulated as a result of an abnormal extension in cellular viability, and not due to increased proliferation (Hockenbery et al., 1990). It is proposed that abnormal extension of viability, due to overexpression of Bcl-2, renders the affected lymphocytes susceptible to the acquisition of secondary genetic lesions. Acquisition of these lesions can cumulatively result in
malignant transformation. Following a latency period of approximately 15 months, the Bcl-2 transgenic mice develop malignant lymphomas. Southern blotting demonstrated that approximately 50% of these lymphomas possess clonal rearrangements of the endogenous c-myc oncogene (McDonnell and Korsmeyer, 1991). Infection of pre-B cells expressing a Eμ-myc transgene with a Bcl-2 retrovirus permits the eventual outgrowth of immortalized pre-B lines (Vaux et al., 1988). Mice that express both myc and Bcl-2 transgenes show hyperproliferation of pre-B and B cells and rapidly develop tumours of primitive lymphoid progenitor cells (Strasser et al., 1990). In addition to these murine examples, cooperation between Bcl-2 and myc has also been seen in human lymphoma. Lee et al. reported a case of untreated NHL with histologic progression over 1 year from a low-grade, small cleaved follicular center cell lymphoma to a high-grade, small noncleaved follicular center cell lymphoma. Both lymphomas possessed identical t(14;18) translocations (indicating their clonality), however, the initial lymphoma had c-myc in germline configuration while the high-grade lymphoma had one c-myc allele rearranged. The presence of the c-myc rearrangement in the high-grade tumour suggests a role for c-myc in the clonal evolution of the low-grade tumour into a more aggressive lymphoma (Lee et al., 1989). These very significant observations suggests that c-myc is likely to be an important secondary event in lymphomagenesis.

Much like deregulated Bcl-2 expression, constitutive expression of c-myc in lymphoblastoid cell lines results in phenotypic changes and enhanced
growth characteristics but does not result in enhanced tumourigenicity (Hotchin et al., 1990). This is likely due to the seemingly contradictory functions of c-myc. c-myc expression directly correlates with cellular proliferation. Cells that are terminally differentiated or resting do not possess detectable amount of c-myc mRNA or protein. Upon stimulation of resting (G₀) cells, c-myc expression rapidly increases and then declines to a plateau that is maintained throughout the cell cycle in proliferating cells. Withdrawal of cells from the cell cycle by growth factor removal leads to downregulation of c-myc expression (Waters et al., 1991). Paradoxically, deregulated c-myc expression also induces apoptosis in cells deprived of certain growth factors in a highly accelerated manner (Askew et al., 1991; Evan et al., 1992). However, when both c-myc and Bcl-2 are overexpressed in the same cell it appears that Bcl-2 substitutes for a survival signal (usually provided by growth factors), thereby abrogating c-myc-induced apoptosis without affecting the c-myc mitogenic function (Bissonnette et al., 1992; Fanidi et al., 1992; Alarcon et al., 1996). Although functional cooperation between Bcl-2 and c-myc has been well documented very little is known about other secondary events capable of cooperating with Bcl-2 in lymphomagenesis.
1.5. **Lymphoblastoid cell lines: molecular and phenotypic properties**

As a model for B cell lymphoma I attempted to recreate B cell neoplasia by introducing oncogenes believed to be involved in progression of this disease into B lymphocytes. However, it was necessary to infect primary B cells with Epstein-Barr virus (EBV) to create this model due to the fact that primary cells rarely survive in culture. Infection of human B cells with transforming EBV *in vitro* results in polyclonal activation and immortalization of the cells but not malignant transformation (reviewed by Nilsson and Klein, 1982). EBV infection *in vivo* leads to spontaneous outgrowth of B cell clones harbouring the virus. *In vitro* transformed lymphoblastoid cells (LCLs) parallel normally activated B lymphoblasts but express latent viral proteins. These expressed proteins are responsible for the immortalization of the cells and for the maintenance of the virus in a latent episomal form. At least nine EBV proteins are usually expressed by LCLs including six EBV nuclear antigens [EBNA 1, 2, 3A, 3B, 3C, and LP (leader protein)] and three latent membrane proteins LMP 1, 2A, and 2B (Allday *et al.*, 1989; Gregory *et al.*, 1991). Similar patterns of EBV protein expression are seen in EBV-associated B cell lymphomas in immuno-compromised patients, however, more restricted expression patterns are associated with other EBV-associated cases of lymphoma (Hodgkin’s disease and Burkitt’s lymphoma).

Induction of cellular RNA, protein and DNA synthesis occurs due to the expression of viral proteins which presumably lead to the
immortalization of the infected cells. LMP 1 and EBNA 2 were found to induce the expression of Bcl-2, perhaps leading to the suppression of apoptosis (Gregory et al., 1991; Henderson et al., 1991). EBNA 5 was reported to bind to p53 and Rb (retinoblastoma) proteins in vitro which may interfere with their functions (Szekely et al., 1993). Although the presence of EBV viral proteins appear to associate and alter the functions of certain host proteins, which are important to normal cellular functioning, they do not seem to block the p53 activated DNA damage response pathway. EBV immortalized LCLs show functional p53 and remain sensitive to DNA-damaging chemotherapeutic drugs such as cisplatin, actinomycin D and adriamycin (Allday et al., 1995).

Phenotypic properties of LCLs are similar between various cell lines, but it has been found that secondary changes of the phenotype may occur upon prolonged passaging (over six months) (reviewed by Nilsson and Klein, 1982). Individual LCL cells are motile and irregular in shape. They adhere to each other in suspension culture forming clusters. The doubling time of LCLs is usually around 30 hrs and they do not survive in low cell culture densities (less than $10^4$ cells/ml). LCLs do not constitute transformed cell lines as they do not form tumours when injected subcutaneously into nude mice, nor do they form colonies in soft agar in vitro (Nilsson et al., 1977).
1.6. Historical methods of oncogene detection

Cell properties are altered when normal cells undergo transformation events. Transformed cells exhibit behavioural and morphological characteristics very different from those of their normal counterparts. These differences form the basis of foci and colony formation assays designed to monitor in vitro cell transformation (DiPaolo et al., 1969; DiPaolo et al., 1972; Heidelberger et al., 1973). Other properties such as the ability to form anchorage-independent colonies in a semisolid medium and to proliferate in serum deficient medium have also been utilized as criteria in assessing the transformed status of a cell population (Holley and Kiernan, 1968; Smith and Scher, 1971).

In the past, the characteristic experiment for detection of transformation-inducing genes was DNA transfection of the NIH 3T3 fibroblast cell line. Following transfection of DNA from carcinomas, known viruses, cell lines or cloned genes, the morphology and behaviour of the 3T3 cells was observed (Blair et al., 1982; Blair et al., 1983; Müller and Müller, 1984; Wahrman et al., 1985). Typical characteristics of transformed 3T3 cells include formation of extensive foci in culture, exhibition of anchorage-independent growth in soft agar and disruption of cytoplasmic F-actin organization (Wahrman et al., 1985). However, since the identification of foci is dependent on cell culture conditions and cell morphology (personal observations which can be quite subjective) an alternative/additional method for screening and
detection of transformed cells was developed. Athymic nude (nu/nu) mice provide an excellent vehicle for measurement of the tumourigenic potential of cells in question. When 3T3 cells were transfected with varying amounts (0.4 ng and 400 ng) of proviral Moloney murine sarcoma virus (MSV) DNA it was found that tumours developed with a latency period of 6 to 8 weeks. When 0.4 ng of DNA was used 50% of the mice formed tumours whereas 80% developed tumours with 400 ng. A portion of each transfected cell population was also assayed in the conventional focus forming assay. Only one focus was observed on cells treated with 0.4 ng of MSV DNA while cells treated with 400 ng yielded approximately 400 foci. Tumourigenicity results were quite similar when the human fibrosarcoma cell line, HT1080, was tested in a similar manner, however, no foci were observed in the in vitro focus forming assay. This implies that the nude mouse assay detects or selects for some transformation events which are not detectable in the traditional focus assays (Blair et al., 1982).

A major drawback of using NIH 3T3 cells to detect human oncogenes is that it is a murine cell line. It has been found that the majority of DNA samples from human tumours do not have focus forming activity on 3T3 cells, possibly due to the fact that many of the genetic elements responsible for tumour formation in humans cannot cooperate with pre-existing oncogenic alterations in murine cells or affect their morphology. To circumvent the problem of relying on subjective morphological changes to determine
whether cells were transformed or not, Blair et al. developed the NIH 3T3 tumourigenicity assay (Blair et al., 1982). Fasano et al. then demonstrated that this approach can lead to the detection of new human tumourigenic genetic loci (Fasano et al., 1984). However, the NIH 3T3 transforming gene assay system suffers from a relatively high spontaneous transformation rate of the cell line. Consequently, tumours arising 12 or more weeks after injection of cells are likely to be due to spontaneous transformation of the 3T3 cells rather than the acquisition of specific human DNA sequences. Therefore, this assay possibly impedes detection of genes that cause slow tumour growth, limiting it instead to genes that induce rapid tumour formation. To avert the aforementioned problems with the use of NIH 3T3 cells for the detection of human oncogenes a human recipient cell line, HOS, was developed for oncogene transfection studies. HOS is a human osteosarcoma cell line that has a very low basal level of spontaneous tumour formation in nude mice and is transformed by genes that are able and unable to transform NIH 3T3 cells as determined by observed anchorage-independent growth and tumour formation (Tainsky et al., 1987).

Recently, molecular cytogenetic tools have been employed to characterize alterations in cancer cells. Chromosomal banding has been widely used to recognize chromosomal abnormalities, however, its resolution has limits and many abnormalities cannot be resolved. A particular chromosome band is identified by its position on a normal chromosome. However, cryptic changes involving small chromosome
segments that appear abnormal do not always have a characteristic band to be accurately identified. Fluorescence in situ hybridization (FISH) is one of the most effective tools for detecting genetic alterations in cancer cells. The most recent addition to the FISH repertoire is comparative genomic hybridization (CGH). CGH detects changes in DNA copy throughout the tumour genome. Based upon hybridization of normal and tumour DNA on normal chromosome spreads, CGH is powerful for detection of DNA deletions and amplifications. Balanced and unbalanced translocations and numerical deviations can easily be identified by painting with whole chromosome probes. CGH, in combination with spectral karyotyping and chromosome banding, allows for the identification of structural and numerical alterations and mapping of the DNA copy number on the entire tumour genome. It also demonstrates the origin of complex rearrangements, and the localization of breakpoints and deletions. Regions of recurrent alterations can be microdissected, isolated, and localized on extended chromosomes or chromatin fibers. This will allow for construction of high resolution maps that are critical for positional cloning and gene isolation (reviewed in Popescu and Zimonjic, 1997).

Although the NIH 3T3 system, and its equivalent human cell line counterparts, and modern molecular cytogenetic tools are capable of detecting novel oncogenes they are not capable of detecting oncogenic cooperation. It is well known that cancer is a multistep process, resulting from the acquisition of at least 2 (but likely many more) genetic alterations that influence cellular
function. Therefore, a system that is capable of detecting oncogenic cooperation between a known oncogene (that is important in a specific cancer) and oncogene "x" would provide a better understanding of the molecular mechanisms responsible for the progression of that neoplasm, possibly leading to new treatments based upon those mechanisms.
CHAPTER 2: MATERIALS AND METHODS
2.1. Cell lines and culture conditions

Human lymphoblastoid cell line, GM607, was obtained from Dr. L. Rubin (University of Toronto, Toronto, ON). It is a non-transformed B cell line which has been immortalized with Epstein-Barr virus (Allday et al., 1995). A Bcl-2 protein overexpressing cell line (GM607/C5-5) was generated by the transfection of GM607 with a Bcl-2/pRcCMV construct (Kuzniar, 1997). GM607 transfected with a pRcCMV vector (GM607/pRcCMV) behaves in the same way as GM607 and is used as a control cell line in these assays. A cell line expressing an ecotropic receptor (C5-5/MCAT) was generated by transfection of GM607/C5-5 with MCAT-1 cDNA expressed in a pCEP4 vector (vector control, C5-5/pCEP4). A cell line overexpressing both Bcl-2 and c-myc (C5-5/MCAT/mycGFP) was generated by transducing subcloned C5-5/MCAT cells with the retroviral producer cell line GP+E-86/mycGFP (control line, C5-5/MCAT/GFP, generated using GP+E-86/GFP).

GP+E-86, an ecotropic packaging cell line, was kindly provided by Dr. L. Penn (OCI, Toronto, ON). Retrovirus secreting cell lines, GP+E-86/mycGFP and GP+E-86/GFP, were generated by transfecting GP+E-86 with retroviral vectors, pBabeMNIreshuc-mycGFP or pBabeMNIresGFP respectively.

A human diffuse large cell lymphoma cell line, OCI LY8 C3, used in this study was obtained from Dr. H. Messner (Princess Margaret Hospital, Toronto, ON). OCI LY8 C3 is a clone of the parent cell line OCI LY8 and possess a t(14;18) translocation resulting in the overexpression of Bcl-2, a
t(8;14) translocation resulting in the overexpression of c-myc and a mutated p53 gene resulting in loss of function (Farrugia et al., 1994; Chang et al., 1995).

All non-adherent cells were cultured in RPMI 1640 (Wisent, St. Bruno, Que.) supplemented with 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 15% bovine calf serum (BCS, Wisent) in the presence of 5% CO₂ at 37°C. GM607/Bcl-2 and the corresponding control, GM607/pRcCMV, were grown in the above media with the addition of 750 µg/ml of G418 (geneticin sulfate, GIBCO BRL, Gaithersburg, MD) while the MCAT-1 expressing cell line, (C5-5/MCAT), its equivalent control, C5-5/pCEP4, and C5-5/MCAT/mycGFP and its equivalent control, C5-5/MCAT/GFP, were cultured in the presence of both 750 µg/ml G418 and 150 µg/ml hygromycin (GIBCO BRL). Adherent cell lines GP+E-86, GP+E-86/myc-GFP, and GP+E-86/GFP were cultured in DMEM (GIBCO BRL) supplemented with 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% bovine calf serum (Wisent). Trypsinization of adherent cell lines was performed with the addition of 0.05% trypsin, 0.53 mM EDTA (GIBCO BRL).

2.2. Plasmids and constructs

The pRcCMV (Invitrogen Corp., San Diego, CA) carrying a G418 resistance gene, was used for the overexpression of Bcl-2 cDNA to create an apoptosis resistant B-cell line (GM607/C5-5). pCEP4 (Invitrogen Corp.) is a Cytomegalovirus (CMV) promoter based and hygromycin resistance
conferring shuttle vector. The EBV origin of replication and the expression of EBNA-1 allow high copy episomal replication of the vector. It was used to express MCAT-1 cDNA (MCAT-1/pCEP4). All ligations were performed using T4 DNA ligase (GIBCO BRL). The ligated products were transfected by heat shock into DH5α Escherichia coli (GIBCO BRL) and plated on Luria Broth (LB) agar plates (with 100 μg/ml ampicillin, Sigma-Aldrich Canada Ltd., Oakville, ON). Large scale plasmid preparations were produced using standard plasmid preparation techniques and Qiagen columns following the company supplied protocol (Qiagen Inc., Santa Clarita, CA).

MCAT-1 cDNA was originally expressed under the SV40 promoter in the pJET vector donated by Dr. J. Cunninham (Howard Hughes Medical Institute, Boston, MA).

pBabeMNIresGFP, obtained from Dr. L. Penn (Ontario Cancer Institute, Toronto, ON), is a Moloney murine leukemia virus LTR promoter-driven retroviral vector carrying a Green Fluorescent Protein (GFP) cassette. pBabeMNIres-huc-mycGFP (Dr. L. Penn), used for the overexpression of c-myc, is identical to pBabeMNIresGFP except that it possesses human c-myc cDNA (~1.6 kb) ligated into the EcoRI and XhoI sites in the vector (Sara Oster, Ontario Cancer Institute, Toronto, ON).

2.3. Transfection of cell lines

Gene transfer into the GM607, GM607/C5-5 and GP+E-86 cell lines were performed by electroporation. Briefly, 3x10⁷ GM607 cells in 0.75 ml of PBS
buffer (5.4 mM KCl, 2.8 mM Na$_2$HPO$_4$, 2.9 mM KH$_2$PO$_4$ and 0.3 M NaCl, pH 7.3) were mixed with 50-100 µg of pRcCMV/Bcl-2 plasmid DNA contained in 100 µl of PBS, pulsed at 700 V and 25 µF and incubated at 5% CO$_2$ and 37°C for 48 hrs in non-selective media. The cells were then plated out at $10^4$, $10^3$, and $10^2$ cells per well in 96 well plates in media with the addition of G418 at 750 µg/ml and grown for 2-3 weeks. Colonies were expanded off plates on which less than 30% of the wells contained growing cells and screened by western blot (Kuzniar, 1997). The C5-5/MCAT cell line was generated in a similar manner, except that MCAT-1 cDNA transfected into GM607/C5-5 cells was expressed via a pCEP4 vector and cells were therefore selected using hygromycin (150 µg/ml). Colonies were expanded off plates on which less than 30% of the wells contained growing cells and were screened by retroviral infection with GP+E-86/pBabeMNIresGFP supernatant.

The GP+E-86 packaging cell line was electroporated with either a pBabeMNIresGFP vector or a pBabeMNIreshuc-mycGFP vector. $3 \times 10^7$ GP+E-86 cells in 0.75 ml of PBS buffer were mixed with 35-65 µg of plasmid DNA contained in 100 µl PBS, pulsed at 420 V and 960 µF and incubated in the presence of 5% CO$_2$ at 37°C for 48 hrs in 20 ml culture media after which new culture media was provided. GFP expression was then analyzed by FACS and GFP positive cells were sorted, collected and expanded. The GFP positive bulk populations were then cloned by limiting dilution in non-selective media (as described previously).
2.4. Infection of cells with murine retrovirus

C5-5/MCAT cells were transduced with either GP+E-86/GFP or GP+E-86/mycGFP supernatant. Briefly, 4x10^5 cells from either packaging cell line were plated in 60mm plates (Falcon) in 5 ml of culture media and incubated at 37°C in the presence of 5% CO₂. When cells reached ~80% confluency the culture media was replaced with 4 ml of RPMI/15%BCS/1% penicillin/streptomycin culture media. Following incubation for 72 hr (5% CO₂ and 37°C) the supernatant was removed and filtered using a 0.45 µm filter (Nalge Nunc International, Denmark). 1x10^6 C5-5/MCAT cells were pelleted by centrifugation at 1,000 rpm and 4°C for 7 min. Cell pellets were resuspended in 1 ml of filtered GP+E-86/GFP or GP+E-86/mycGFP supernatant, aliquoted to 60mm plates to which Polybrene (13 µg/ml) (Sigma-Aldrich) was added. Plates were incubated at 37°C in the presence of 5% CO₂ for 2 hrs with occasional rocking. Four ml of culture media was then added and plates were incubated (37°C and 5% CO₂) for 48 hrs. Cells were then expanded and GFP expression was monitored at various time points by FACS. These bulk populations, C5-5/MCAT/GFP and C5-5/MCAT/mycGFP, were then cloned by limiting dilution (as described previously).
2.5. Cloning efficiency assay

GM607/C5-5 and OCI LY8 C3 cells were mixed together at ratios of 250:1, 2,500:1, and 25,000:1 (GM607/C5-5:OCI LY8 C3) in culture media containing either 15%, 5%, 2.5%, 1% or 0.1% BCS and plated at either 10,000, 5,000 or 1000 cells, in 100 µl of media, per well in 96 well plates. Plates were incubated in the presence of 5% CO₂ and 37°C for 1 week, at which time an additional 100 µl of media was added to each well. Colonies were expanded after an incubation period of 2-3 weeks. Cells were stained with purified mouse anti-human Ig light chain, kappa (κ) monoclonal antibody and purified mouse anti-human Ig light chain, lambda (λ) monoclonal antibody (PharMingen, San Diago, CA) and then analyzed by flow cytometry.

C5-5/MCAT and C5-5/MCAT/GFP cells were mixed together at ratios of 250:1, 2,500:1, and 25,000:1 (C5-5/MCAT:C5-5/MCAT/GFP) in culture media containing 1% BCS and plated at 1000 cells per well in 96 well plates. Plates were incubated at 37°C in the presence of 5% CO₂ for 3 weeks after which colonies were expanded. Cells were stained with mouse anti-human c-myc monoclonal antibody (Chemicon, Temecule, CA) (isotype control; mouse IgG₁κ, clone MOPC-21, PharMingen) and then analyzed for both c-myc and GFP expression by flow cytometry. This protocol was repeated for: 1) C5-5/MCAT and C5-5/MCAT/mycGFPc3 (C5-5/MCAT:C5-5/MCAT/mycGFPc3, 2) C5-5/MCAT and C5-5/MCAT/mycGFPc4 (C5-5/MCAT:C5-5/MCAT/mycGFPc4), 3) C5-5/MCAT/GFP and C5-5/MCAT/mycGFPc3 (C5-
5/MCAT/GFP:C5-5/MCAT/mycGFPc3), 4) C5-5/MCAT/GFP and C5-5/MCAT/mycGFPc4 (C5-5/MCAT/GFP:C5-5/MCAT/mycGFPc4) at dilutions of 250:1, 2,500:1 and 25,000:1 (Table I).

2.6. Soft agar assay

GM607/C5-5 and OCI LYS C3 cells were mixed together at ratios of 250:1, 2,500:1, and 25,000:1 (GM607/C5-5:OCI LYS C3) in 1X MEM (GIBCO BRL), 15% BCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. 1x10^6 cells in 10 ml of media were added to 100-mm plastic Petri dishes (Falcon) that had been base-coated (2-3 mm) with 0.75% Noble agar (GIBCO BRL) in complete MEM. Plates were incubated at 37°C in the presence of 5% CO₂ for 30 days, with media being changed every 7 days (Yuhas et al., 1977). Colonies (>40 cells) were removed from agar using a Pasteur pipette and expanded. Cells were stained with mouse anti-human Ig κ and mouse anti-human Ig λ antibodies and analyzed by flow cytometry.

Table I: Summary of cloning efficiency assay protocol

<table>
<thead>
<tr>
<th>Cell Line A:B</th>
<th>Ratio A:B</th>
<th># of A &amp; B cells per plate</th>
<th>Total # of cells per well</th>
<th>% BCS in culture media</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM607/Bcl-2:OCI LY8 C3</td>
<td>250:1</td>
<td>95,616 &amp; 384</td>
<td>10,000, 5,000 and 1,000</td>
<td>15, 5, 2.5, 1 and 0.1</td>
</tr>
<tr>
<td></td>
<td>2,500:1</td>
<td>95,961.6 &amp; 38.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>25,000:1</td>
<td>95,996.16 &amp; 3.84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bcl-2/MCAT-1:Bcl-2/MCAT/GFP</td>
<td>250:1</td>
<td>95,616 &amp; 384</td>
<td>1,000</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2,500:1</td>
<td>95,961.6 &amp; 38.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>25,000:1</td>
<td>95,996.16 &amp; 3.84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bcl-2/MCAT:Bcl-2/MCAT/mycGFPc2-3</td>
<td>250:1</td>
<td>95,616 &amp; 384</td>
<td>1,000</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2,500:1</td>
<td>95,961.6 &amp; 38.4</td>
<td></td>
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<tr>
<td></td>
<td>25,000:1</td>
<td>95,996.16 &amp; 3.84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bcl-2/MCAT:Bcl-2/MCAT/mycGFPc2-4</td>
<td>250:1</td>
<td>95,616 &amp; 384</td>
<td>1,000</td>
<td>1</td>
</tr>
<tr>
<td></td>
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<td>Bcl-2/MCAT/GFP:Bcl-2/MCAT/mycGFPc2-3</td>
<td>250:1</td>
<td>95,616 &amp; 384</td>
<td>1,000</td>
<td>1</td>
</tr>
<tr>
<td></td>
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<td></td>
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<td>250:1</td>
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<td>1,000</td>
<td>1</td>
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<td></td>
<td>25,000:1</td>
<td>95,996.16 &amp; 3.84</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
(isotype control, IgG1,κ, clone MOPC-21) and analyzed for both c-myc and GFP by flow cytometry (Table II).

2.7. Growth curve assay

2x10⁶ C5-5/MCAT, C5-5/MCAT/GFP, C5-5/MCAT/mycGFP 2-3 and C5-5/MCAT/mycGFP 2-4 cells were seeded in 2 ml of culture media containing either 15% BCS or 4% BCS in 24 well plates (Falcon). Cell counts were taken from each of two separate wells for each cell line in the two different culture conditions, by trypan blue (Sigma-Aldrich) exclusion, every day from days 1 - 11 and then on day 15, 16 and 17. Cellular DNA, from each cell line, was also stained with propidium iodide on days 2, 4, 6, 8, 10, 11 and 15 and cell cycle analysis was performed by FACS.

2.8. Cell cycle assay

On days 2, 4, 6, 8, 10, 11 and 15, 0.5 - 1 x 10⁶ cells from each cell line in the growth curve assay were spun down for 7 min at 1,000 rpm and 4°C. The supernatant was discarded and the cell pellets were washed with 1X PBS buffer and fixed in 1 ml of 80% ethanol for 1 hr on ice. The cells were then centrifuged at 3,000 rpm and 4°C and the supernatant was discarded. The pellets were then washed, first with 1X PBS and then with propidium iodide staining buffer (PIB: 1X PBS buffer with the addition of 0.12% Triton X-100 and 0.12 mM EDTA). The cell pellets were resuspended in 0.5 ml
Table II: Summary of soft agar assay protocol

<table>
<thead>
<tr>
<th>Cell Line A:B</th>
<th>Ratio A:B</th>
<th># of A &amp; B cells per plate</th>
<th>Total # of cells per plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>GM607/Bcl-2:OCI LY8 C3</td>
<td>250:1</td>
<td>996,000 &amp; 4,000</td>
<td>1x10^6</td>
</tr>
<tr>
<td></td>
<td>2,500:1</td>
<td>999,600 &amp; 400</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25,000:1</td>
<td>999,960 &amp; 40</td>
<td></td>
</tr>
<tr>
<td>Bcl-2/MCAT-1:Bcl-2/MCAT/GFP</td>
<td>250:1</td>
<td>996,000 &amp; 4,000</td>
<td>1x10^6</td>
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<tr>
<td></td>
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<td>999,600 &amp; 400</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25,000:1</td>
<td>999,960 &amp; 40</td>
<td></td>
</tr>
<tr>
<td>Bcl-2/MCAT:Bcl-2/MCAT/mycGFPc2-3</td>
<td>250:1</td>
<td>996,000 &amp; 4,000</td>
<td>1x10^6</td>
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<tr>
<td></td>
<td>2,500:1</td>
<td>999,600 &amp; 400</td>
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</tr>
<tr>
<td>Bcl-2/MCAT/GFP:Bcl-2/MCAT/mycGFPc2-4</td>
<td>250:1</td>
<td>996,000 &amp; 4,000</td>
<td>1x10^6</td>
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<tr>
<td></td>
<td>2,500:1</td>
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</tr>
<tr>
<td></td>
<td>25,000:1</td>
<td>999,960 &amp; 40</td>
<td></td>
</tr>
</tbody>
</table>
RNAseA/PIB (10 μg/ml, Boehringer Mannheim Canada, Mississauga, ON) and incubated at 37°C for 45 min. Propidium iodide (PI) solution (5 mg/ml propidium iodide dissolved in PIB) was added at 50 μg/ml and the reactions were incubated at RT in the dark for 1 hr. The analysis was performed immediately using the FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA).

2.9. Cell staining and FACS analysis

For staining of cell surface markers, 10⁶ cells were dispensed into a 4 ml Falcon tube (12x75 mm, polystyrene, round-bottom) (Beckton Dickinson, New Jersey) and centrifuged at 1,000 rpm at 4°C for 7 min. Supernatant was discarded and 1 μg of either mouse anti-human Igκ or mouse anti-human Igλ antibody was added (Pharmingen). After a 20 min incubation on ice, cells were washed twice with 2 ml of serum-free RPMI and centrifuged at 1,000 rpm at 4°C for 7 min for each wash. After the supernatant was discarded from the second wash, 1 μg of Fluorescein Isothiocyanate (FITC)-conjugated polyclonal goat anti-mouse Ig antibody (Pharmingen) was added and cells were incubated on ice for 20 min. Cells were then washed twice with serum-free RPMI and resuspended in 1 ml of 3.6% para-formaldehyde. Tubes were stored at 4°C and in the dark until analysis.

For intracellular proteins, 1x10⁶ cells were aliquoted into a 5 ml Falcon tube and centrifuged at 1,000 rpm at 4°C for 7 min. Supernatant was discarded
and 1 μg of mouse anti-c-myc, 1 μg of mouse IgGκ clone MOPC-21 or mouse anti-human Bcl-2 clone 100 (Pharmingen) diluted 1:50 in PBS, 1% Bovine Serum Albumin (BSA, Sigma-Aldrich), and 0.3% Saponin (Sigma-Aldrich) was added. After a 20 min incubation on ice, cells were washed twice with 2 ml of serum-free RPMI with 0.1% Saponin and centrifuged at 1,000 rpm at 4°C for 7 min. After the supernatant was discarded from the second wash, 1 μg of R-Phycoerythrin (PE)-conjugated F(ab')2 fragment affinity purified goat anti-mouse antibody (Chemicon) diluted 1:50 in PBS, 1% BSA, and 0.3% Saponin was added and cells were incubated on ice in the dark for 20 min. Cells were then washed twice with serum-free RPMI with 0.1% Saponin and resuspended in 0.5 ml PBS with 1% BSA. Tubes were kept on ice and analyzed immediately by flow cytometry.

For GFP analysis, 1x10⁶ cells were aliquoted into a 5 ml Falcon tube and centrifuged at 1,000 rpm at 4°C for 7 min. Supernatant was discarded and cells were resuspended in 0.5 ml of cold 1X PBS. Tubes were kept on ice and analyzed immediately by flow cytometry.

Cells were analyzed using a FACScan or FACScalibur flow cytometer (Becton Dickinson) equipped with an Argon laser tuned at 488 nm. Green fluorescence was recorded in the FL1 emission channel using a standard 530/30 nm filter, while PI and PE were recorded in the FL2 emission channel using a standard 585/42 nm filter.
2.10. Tumourigenicity assay

Female BALB/c nu/nu mice aged 8-10 weeks were purchased from Charles River (St. Constant, Que.). The mice were housed in the Animal Facility at the University of Toronto. All mice were maintained in sterile conditions in microisolation boxes and in accordance with the Guide to the Care and Use of Experimental Animals, Canadian Council on Animal Care (Olfert et al., 1993).

Approximately $2 \times 10^7$ C5-5/MCAT, C5-5/MCAT/GFP, C5-5/MCAT/mycGFPc3 and C5-5/MCAT/mycGFPc4 were pelleted by centrifugation at 1,000 rpm at 4°C for 7 min. Supernatant was discarded and cells were washed twice in serum free RPMI. Final cell pellets were resuspended in 4 ml of serum free RPMI. Approximately $1 \times 10^6$ cells (200 μl) were injected subcutaneously into the right flank of the BALB/c nu/nu mice, with an n=6 in each of 4 experimental groups. Mice were checked for tumour formation at least 3 times weekly throughout the experimental period, with daily checks at times of peak tumour incidence. Mice were sacrificed when tumours reached 2 cm in diameter in accordance with the policies and guidelines set by the University of Toronto.
CHAPTER 3: RESULTS
3.1. **Development of transformation assays**

The overall objective of the transformation assays was to find growth conditions that supported the survival and growth of the transformed cell line, OCI LY8 c3 (overexpresses Bcl-2, c-myc and possesses mutated p53), but did not support the survival nor growth of the non-transformed line, GM607/C5-5 (overexpresses Bcl-2 only).

3.1.a. **Cloning efficiency assay**

I set out to determine which plating conditions would or would not support the growth of the OCI LY8 c3 and GM607/C5-5 cell lines in normal serum conditions (15% BCS). Both cell lines were plated at 10, 1 and 0.1 cells per well in 96 well flat bottom plates. The OCI LY8 c3 cell line produced multiple colonies in every well when plated at 10 cells per well. When plated at 1 and 0.1 cells per well, 60 and 10 wells respectively, contained one colony. However, under these conditions, the GM607/C5-5 cell line did not produce any viable colonies. Therefore, GM607/C5-5 cells were plated under the same conditions but at concentrations of 250, 100 and 50 cells per well. When GM607/C5-5 was plated at 250 and 100 cells per well, all 96 wells contained a colony. When plated at 50 cells per well, 10 wells contained a colony (Table III).

The two cell lines were then mixed together at ratios of 1:250, 1:2,500 and 1:25,000 (OCI LY8 c3:GM607/C5-5) and plated at 10,000, 5,000 or 1,000 cells per well in 96 well flat bottom plates in the presence of either 15%, 5%, 2.5%,
Table III: Cloning efficiency of OCI LY8 c3 and GM607/C5-5

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Number of cells per well</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>250</td>
</tr>
<tr>
<td>OCI LY8 c3</td>
<td>nd</td>
</tr>
<tr>
<td>GM607/C5-5</td>
<td>96</td>
</tr>
</tbody>
</table>

Table IV: Colony forming ability of OCI LY8 c3 and GM607/C5-5 in soft agar

<table>
<thead>
<tr>
<th># of cells /plate</th>
<th>Cell Line</th>
<th>OCI LY8 c3</th>
<th>GM607/C5-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^6</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>10^5</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>10^4</td>
<td>+</td>
<td>-</td>
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</tr>
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<td>10^3</td>
<td>+</td>
<td>-</td>
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<td>nd</td>
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</tr>
<tr>
<td>10</td>
<td>-</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>nd</td>
<td></td>
</tr>
</tbody>
</table>

nd = not determined
Table III: Cloning efficiency of OCI LY8 c3 and GM607/C5-5. OCI LY8 c3 cells were plated at 10, 1 and 0.1 cells per well and GM607/C5-5 cells at 250, 100, 50, 10, 1 and 0.1 cells per well in 100 μl of complete culture media in 96 well flat bottom plates. Numbers indicate how many wells, out of 96, that had colonies growing in them.

nd = not done

Table IV: Colony forming ability of OCI LY8 c3 and GM607/C5-5 in soft agar. OCI LY8 c3 cells were plated at $10^6$, $10^5$, $10^4$, $10^3$, $10^2$, 10 and 1 cells per 100 mm plate in 10 ml of complete culture media on a base of 0.75% Noble agar. GM607/C5-5 cells were plated at $10^6$, $10^5$, $10^4$ and $10^3$ cells per 100 mm plate in the same conditions.

^ = exponentiation (e.g. $10^6 = 10^6$)
+ = colonies formed in agar
- = no colonies formed in agar
nd = not done
1%, or 0.1% BCS. When 10,000 or 5,000 cells were plated per well in the presence of 15%, 5%, 2.5% or 1% BCS multiple colonies were seen in every well by day 7 (data not shown). These plating conditions and the colonies derived from them were not chosen for further analysis. When 1,000 cells were plated per well in the presence of 15% and 5% BCS, colonies were seen in all 96 wells by day 19 (data not shown). For each experimental condition, 10 colonies were picked from different wells, expanded and stained with anti-κ and anti-λ antibodies. Figure 3.1 shows the staining profiles for OCI LY8 c3 and GM607/C5-5. OCI LY8 c3 is λ positive while GM607/C5-5 is κ positive. The representative staining profiles show that the majority of colonies picked were comprised of both κ and λ positive (GM607/C5-5 and OCI LY8 c3) cells, regardless of the ratio of OCI LY8 c3:GM607/C5-5 cells plated (Figure 3.2). However, when the same ratios of OCI LY8 c3:GM607/C5-5 were plated at 1,000 cells per well in media containing 1% BCS, by day 19, 96 wells contained a colony when the cells were plated at the 1:250 ratio, 35 wells contained a colony when the cells were plated at 1:2,500 and 4 wells contained a colony in the 1:25,000 plating condition (data not shown). For the 1:250 and 1:2,500 plating conditions, 10 colonies from different wells were picked from each plate and all 4 colonies resulting from the 1:25,000 condition were picked. All colonies were expanded and stained with anti-κ and anti-λ antibodies. The resulting representative staining profiles show that all colonies consisted of λ positive (OCI LY8 c3) cells only (Figure 3.2). When cells were plated in the
FIGURE 3.1: Profile of immunoglobulin light chain surface expression on OCI LY8 c3 and GM607/C5-5. 1 x 10^6 OCI LY8 c3 and GM607/C5-5 cells were stained with anti-human kappa, anti-human lambda and anti-mouse FITC antibodies and analyzed by FACS. Resulting histograms show fluorescence profiles of unstained cells, cells stained with anti-kappa, anti-lambda or FITC only and anti-kappa + FITC and anti-lambda + FITC.
FIGURE 3.2

1:250

kappa

15%

lambda

5%

kappa

1%

lambda
FIGURE 3.2: Outgrowth of OCI LY8 c3 cells, when mixed with GM607/C5-5 cells at ratios of 1:250, 1:2,500 and 1:25,000 in reduced serum conditions, as identified by immunoglobulin light chain staining and FACS analyses. OCI LY8 c3 and GM607/C5-5 cells were mixed together at ratios of 1:250, 1:2,500 and 1:25,000 and plated at 1,000 cells per well in 96 well flat bottom plates. Cells were plated in media containing either 15%, 5%, 1% or 0.1% BCS. A proportion of colonies seen were picked, expanded and stained with anti-kappa and anti-lambda antibodies and analyzed by FACS. Resulting histograms show the surface immunoglobulin light chain profiles of daughter cells evolving from the colonies derived from the various plating conditions.
above mentioned ratios in the presence of 0.1% BCS, no colonies were observed in any of the wells by day 21 and the experiment was terminated (data not shown).

3.1.b. Soft agar assay

To determine under what conditions OCI LY8 c3 and GM607/C5-5 cells would or would not grow on soft agar, I plated each cell type in 100 mm dishes, at concentrations of $10^6$, $10^5$, $10^4$, $10^3$, and $10^2$ (and 10 and 1 for OCI LY8 c3) cells in 10 ml of complete media, on a agar base. When plated at $10^6$ - $10^4$ cells per plate, OCI LY8 c3 produced innumerable colonies (>40 cells) on/in the agar base (so many that the colony borders were not defined and resembled a lawn). When $10^3$ cells were plated approximately 40 distinct, separate colonies could be identified. However, when $10^2$, 10 or 1 cells were plated, no colonies could be seen. In contrast, GM607/C5-5 failed to produce any colonies at any of the plating concentrations (Table IV). The OCI LY8 c3 and GM607/C5-5 cell lines were then mixed together at the previously mentioned ratios of 1:250, 1:2,500 and 1:25,000 (OCI LY8 c3:GM607/C5-5, respectively) and plated at $10^5$ and $10^6$ cells per plate. When $10^5$ cells were plated, colonies were seen in the agar at the 1:250 and 1:2,500 ratio but not at the 1:25,000 ratio by day 33 (data not shown). When $10^6$ cells were plated, colonies were seen on the agar for all plating ratios by day 33 (data not shown). Colonies were picked from the agar, expanded and then stained for $\kappa$ and $\lambda$ expression. The resulting representative histograms show that for all
plating conditions, colonies that formed on the agar consisted of λ positive cells only (Figure 3.3).

3.2. Generation of C5-5/MCAT/mycGFP, a Bcl-2 and c-myc overexpressing cell line

Generation of a c-myc overexpressing C5-5 cell line (C5-5/MCAT/mycGFP) was accomplished in 3 steps. The first step was to introduce MCAT-1 cDNA into GM607/C5-5 cells, the second was to generate GP+E-86 cells that secrete murine retrovirus containing c-myc and the third was to transduce C5-5/MCAT with murine retrovirus containing c-myc (Figure 3.4).

3.2.a. Generation of a MCAT-1 expressing cell line

To create a human B-cell line that was permissive to murine retrovirus we obtained the pJET vector, containing MCAT-1 cDNA, from Dr. J. Cunningham (Howard Hughes Medical Institute, Boston, MA). pJET, however, does not contain a drug resistance marker so we sought to subclone the MCAT-1 cDNA into a vector that did. Figure 3.5 depicts the generation of the pCEP4/MCAT-1 vector. In pJET, the MCAT-1 cDNA is flanked at both the 5’ and 3’ ends by Eco RI restriction enzyme sites and was therefore digested out of the pJET vector using Eco RI. The resulting sticky ends were then
FIGURE 3.3
FIGURE 3.3: Outgrowth of OCI LY8 c3 cells, when mixed with GM607/C5-5 cells at ratios of 1:250, 1:2,500 and 1:25,000 and plated on soft agar, as identified by immunoglobulin light chain staining and FACS analyses. OCI LY8 c3 and GM607/C5-5 cells were mixed together at ratios of 1:250, 1:2,500 and 1:25,000 and plated at $10^6$ and $10^5$ cells, in 10 ml of complete media in 100 mm plates, over a base of 0.75% Noble agar. A proportion of colonies seen were picked, expanded and stained with anti-kappa and anti-lambda antibodies and analyzed by FACS. Resulting histograms show the surface immunoglobulin light chain profiles of daughter cells evolving from the colonies derived from the various plating conditions.
FIGURE 3.4: Generation of cell lines. GM607, a human, mature, EBV+ B-cell line, was transfected with human Bcl-2 cDNA, resulting in its overexpression (Kuzniar, 1997). This cell line, GM607/C5-5, was then transfected with pCEP4/MCAT-1, resulting in MCAT-1 receptors being expressed on the surface of the cell (C5-5/MCAT). MCAT-1, an ecotropic receptor, makes human cells permissive to infection with murine retrovirus. GP+E-86 cells were transfected with either pBabeIresMNGFP (GP+E-86/GFP) or pBabeIresMNhuc-mycGFP (GP+E-86/mycGFP) resulting in new lines that packages and secretes murine retrovirus containing GFP or c-myc and GFP, respectively. C5-5/MCAT was then transduced with the supernatant from GP+E-86/GFP or GP+E-86/mycGFP cells resulting in cells overexpressing Bcl-2 and GFP (C5-5/MCAT/GFP) or Bcl-2, c-myc and GFP (C5-5/MCAT/mycGFP).
FIGURE 3.5: Generation of pCEP4/MCAT-1 vector. This schematic diagram outlines the various steps involved in the generation of the pCEP4/MCAT-1 vector. MCAT-1 cDNA was digested out of the pJET vector using Eco RI. The resulting sticky ends were blunted by the addition of dNTPs and Klenow. pCEP4, a mammalian expression vector containing a hygromycin resistance cassette, was blunt ended (in the multiple cloning site) by digestion of the plasmid with Pvu II. MCAT-1 cDNA was then subcloned into the blunt ended pCEP4 with the addition of T4 ligase. The resulting plasmid population was transfected into and propagated by E. coli and plated on LB agar plates. 4 resulting colonies were picked, expanded and small scale plasmid preparations were produced by standard plasmid preparation techniques. The 4 separate preparations were then analyzed for correct orientation of the MCAT-1 insert by digestion with Bam HI. Two of the 4 plasmid preparations had the MCAT-1 insert in the correct orientation and one, pCEP4/MCAT-1, was selected for further use.
blunted by the addition of dNTPs and Klenow. The pCEP4 mammalian expression vector, containing a hygromycin resistance cassette, was blunt ended by digestion of the vector with Pvu II. The blunt ended MCAT-1 cDNA was subcloned into the blunt ended pCEP4 with the addition of T4 ligase. The resulting plasmid DNA was transfected by heat shock into DH5α Escherichia coli and plated on LB agar plates. The 4 colonies produced on the agar plates were picked and expanded. Small scale plasmid preparations were produced using standard plasmid preparation techniques. The 4 separate preparations were then analyzed for correct orientation of the MCAT-1 insert by digestion with Bam HI. Two of the 4 plasmid preparations had the MCAT-1 insert in the correct orientation and one, pCEP4/MCAT-1, was selected for further use (data not shown). pCEP4/MCAT-1 was then transfected into GM607/C5-5 under conditions described in Material and Methods 2.3. The resulting cell line, C5-5/MCAT, was screened by retroviral infection to determine its permissiveness to a murine retrovirus.

3.2.b. Generation of retrovirus-secreting cell lines

The murine fibroblast packaging cell line, GP+E-86, was transfected with either the pBabeIresMNGFP or pBabeIresMNhuc-mycGFP viral vector under conditions describe in Materials and Methods 2.3. The resulting bulk cultures and clones were then analyzed for GFP expression. Two days following transfection GFP expression by GP+E-86/GFP bulk and GP+E-86/mycGFP bulk cells was assessed. Approximately 10.5% of the GP+E-
86/GFP bulk culture showed increased levels of fluorescence when compared to the control cells (non-transfected GP+E-86 cells) (Figure 3.6.a). A very small percentage, approximately 0.9%, of the GP+E-86/mycGFP bulk culture demonstrated increased fluorescence when compared with non-transfected controls (Figure 3.7.a). Both the GP+E-86/GFP and GP+E-86/mycGFP bulk cultures were then sorted by FACS for high GFP expressers and the resulting GFP expression for the sorted bulk populations, compared to non-transfected GP+E-86 cells, are shown in Figures 3.6.b and 3.7.b. The sorted GP+E-86/GFP and GP+E-86/mycGFP bulk populations were then cloned by limiting dilution. GFP expression of the resulting clones, compared to non-transfected GP+E-86 cells, are shown in Figures 3.6.c and 3.7.c. GP+E-86/GFPc7 and GP+E-86/mycGFPc2 were chosen for future experiments.

3.2.c. Generation of a Bcl-2 and c-myc overexpressing cell line

The C5-5/MCAT cell line was transduced with the supernatant from either GP+E-86/GFPc7 or GP+E-86/mycGFPc2 cells (Materials and Methods 2.4.). The resulting bulk cultures, C5-5/MCAT/GFP bulk, C5-5/MCAT/mycGFP bulk#1 and C5-5/MCAT/mycGFP bulk#2 (2 separate transductions) were expanded in non-selective conditions. GFP expression was analyzed at various time points by FACS and compared with C5-5/MCAT cells transduced by GP+E-86 supernatant (Figure 3.8.a and Table V). On day 2, 10.1% of the C5-5/MCAT/GFP bulk population was positive for GFP expression while the GFP expression of both C5-5/MCAT/mycGFP bulk
FIGURE 3.6

a) GP+E-86 transfected with pBabelresMNGFP

b) GP+E-86/GFP sorted (bulk population)

d) GP+E-86/GFPc1, GP+E-86/GFPc2, GP+E-86/GFPc3, GP+E-86/GFPc4, GP+E-86/GFPc5, GP+E-86/GFPc6, GP+E-86/GFPc7, GP+E-86/GFPc8, GP+E-86/GFPc9, GP+E-86/GFPc10
FIGURE 3.6: Increase in GFP expression in pBabeIresMNGFP transfectants as measured by FACS analysis.

a) GFP expression of non-transfected GP+E-86 cells (shaded histogram) and GP+E-86 cells transfected with the retroviral vector pBabeIresMNGFP (GP+E-86/GFP) (open histogram).

b) GFP expression of non-transfected GP+E-86 cells (open histograms) and GP+E-86/GFP cells after FACS sorting for high GFP expressers (open histogram).

c) GFP expression of independent clones derived, by limiting dilution, from the sorted GP+E-86/GFP bulk population (open histograms) compared to non-transfected GP+E-86 cells (shaded histograms).
FIGURE 3.7

a) GP+E-86 transfected with pBabelresMNhuc-mycGFP

b) GP+E-86/mycGFP sorted (bulk population)

d) GP+E-86/mycGFPc1  GP+E-86/mycGFPc2  GP+E-86/mycGFPc3

GP+E-86/mycGFPc7  GP+E-86/mycGFPc8  GP+E-86/mycGFPc9

GP+E-86/mycGFPc10 GP+E-86/mycGFPc11 GP+E-86/mycGFPc12
FIGURE 3.7: Increase in GFP expression in pBabeIresMNhuc-mycGFP transfectants as measured by FACS analysis.

a) GFP expression of non-transfected GP+E-86 cells (shaded histogram) and GP+E-86 cells transfected with the retroviral vector pBabeIresMNhuc-mycGFP (GP+E-86/mycGFP) (open histogram).

b) GFP expression of non-transfected GP+E-86 cells (open histograms) and GP+E-86/mycGFP cells after FACS sorting for high GFP expressers (open histogram).

c) GFP expression of independent clones derived, by limiting dilution, from the sorted GP+E-86/mycGFP bulk population (open histograms) compared to non-transfected GP+E-86 cells (shaded histograms).
FIGURE 3.8: Increasing GFP expression of C5-5/MCAT/mycGFP bulk populations, compared with vector control bulk population (C5-5/MCAT/GFP), as a function of time in non-selective culture conditions. GFP expression of C5-5/MCAT bulk populations transduced with virus-containing supernatants produced by either GP+E-86/GFPc7 (C5-5/MCAT/GFP) or GP+E-86/mycGFPc2 cells (C5-5/MCAT/mycGFP bulk #1 and #2) (open histograms) compared with C5-5/MCAT cells transduced with GP+E-86 parental, virus-free supernatant (shaded histograms). Cells were analyzed for GFP expression by FACS at various time points.
Table V: GFP expression of C5-5/MCAT cells transduced with either pBabeMNIResGFP or pBabeMNIReshuc-mycGFP virus-containing supernatant

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>day 2</th>
<th>day 8</th>
<th>day 14</th>
<th>day 22</th>
<th>day 28</th>
<th>day 34</th>
<th>day 49</th>
<th>day 52</th>
<th>day 56</th>
<th>day 63</th>
<th>day 67</th>
<th>day 76</th>
<th>day 94</th>
</tr>
</thead>
<tbody>
<tr>
<td>C5-5/MCAT/GFP bulk</td>
<td>10.1</td>
<td>25.56</td>
<td>30.04</td>
<td>33.92</td>
<td>33.22</td>
<td>29.31</td>
<td>34.06</td>
<td>35.3</td>
<td>34.26</td>
<td>43.44</td>
<td>47.94</td>
<td>34.43</td>
<td>41.64</td>
</tr>
<tr>
<td>C5-5/MCAT/mycGFP bulk#1</td>
<td>n/d</td>
<td>0.92</td>
<td>3.49</td>
<td>9.56</td>
<td>20.55</td>
<td>34.38</td>
<td>75.5</td>
<td>83.67</td>
<td>90.28</td>
<td>93.91</td>
<td>95.74</td>
<td>99.07</td>
<td>99.01</td>
</tr>
<tr>
<td>C5-5/MCAT/mycGFP bulk#2</td>
<td>n/d</td>
<td>2.58</td>
<td>6.05</td>
<td>18.99</td>
<td>35.3</td>
<td>48.53</td>
<td>85.53</td>
<td>90.77</td>
<td>95.61</td>
<td>97.35</td>
<td>98.12</td>
<td>99.15</td>
<td>99.33</td>
</tr>
</tbody>
</table>

FIGURE 3.9

![Graph showing GFP expression over time](image-url)
Table V: GFP expression of C5-5/MCAT cells transduced with either pBabeIresMNGFP or pBabeIresMNhuc-mycGFP virus-containing supernatant. An aliquot of cells from each bulk culture, grown in non-selective conditions, was analyzed by FACS on days indicated. Numbers indicate percentage of C5-5/MCAT/GFP bulk, C5-5/MCAT/mycGFP bulk #1 or #2 populations that are positive for GFP expression.

FIGURE 3.9: Percentage of C5-5/MCAT/GFP bulk, C5-5/MCAT/mycGFP bulk #1 and #2 populations that are GFP positive, as a function of time, in non-selective culture conditions. Bulk cultures (C5-5/MCAT/GFP, blue triangle; C5-5/MCAT/mycGFP bulk#1, green square; C5-5/MCAT/mycGFP bulk#2, red circle) were maintained for 94 days in non-selective conditions with aliquots of cells being FACS'd for GFP expression on the days indicated. Each point represents the percent of the population that is positive for GFP expression.
populations was undetectable. On day 8, GFP expression rose to 25.56% for the C5-5/MCAT/GFP bulk population and 0.92% and 2.58% for the C5-5/MCAT/mycGFP bulk #1 and #2 populations respectively. By day 22, GFP expression rose to 33.92% for the C5-5/MCAT/GFP bulk population and 9.56% and 18.99% for the C5-5/MCAT/mycGFP bulk #1 and #2 populations respectively. GFP expression for the C5-5/MCAT/GFP bulk population remained relatively stable for the rest of the time in culture while that of the 2 C5-5/MCAT/mycGFP bulk populations continued to rise. By day 76 both C5-5/MCAT/mycGFP bulk populations were 100% positive for GFP expression and remained 100% positive until the end of the experiment (day 94) (Table V and Figure 3.9). The C5-5/MCAT/GFP bulk and C5-5/MCAT/mycGFP bulk#2 populations were then cloned by limiting dilution. One C5-5/MCAT/GFP clone (c7-3) and two C5-5/MCAT/mycGFP (c2-3 and c2-4) clones were selected on the basis of high, uniform GFP expression for future use (data not shown).

To ensure that our cell lines possessing the pBabeIresMNhc-mycGFP vector truly did express c-myc, all relevant cell lines were stained with an anti-c-myc antibody and analyzed by FACS. Compared with positive control cell lines, Karpas and OCI LY8 c3 which possess a c-myc translocation, GP+E-86/mycGFPc2, C5-5/MCATmycGFP bulk, C5-5/MCAT/mycGFPc2-3 and C5-5/MCAT/mycGFPc2-4 expressed very high levels of c-myc. Cells possessing the pBabeIresMNGFP vector, GP+E-86/GFPc7 and C5-5/MCAT/GFP bulk, did
not show increased levels of c-myc compared to the isotype control (Figure
3.10.a and b).

3.3. Transformation assays

3.3.a. Cloning efficiency

When C5-5/MCAT/GFPc7-3, C5-5/MCAT/mycGFPc2-3 and C5-
5/MCAT/mycGFPc2-4 cells were mixed with C5-5/MCAT cells in ratios of
1:250, 1:2,500 and 1:25,000 and plated in 96 well plates at 1,000 cells per well in
the presence of 1% BCS no colonies were observed in any of the wells by day
14. After 39 days, no colonies were observed and the experiment was
terminated (Table VI).

3.3.b. Soft agar assay

When C5-5/MCAT/GFPc7-3, C5-5/MCAT/mycGFPc2-3 and C5-
5/MCAT/mycGFPc2-4 cells were mixed with C5-5/MCAT cells in ratios of
1:250, 1:2,500 and 1:25,000 and plated at $10^6$ cells in 10 ml of media on a soft
agar base no colonies were observed by day 30. After 45 days, no colonies were
observed and the experiment was terminated (Table VII).
FIGURE 3.10

a)  
- Karpas

- OCI-ly8 c3

- GP+E-86/GFP c7

- GP+E-86/mycGFP c2

b)  
- Karpas

- C5-5/MCAT

- C5-5/MCAT/GFP bulk

- C5-5/MCAT/mycGFP bulk

- C5-5/MCAT/mycGFP 2-3

- C5-5/MCAT/mycGFP 2-4

c)  
- C5-5/MCAT

- C5-5/MCAT/mycGFP c2-3

- C5-5/MCAT/mycGFP c2-4
FIGURE 3.10: Overexpression of c-myc in GP+E-86/mycGFPc2, C5-5/MCAT/mycGFP bulk, C5-5/MCAT/mycGFPc2-3 and c2-4 compared to negative and positive control cell lines. 1 x 10^6 cells were stained with isotype control, mouse IgG\_\kappa clone MOPC-21 (Pharmingen, shaded histogram) or with anti-c-myc (Chemicon, open histogram) plus goat anti-mouse PE (Chemicon) antibodies and analyzed by FACS.

a) Karpas and OCI LYS c3, both possessing c-myc translocations, represent positive controls for c-myc expression. GP+E-87/GFPc7 and GP+E-86/mycGFPc2 are the retrovirus-secreting cell lines used to infect C5-5/MCAT cells.

b) c-myc expression in C5-5/MCAT cells transduced by supernatant produced by GP+E-86 (C5-5/MCAT), GP+E-86/GFPc7 (C5-5/MCAT/GFP bulk) or GP+E-86/mycGFPc2 (C5-5/MCAT/mycGFP bulk, C5-5/MCAT/mycGFPc2-3 and c2-4) cells.

c) c-myc expression of C5-5/MCAT, C5-5/MCAT/mycGFPc2-3 and c2-4 cells that were used in the cloning efficiency, soft agar, tumourgenecity and growth curve assays. Cells were kept in culture until the end of the above assays and then c-myc expression was assessed.
Table VI: Oncogenic cooperation between Bcl-2 and c-myc could not be detected by the cloning efficiency assay in which cells were plated at various ratios in media containing 1% BCS.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Plating ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:250</td>
</tr>
<tr>
<td>C5-5/MCAT/GFPc7-3:C5-5/MCAT</td>
<td>0</td>
</tr>
<tr>
<td>C5-5/MCAT/mycGFPc2-3:C5-5/MCAT</td>
<td>0</td>
</tr>
<tr>
<td>C5-5/MCAT/mycGFPc2-4:C5-5/MCAT</td>
<td>0</td>
</tr>
</tbody>
</table>

Table VII: Oncogenic cooperation between Bcl-2 and c-myc could not be detected by the soft agar assay in which 10^6 cells were plated at various ratios on soft agar.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Plating ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1:250</td>
</tr>
<tr>
<td>C5-5/MCAT/GFPc7-3:C5-5/MCAT</td>
<td>0</td>
</tr>
<tr>
<td>C5-5/MCAT/mycGFPc2-3:C5-5/MCAT</td>
<td>0</td>
</tr>
<tr>
<td>C5-5/MCAT/mycGFPc2-4:C5-5/MCAT</td>
<td>0</td>
</tr>
</tbody>
</table>

Table VIII: Oncogenic cooperation between Bcl-2 and c-myc could not be detected by the tumourigenicity assay in which 10^6 cells were injected into BALB/c nu/nu mice.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumour formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>C5-5/MCAT</td>
<td>0 / 4</td>
</tr>
<tr>
<td>C5-5/MCAT/GFPc7-3</td>
<td>0 / 4</td>
</tr>
<tr>
<td>C5-5/MCAT/mycGFPc2-3</td>
<td>0 / 4</td>
</tr>
<tr>
<td>C5-5/MCAT/mycGFPc2-4</td>
<td>0 / 4</td>
</tr>
</tbody>
</table>
Table VI: Oncogenic cooperation between Bcl-2 and c-myc could not be detected by the cloning efficiency assay in which cells were plated at various ratios in media containing 1% BCS. C5-5/MCAT/GFPc7-3, C5-5/MCAT/mycGFPc2-3 and c2-4 were mixed together with C5-5/MCAT cells at ratios of 1:250, 1:2,500 and 1:25,000 and plated at 1,000 cells per well in 96 well plates in media containing 1% BCS. Indication of the number of wells that contained a colony, for each plating condition, after 39 days.

Table VII: Oncogenic cooperation between Bcl-2 and c-myc could not be detected by the soft agar assay in which $10^6$ cells were plated at various ratios on soft agar. C5-5/MCAT/GFPc7-3, C5-5/MCAT/mycGFPc2-3 and c2-4 were mixed together with C5-5/MCAT cells at ratios of 1:250, 1:2,500 and 1:25,000 and plated at $10^6$ cells in 10 ml of complete media overlayed on an agar base in 100 mm plates. Indication of the number of colonies formed in the soft agar, for each plating condition, after 45 days.

$^\wedge =$ exponentiation (e.g. $10^6 = 10^6$)

Table VIII: Oncogenic cooperation between Bcl-2 and c-myc could not be detected by the tumourigenicity assay in which $10^6$ cells were injected into BALB/c nu/nu mice. $10^6$ C5-5/MCAT, C5-5/MCAT/GFPc7-3, C5-5/MCAT/mycGFPc2-3 and c2-4 cells were injected subcutaneously into BALB/c nu/nu mice. Indication of the number of mice, out of 4 in each group, that formed tumours by day 80.

$^\wedge =$ exponentiation (e.g. $10^6 = 10^6$)
3.3.c. Tumourigenicity assay

BALB/c nu/nu mice were injected subcutaneously with $10^6$ C5-5/MCAT, C5-5/MCAT/GFPc7-3, C5-5/MCAT/mycGFPc2-3 or C5-5/MCAT/mycGFPc2-4 cells to assess the ability of the c-myc and Bcl-2 overexpressing cells to form tumours. After 81 days, no tumours were observed, in any of the groups, and the experiment was terminated (Table VIII).

To ensure that our lack of colony or tumour formation was not due to the C5-5/MCAT/mycGFPc2-3 and c2-4 cell lines losing c-myc expression, these cell lines (that had been kept in culture for the length of the assays) were stained with anti-c-myc and then analyzed by FACS. Figure 3.10.c shows that both C5-5/MCAT/myc GFPc2-3 and c2-4 cells still expressed very high levels of c-myc at the time of assay completion.

3.4. Growth characteristics of C5-5/MCAT, C5-5/MCAT/GFPc7-3 and C5-5/MCAT mycGFPc2-3 and c2-4 cultured in normal (15%) and reduced (4%) serum conditions.

To determine the growth characteristics of the 4 cell lines used in the cloning efficiency, soft agar and tumourigenecity assays, the cells were plated
in media containing either normal (15% BCS) or reduced (4% BCS) serum levels (Materials and Methods 2.7).

3.4.a. Growth curve assay

Cell counts were obtained, in duplicate, for each cell line on days 1 - 11 and 15 - 17. Each data point represents the average cell count for each cell line on that day, with error bars indicating the standard deviation of the 2 cell counts (Figures 3.11 and 3.12).

The resulting growth curves are shown in Figure 3.11 for cells that were cultured in media containing 15% BCS. The doubling time, in hours, of the cell lines are as follows; C5-5/MCAT = 43.5, C5-5/MCAT/GFPc7-3 = 33, C5-5/MCAT/mycGFPc2-3 = 46.5 and C5-5/MCAT/mycGFPc2-4 = 48. The doubling time of the cell lines was calculated as the time, in hours, required for the cells to increase from $3.5 \times 10^5$ to $7.0 \times 10^5$ cells/ml. There are no differences in doubling times between C5-5/MCAT/mycGFPc2-3 and c2-4, and C5-5/MCAT is only slightly lower than either c-myc line. However, the doubling time for the vector control, C5-5/MCATGFPc7-3, is much lower than the other 3 lines. C5-5/MCAT reached the highest maximum density of all of the cell lines, $1.24 \times 10^6$ cells/ml, on day 7 with cell numbers declining thereafter (with the exception of day 9). C5-5/MCAT/GFPc7-3 and C5-5/MCAT/mycGFPc2-3 and c2-4 reached maximum densities on day 6 and then cell numbers declined (with the exception of day 8) until the end of the assay. C5-5/MCAT/GFPc7-3 reached a maximum density of $1.18 \times 10^6$.
FIGURE 3.11

![Graph showing cell growth over time (days)]

FIGURE 3.12

![Graph showing cell growth over time (days)]
FIGURE 3.11: Growth curves of C5-5/MCAT, C5-5/MCAT/GFPc7-3, C5-5/MCAT/mycGFPc2-3 and c2-4 cells cultured in media containing 15% BCS. 2 x 10^5 C5-5/MCAT, C5-5/MCAT/GFPc7-3, C5-5/MCAT/mycGFPc2-3 and c2-4 cells were plated in 2 ml of culture media containing 15% BCS (per well) in 24 well plates. Cell counts were performed by trypan blue exclusion, in duplicate, on days 1 - 11 and 15 - 17. Each point represents the average of the 2 cell counts for a given day with error bars indicating the standard deviation of the 2 counts.

FIGURE 3.12: Growth curves of C5-5/MCAT, C5-5/MCAT/GFPc7-3, C5-5/MCAT/mycGFPc2-3 and c2-4 cells cultured in media containing 4% BCS. 2 x 10^5 C5-5/MCAT, C5-5/MCAT/GFPc7-3, C5-5/MCAT/mycGFPc2-3 and c2-4 cells were plated in 2 ml of culture media containing 4% BCS (per well) in 24 well plates. Cell counts were performed by trypan blue exclusion, in duplicate, on days 1 - 11 and 15 - 17. Each point represents the average of the 2 cell counts for a given day with error bars indicating the standard deviation of the 2 counts.
cells/ml, while C5-5/MCAT/mycGFPc2-3 and c2-4 reach lower maximum densities, of 9.36 and 8.91 x 10^5 cells/ml respectively (Figure 3.11).

The resulting growth curves are shown in Figure 3.12 for cell lines that were cultured in media containing 4% BCS. The doubling times, in hours, of the cell lines are as follows; C5-5/MCAT = 36, C5-5/MCAT/GFPc7-3 = 34.5, C5-5/MCAT/mycGFPc2-3 = 30 and C5-5/MCAT/mycGFPc2-4 = 18. The doubling time of the cell lines was calculated as the time, in hours, required for the cells to increase from 3.0 x 10^5 to 6.0 x 10^5 cells/ml. There is no difference in doubling time between C5-5/MCAT and C5-5/MCAT/GFPc7-3 and that of C5-5/MCAT/mycGFPc2-3 is only slightly lower than the parental and vector controls. However, the doubling time of C5-5/MCAT/mycGFPc2-4 is much lower than the other 3 cell lines. The doubling times of C5-5/MCAT and C5-5/MCAT/mycGFPc2-3 and c2-4 cells are considerably lower when cells are cultured in media containing 4% BCS compared to 15% BCS. C5-5/MCAT reached the lowest maximum density, 7.5 x 10^5 cells/ml, on day 7, compared to the other 3 cell lines. Cell numbers declined thereafter until the end of the assay. C5-5/MCAT/GFPc7-3 and C5-5/MCAT/mycGFPc2-3 reached similar maximum densities of 7.65 and 7.8 x 10^5 cells/ml, respectively, on day 6 with cell numbers declining (with the exception of days 9 and 10 and 8 and 9 respectively) until the end of the assay. C5-5/MCAT/mycGFPc2-4, however, reached the highest maximum density, 9.12 x 10^5 cells/ml, out of all cell lines on day 6 with cell numbers declining (with the exception of days 8 and 9) until the end of the assay (Figure 3.12).
When the cell lines were cultured in the presence of 15% BCS, the percent viability [# alive cells ÷ (# alive + # dead cells) x 100%] of all 4 cell lines was essentially the same for days 1 - 6. On day 7, however, the percent viability for C5-5/MCAT/GFPc7-3, C5-5/MCAT/mycGFPc2-3 and C5-5/MCAT/mycGFPc2-4 decreased with respect to C5-5/MCAT. On day 7, there was no difference in percent viability between C5-5/MCAT/GFPc7-3 and C5-5/MCAT/mcyGFPc2-3. However, the percent viability of C5-5/MCAT/mycGFPc2-4 was substantially lower than the other 3 cell lines. On days 8 - 17, all 4 lines showed decreases in percent viability however, the percent viability of both c-myc lines remained considerably lower compared to C5-5/MCAT and C5-5/MCAT/GFPc7-3 at all time points (Figure 3.13 and Table IX).

When the cell lines were cultured in the presence of 4% BCS, the percent viability of all 4 cell lines was essentially the same for days 1 - 6. On day 7, all 4 cell lines showed reductions in percent viability. The percent viability of both C5-5/MCAT/mcyGFPc2-3 and c2-4 cell lines were very similar to each other and much lower compared to the control cell lines (C5-5/MCAT and C5-5/MCAT/GFPc7-3). On day 8, the percent viability of the c-myc cell lines remained similar to each other and much lower than the control cell lines, which were similar to each other. On day 9, the percent viability of the c-myc lines remained essentially unchanged while that of C5-5/MCAT/GFPc7-3 decreased, reaching the level of the c-myc lines. Percent viability of C5-5/MCAT decreased slightly but remained much higher than
FIGURE 3.13

Percent viability vs. time (days)

- C5-5/MCAT
- C5-5/MCAT/GFPc7-3
- C5-5/MCAT/mycGFP 2-3
- C5-5/MCAT/mycGFP 2-4

FIGURE 3.14

Percent viability vs. time (days)

- C5-5/MCAT
- C5-5/MCAT/GFPc7-3
- C5-5/MCAT/mycGFP 2-3
- C5-5/MCAT/mycGFP 2-4
FIGURE 3.13: Decrease in percent viability of C5-5/MCAT/mycGFPc2-3 and c2-4 cells, compared to control cell lines, during the growth curve assay in which cells were cultured in media containing 15% BCS. 2 x 10^5 cells, C5-5/MCAT/, C5-5/MCAT/GFPc7-3, C5-5/MCAT/mycGFPc2-3 and c2-4, were plated in 2 ml of culture media containing 15% BCS (per well) in 24 well plates. Alive and dead cell counts were performed, by trypan blue exclusion, in duplicate on days 1 - 11 and 15 - 17. Each data point represents the percent viability of a cell line on a given day. Percent viability was calculated using the following formula:

# alive cells ÷ (# alive + # dead cells) x 100%

FIGURE 3.14: Decrease in percent viability of C5-5/MCAT/mycGFPc2-3 and c2-4 cells, compared to control cell lines, during the growth curve assay in which cells were cultured in media containing 4% BCS. 2 x 10^5 cells, C5-5/MCAT/, C5-5/MCAT/GFPc7-3, C5-5/MCAT/mycGFPc2-3 and c2-4, were plated in 2 ml of culture media containing 4% BCS (per well) in 24 well plates. Alive and dead cell counts were performed, by trypan blue exclusion, in duplicate on days 1 - 11 and 15 - 17. Each data point represents the percent viability of a cell line on a given day. Percent viability was calculated using the following formula:

# alive cells ÷ (# alive + # dead cells) x 100%
Table IX: Summary of percent viability and actual number of alive and dead CS-5/MCAT, /GFP c7-3 and /mycGFPc2-3 and c2-4 cells counted on indicated days of growth curve assay. All cells were cultured in 15% BCS.

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90
Table IX: Summary of percent viability and actual number of alive and dead C5-5/MCAT, C5-5/MCAT/GFPc7-3, and C5-5/MCAT/mycGFPc2-3 and c2-4 cells counted on indicated days of a growth curve assay. All cells were cultured in the presence of 15% BCS. Summary table showing the percent viability and actual number of alive and dead cells for each cell line on each day of the growth curve assay. All cell lines were cultured in the presence of 15% BCS and cell counts were performed, on days indicated, by trypan blue exclusion. Number of alive and dead cells is the combined total from duplicate wells for each cell line on each day. Percent viability was calculated using the combined total number of alive and dead cells and the following formula:

\[ \text{Percent viability} = \frac{\text{# alive cells}}{\text{# alive} + \text{# dead cells}} \times 100\% \]
the other 3 lines. On day 10, the percent viability of all 4 lines decreased (with the exception of C5-5/MCAT/GFPc7-3) and continued to do so until the end of the assay (with the exception of day 15, and 16 and 17 for C5-5/MCAT). The percent viability of C5-5/MCAT/GFPc7-3 was higher than that of C5-5/MCAT from day 10 - 16. With the exception of day 15 the c-myc cell lines had much lower percent viability compared to the control lines (C5-5/MCAT and C5-5/MCAT/GFPc7-3) and were very similar to each other (Figure 3.14 and Table X).

3.4.b. Cell cycle assay

On days 2, 4, 6, 8, 10, 11 and 15 of the growth curve assay, cellular DNA from each of the 4 cell lines were stained with propidium iodide and analyzed by FACS.

When cells were cultured in the presence of 15% BCS, cell cycle analysis revealed that C5-5/MCAT/mycGFPc2-3 and c2-4 cells consistently demonstrate increased amounts of both apoptotic and "S phase" cells compared to both control lines (C5-5/MCAT and C5-5/MCAT/GFPc7-3) at every time point, with the exception of day 2 (Figure 3.15). C5-5/MCAT/mycGFPc2-3 showed increases in apoptosis ranging from 0.83 - 7.9X compared to control cells. Increases ≥2X compared with control cells, in the apoptotic population, were seen on days 6, 8, 10 and 11. The most notable increases occurred on days 10 (6.2X over C5-5/MCAT and 5.9X over C5-5/MCAT/GFPc7-3) and 11 (7.9X over C5-5/MCAT and 4.2X over C5-
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Table X: Summary of percent viability and actual number of alive and dead CS/MCAT/GCPF 2.4 and CS/MCAT/myc GCPF 2.4.
Table X: Summary of percent viability and actual number of alive and dead C5-5/MCAT, C5-5/MCAT/GFPc7-3, and C5-5/MCAT/mycGFPc2-3 and c2-4 cells counted on indicated days of a growth curve assay. All cells were cultured in the presence of 4% BCS. Summary table showing the percent viability and actual number of alive and dead cells for each cell line on each day of the growth curve assay. All cell lines were cultured in the presence of 4% BCS and cell counts were performed, on days indicated, by trypan blue exclusion. Number of alive and dead cells is the combined total from duplicate wells for each cell line on each day. Percent viability was calculated using the combined total number of alive and dead cells and the following formula:

\[
\text{Percent viability} = \frac{\# \text{ alive cells}}{\# \text{ alive cells} + \# \text{ dead cells}} \times 100\%
\]
FIGURE 3.15: Increases in apoptotic and "S phase" populations of C5-5/MCAT/mycGFPc2-3 and c2-4 cells compared to control cell lines, during the growth curve assay in which cells were cultured in the presence of 15% BCS, as measured by cell cycle assay. In the growth curve assay, $2 \times 10^5$ C5-5/MCAT/, C5-5/MCAT/GFPc7-3, C5-5/MCAT/mycGFPc2-3 and c2-4 cells were plated in 2 ml of culture media containing 15% BCS (per well) in 24 well plates. Cell cycle of all 4 cell lines was assayed on days indicated by propidium iodide staining of the DNA and then FACS analysis. Numbers indicate percent of cell population that is apoptotic or in "S phase" (apoptotic/S phase).
C5-5/MCAT/mycGFPc2-4 cells showed increases in apoptosis ranging from 0.67 - 7.6X compared to control cells. Increases ≥2X compared with control cells, in the apoptotic population, were observed on days 6, 8, 10, 11 and 15. The most notable increases occurred on days 10 (5.7X over C5-5/MCAT and 5.4X over C5-5/MCAT/GFPc7-3) and 11 (7.6X over C5-5/MCAT and 4.1X over C5-5/MCAT/GFPc7-3).

Although there were increases in the "S phase" populations, in both c-myc cell lines (compared to C5-5/MCAT and C5-5/MCAT/GFPc7-3 control lines), they were not nearly as dramatic as for the apoptotic populations. Increases for C5-5/MCAT/mycGFPc2-3 (compared with controls) ranged from 1.1 - 1.4X and were maximal on day 11. Increases for C5-5/MCAT/mycGFPc2-4 (compared with controls) ranged from 1.1 - 1.8X and were maximal on days 10 and 11.

When cells were cultured in the presence of 4% BCS, cell cycle analysis reveals that C5-5/MCAT/mycGFPc2-3 and c2-4 cells consistently demonstrate increased amounts of both apoptotic and "S phase" cells compared to both control lines at every time point, with the exception of day 4 and 6 for C5-5/MCAT/mycGFPc2-3 (Figure 3.16).

C5-5/MCAT/mycGFPc2-3 showed increases in apoptosis ranging from 0.93 - 3.8X compared to control cells when cultured in the presence of 4% BCS. Increases ≥2X compared with control cells, in the apoptotic population, were observed on days 6 (over C5-5/MCAT only), 8, 10, 11 and 15. The most striking increases occurred on days 8 (3.8X over C5-5/MCAT and 3.0X compared with
FIGURE 3.16: Increases in apoptotic and "S phase" populations of C5-5/MCAT/mycGFPc2-3 and c2-4 cells compared to control cell lines, during the growth curve assay in which cells were cultured in the presence of 4% BCS, as measured by cell cycle assay. In the growth curve assay, $2 \times 10^5$ C5-5/MCAT/, C5-5/MCAT/GFPc7-3, C5-5/MCAT/mycGFPc2-3 and c2-4 cells were plated in 2 ml of culture media containing 4% BCS (per well) in 24 well plates. Cell cycle of all 4 cell lines was assayed on days indicated by propidium iodide staining of the DNA and then FACS analysis. Numbers indicate percent of cell population that is apoptotic or in "S phase" (apoptotic/S phase).
C5-5/MCAT/GFPc7-3) and 10 (3.4X over C5-5/MCAT and 3.2X over C5-5/MCAT/GFPc7-3). C5-5/MCAT/mycGFPc2-4 cells showed increases in apoptosis ranging from 1.2 - 11.9X compared to control cells. Increases ≥2X compared with control cells, in the apoptotic population, were observed on days 6, 8, 10, 11 and 15 (over C5-5/MCAT/GFPc7-3 only). The most notable increases occurred on days 6 (11.9X over C5-5/MCAT) and 10 (3.3X over C5-5/MCAT and 3.0X over C5-5/MCAT/GFPc7-3).

For both c-myc lines, increases in the "S phase" populations (compared to C5-5/MCAT and C5-5/MCAT/GFPc7-3 control lines) were, once again, not nearly as large as for the apoptotic populations. However, the increases were higher than those observed for cells cultured in 15% BCS. Increases for C5-5/MCAT/mycGFPc2-3 (compared with controls) ranged from 1.1 - 2.2X and were maximal on day 11. Increases for C5-5/MCAT/mycGFPc2-4 (compared with controls) ranged from 1.05 - 2.5X and were maximal on day 11.
CHAPTER 4: DISCUSSION
4.1. Cloning efficiency and soft agar assays are capable of detecting oncogenic cooperation with Bcl-2 at a frequency as low as 1:25,000

4.1.a. Assays used to detect oncogenic transformation

Historically, novel oncogenes were usually discovered by their ability to transform murine NIH 3T3 fibroblasts. The morphology and behaviour of 3T3 cells was observed following transfection of DNA from carcinomas, known viruses, cell lines or cloned genes (Blair et al., 1982; Blair et al., 1983; Müller and Müller, 1984; Wahrman et al., 1985). Properties such as focus formation, growth in soft agar, low serum and tumour formation in nude mice are routinely looked at as measures of transformation (Holley and Kieman, 1968; Heidelberger et al., 1973; Lu et al., 1997; Qiu et al., 1997). Major drawbacks of using the NIH 3T3 system are; 1) morphological observations are subjective, 2) a murine cell line may not be suitable for detection of human oncogenes, 3) suffers from high spontaneous transformation rate and 4) requires "one hit" for full transformation (Blair et al., 1982; Tainsky et al., 1987). Although introduction of cDNA expression libraries into the NIH 3T3 system have yielded novel oncogenes, this system presents the same limitations mentioned above (Chan et al., 1993; Whitehead et al., 1995; Whitehead et al., 1995; Chan et al., 1996). The genes discovered are only capable of transforming NIH 3T3 fibroblasts, which as mentioned previously only require one hit for transformation and therefore does not accurately depict the multistep nature of oncogenic cooperation in cancer development. Due to the fact that I am concerned primarily with human B-cell lymphoma, the fact that transformation of NIH 3T3 cells can be achieved by numerous different genes, most of which are not important in human
lymphoma, is also disconcerting. The system I have developed is superior to the standard NIH 3T3 system due to the fact that it is capable of detecting oncogenic cooperation of genes important in human B-cell lymphoma.

4.1.b. Sensitivity of transformation assays developed

To look at oncogenic cooperation in a lymphoma model system I made use of human B-cell lines that differed in the number of oncogenic "hits" they possessed. GM607/C5-5 overexpresses Bcl-2 and served as my non-transformed control. OCI LY8 c3, which overexpresses Bcl-2, c-myc and possesses a mutated p53 gene, served as our transformed control. In developing my assays I capitalized on strategies that have been shown previously to detect transformation; growth in low serum and soft agar. Normal untransformed cells require culture media to contain certain growth factors for survival and proliferation. Transformed cells, however, frequently possess the ability to survive in conditions in which their required factors are missing, due to resistance to apoptosis or an increase in proliferation. In addition, transformed cells are commonly capable of forming colonies on soft agar, whereas their non-transformed counterparts are not. Colony formation on soft agar is due to loss of contact inhibition and the ability for anchorage-independent growth (Smith and Scher, 1971; Hamburger and Salmon, 1977; Yuhas et al., 1978; Lu et al., 1997; Qiu et al., 1997). Due to these reports, I hypothesized that if I mixed non-transformed and transformed cells together and then subjected them to low serum or soft agar conditions, the non-transformed population would die off and we would select for the transformed cell population. The long term objective was to eventually introduce a human lymphoma cDNA expression library into a
Bcl-2 overexpressing cell line and then use the assays developed to detect a gene(s) that was capable of cooperation with Bcl-2. The premise of the assays developed was that a single transformed cell (possessing Bcl-2 and an oncogene that was capable of cooperation with Bcl-2, "x") would be able to be detected and isolated out of a non-transformed population (possessing Bcl-2 and a gene not capable of cooperation with Bcl-2). In both assays developed, two cell lines were mixed together at ratios of 1:250, 1:2,500 and 1:25,000 (transformed:non-transformed). These ratios were chosen because approximately 20,000 - 30,000 housekeeping genes are expressed in a given cell at a given time (Hess et al., 1998). I hypothesized that one of these genes is possibly capable of cooperation with Bcl-2. Therefore, the assays needed to be sensitive enough to detect one transformed cell (possessing Bcl-2 and oncogene "x") out of 25,000 non-transformed cells.

In the cloning efficiency assay, OCI LY8 c3 and GM607/C5-5 cells were mixed together at ratios of 1:250, 1:2,500 and 1:25,000 (respectively), plated at 10,000, 5,000 or 1,000 cells per well in 96 well plates in media containing 15%, 5%, 1% or 0.1% BCS. When the cells were plated at 10,000 or 5,000 cells per well multiple colonies were seen in every well for each serum condition by day 7 (data not shown). Due to the fact that multiple colonies were seen at an early time point in every well when the cells were plated at the 1:25,000 (OCI LY8 c3:GM607/C5-5) ratio these conditions were not chosen for further analysis. This is because in the 1:25,000 ratio only 0.4 OCI LY8 c3 cells were plated per well (i.e. 1 cell in every 2.5 wells) yet multiple colonies were observed in all 96 wells indicating that the colonies were not derived from OCI LY8 c3 but from GM607/C5-5.
When 1,000 cells were plated per well in normal (15% BCS) and slightly reduced (5% BCS) serum conditions, colonies were seen in every well for each plating ratio of the 96 well plates. When a proportion of these colonies were expanded and stained with anti-κ and anti-λ antibodies (surface molecules that differed between the two cell lines) and analyzed by FACS, I saw that all of the colonies formed consisted of both κ positive and λ positive cells (Figure 3.2). This indicates that under these conditions both GM607/C5-5 and OCI LY8 c3 cells survived and expanded. Therefore, when cells were plated at the 3 ratios in 15% BCS or 5% BCS I was not able to detect and isolate a single transformed cell (OCI LY8 c3) out of the non-transformed population (GM607/C5-5).

However, when the cells were plated at 1,000 cells per well in media containing 1% BCS this assay was able to identify transformed cells. Colonies were seen in 96 wells when the cells were plated at 1:250, 35 wells at 1:2,500 and 4 wells at 1:25,000 (OCI LY8 c3:GM607/C5-5). The number of wells containing colonies directly correlates with the actual number of OCI LY8 c3 cells plated for the 3 ratios (1:250 = 4 cells/well, 1:2,500 = 38.4 cells/plate and 1:25,000 = 3.84 cells/plate). When a proportion of these colonies were expanded and stained with anti-κ and anti-λ antibodies and analyzed by FACS I saw that all of the colonies formed consisted of λ positive cells only (Figure 3.2). This indicates that under this condition (plating in 1% BCS) only OCI LY8 c3 cells survived and expanded. Therefore, when cells are plated at 1,000 cells per well in media containing 1% BCS I was able to detect and isolate one transformed cell (OCI LY8 c3) out of a population of 250, 2,500 and 25,000 non-transformed cells.
At 0.1\% BCS, no cells survived indicating that even the OCI LY8 c3 cell line could not survive and expand at a very, very low serum level.

When the two cell lines were mixed together (at the same ratios as used in the cloning efficiency assay) and plated on soft agar, colonies formed in the agar for each of the plating ratios. When a proportion of these colonies were picked, expanded and stained with anti-\(\kappa\) and anti-\(\lambda\) antibodies it was found that all colonies consisted of \(\lambda\) positive (i.e. OCI LY8 c3) cells only (Figure 3.3). Therefore, using the soft agar assay I could detect and isolate one transformed cell out of 250, 2,500 and 25,000 non-transformed cells.

4.2. MCAT-1 makes GM607/C5-5 permissive to infection with murine retrovirus

The use of retroviral vectors to transfer and express genes has become increasingly popular due to the fact that they have the ability to efficiently infect a wide range of cell types and precisely integrate genetic material carried by the vector into recipient cells. In addition, there is lack of vector spread or production of viral proteins after infection and lack of toxicity of these viruses in infected cells. They also have the potential to be used as vectors for introduction of cDNA libraries. This technique entails construction of a cDNA library in a retroviral vector, followed by transfection of the DNA into retroviral packaging cells to generate virus carrying the inserted cDNAs. Target cells are then transduced with the resultant virus. The advantage of using retroviral vectors in
an application such as this is that cells which are difficult to transfect can become efficient recipients of the library (Miller, 1992).

To study oncogenic cooperation with Bcl-2 I sought to infect GM607/C5-5, a human, mature B-cell line, with a cDNA expression library. However, due to the fact that GM607/C5-5 is a human cell line there were several concerns associated with the protocol. The first has to do with safety. The cDNA expression library was going to be derived from a human lymphoma sample it was obviously going to contain oncogenes. My concern centered around the fact that we were going to be putting oncogenes into a vector system that was infectable to human cells (i.e. infectable to the person performing the transduction). The second concern pertains to the efficiency of human packaging cell lines. Human packaging cell lines have a much lower efficiency compared with murine packaging cells, with GP+E-86 being the least likely to produce helper virus (Miller, 1992).

Therefore, to circumvent both of these problems we decided to make GM607/C5-5 permissive to infection with a murine retrovirus. To accomplish this we transfected GM607/C5-5 with MCAT-1 cDNA. MCAT-1 (mouse cationic amino acid transporter) was derived from NIH 3T3 cells and encodes a multiple membrane-spanning protein and confers susceptibility to virus infection (Albritton et al., 1989). Transfection experiments showed that MCAT-1 expression makes non-permissive cells susceptible to ecotropic retroviruses (Closs et al., 1993; Ott et al., 1998).

Here I show that introduction of MCAT-1 cDNA into GM607/C5-5 cells allows these cells, C5-5/MCAT, to be transduced by the ecotropic retroviral vectors pBabeIresMNGFP and pBabeIresMNhuc-mycGFP. After transfection of
MCAT-1, the resulting bulk cultures and clones were infected with supernatant from GP+E-86/GFP and GP+E-86/mycGFP cells. Analysis of GFP and c-myc expression revealed that, in agreement with the work done by others, introduction of MCAT-1 cDNA into GM607/C5-5 made them permissive to infection by ecotropic retrovirus (Figures 3.8 and 3.10).

4.3. GFP expression is an efficient indicator of gene expression

GFP, from the jellyfish *Aequorea victoria*, has become an important marker of gene expression. As it requires no other substrates or cofactors to fluoresce, it is superior to most other reporter systems (Kain et al., 1995). Long-term, stable expression of GFP in mammalian cells has been reported by various groups (Cheng et al., 1996; Gubin et al., 1997; Persons et al., 1997). Therefore, when I sought to introduce c-myc into our C5-5/MCAT cells GFP appeared to be an excellent choice for monitoring c-myc overexpression.

Two retroviral vectors were used to transduce C5-5/MCAT; pBabeIresMNhuc-mycGFP, which contains c-myc cDNA and a GFP cassette and pBabeIresMNGFP, which serves as a vector control containing the GFP cassette only. Transfection experiments resulted in two cell lines: the first, GP+E-86/GFP, packages and secretes murine retrovirus containing a GFP cassette, while the second line, GP+E-86/mycGFP, packages and secretes murine retrovirus containing both c-myc cDNA and a GFP cassette. GFP expression of GP+E-86/GFP and GP+E-86/mycGFP was assessed shortly after transfection of the plasmids, high GFP expressers were FACS sorted out and then these sorted populations were cloned by limiting dilution. GFP expression was assessed once
again, and one representative clone for each line, GP+E-86/GFPc7 and GP+E-86/mycGFPc2, were chosen, based upon high and uniform GFP expression, to use in future experiments (Figures 3.6 and 3.7). Although these cells expressed high levels of GFP, which should be indicative of c-myc expression, we wanted to confirm this. c-myc expression was then confirmed by staining the cells with anti-c-myc and analyzing them by FACS. Karpas and OCI LY8 c3, which have previously been shown to overexpress c-myc (Farrugia, 1993), were used as positive controls. Figure 3.10.a. shows that GP+E-86/mycGFPc2 markedly overexpresses c-myc (higher levels than both positive controls) while the vector control line, GP+E-86/GFPc7, does not. Therefore, GFP expression was indicative of efficient vector transduction and gene expression.

Similar results were observed when C5-5/MCAT cells were transduced with supernatant from GP+E-86/GFP or GP+E-86/mycGFP cells (Figures 3.8 and 3.9 and Table V). While all of the cells expressed high levels of GFP and cells that were infected with the mycGFP retroviral vector biologically demonstrated c-myc expression we decided to confirm c-myc expression by FACS. Figure 3.10.b shows that the c-myc lines, C5-5/MCAT/mycGFP bulk, C5-5/MCAT/mycGFPc2-3 and c2-4, all overexpress c-myc while the control lines, C5-5/MCAT and C5-5/MCAT/GFP bulk do not express c-myc at all. Once again, GFP expression was indicative of efficient vector transduction and gene expression.

An important factor to consider when using any reporter system is whether the system changes the growth characteristics of the cells. Introduction of GFP into the cells did not confer any detrimental or enhanced growth characteristics as illustrated by the growth curve and cell cycle assays. C5-
5/MCAT/GFPc7-3 did not demonstrate differences in doubling time, proliferation or apoptosis compared to the parental line, C5-5/MCAT, when cultured in 15% BCS or 4% BCS (Figures 3.11, 3.12, 3.15 and 3.16). This is in agreement with other groups that report that stable GFP expression does not interfere with the biological properties of their cells (Bierhuizen et al., 1997; Cheng et al., 1996; Gubin et al., 1997).

4.4. Full cellular transformation requires multiple genetic alterations that cooperate with Bcl-2 overexpression

Although two assays were developed, capable of detecting transformed phenotypes at a frequency as low as 1:25,000, I had developed them using a transformed cell line that had more than two "hits" in it. OCI LY8 c3 not only overexpresses Bcl-2 and c-myc but also possesses a mutated p53 gene. Therefore there are at least 3 genes (and possibly more that have not been identified yet) that are cooperating with each other to give this cell line its ability to survive and proliferate at low densities, in reduced serum conditions and to form colonies in soft agar. However, when introducing a cDNA expression library into the GM607/C5-5 cell line I was only going to be looking for one gene that can cooperate with Bcl-2, resulting in a transformed phenotype. Consequently, OCI LY8 c3 is not a relevant "transformed" control to be used in these assays. In order to ensure that the assays are capable of detecting transformed cells resulting from "two hits" I generated a new transformed control for these assays.

I repeated the assays that were developed using OCI LY8 c3 and GM607/C5-5 with C5-5/MCAT/mycGFP, which overexpresses both Bcl-2 and c-
*myc*, and its corresponding control, C5-5/MCAT/GFP, which only overexpresses Bcl-2.

When the cloning efficiency and soft agar assays were repeated using these new cell lines the results obtained were very unexpected and strikingly different than the results obtained using OCI LY8 c3 as the transformed cell line. In both assays, for all of the different plating conditions, no colonies were observed (Tables VI and VII). Although the tumourigenicity assay was not performed using the OCI LY8 c3 and GM60/C5-5 cell lines, I, with assistance from Dr. B. Guinn, decided to see if our Bcl-2/c-*myc* overexpressing lines could form tumours in nude mice. None of the 4 cell lines injected, C5-5/MCAT, C5-5/MCAT/GFPc7-3, C5-5/MCAT/mycGFPc2-3 and c2-4, resulted in tumour formation (Table VIII). Taken together, these results indicate that the oncogenic cooperation between Bcl-2 and c-*myc* was not sufficient to transform the cells and give them enough of a growth advantage to survive and expand when plated at low density in reduced serum conditions, in soft agar or form tumours.

The results from the cloning efficiency assay were very surprising due to the fact that numerous groups have demonstrated the cooperative effects that overexpressing both c-*myc* and Bcl-2 have on growth kinetics. Overexpression of c-*myc* induces an increase in both cellular proliferation and death. In resting or terminally differentiated cells c-*myc* mRNA and protein are normally undetectable. Upon stimulation of quiescent G0 cells, c-*myc* expression is rapidly up-regulated with peak expression occurring about 3 hrs after stimulation driving cells into "S phase" of the cell cycle. c-*myc* expression then declines to a level that is maintained throughout the rest of the cell cycle (Waters et al., 1991). In addition to c-*myc*'s proliferative role, deregulated c-*myc* expression also
induces apoptosis in cells that are deprived of growth factors (e.g. serum) (Askew et al., 1991; Evan et al., 1991). However, when Bcl-2 is overexpressed in the same cells that have deregulated c-myc expression, c-myc induced apoptosis is prevented (Bissonnette et al., 1992; Fanidi et al., 1992; Wagner et al., 1993). Due to these previous findings we expected that C5-5/MCAT/mycGFPc2-3 and c2-4 cells would have high proliferation rates and low levels of apoptosis (and therefore be detected) when plated in low serum conditions in 96 well plates. A possible reason for these cells not surviving or proliferating may be due to the fact that the cells were plated in very low densities, ranging from 4 cells/well to 4 cells/plate. Perhaps under these conditions, even cells overexpressing both Bcl-2 and c-myc, could not survive. Another feasible explanation is that Bcl-2 overexpression did not completely abrogate c-myc-induced apoptosis. Therefore, due to the fact that the cells were plated at such a low density, increased amounts of apoptosis may have annihilated the Bcl-2/c-myc population before it had a chance to increase its numbers by c-myc-induced proliferation.

Lack of colony formation in the soft agar assay was not entirely unexpected. Previous studies have shown that Bcl-2/c-myc cooperation does not necessarily result in anchorage independent growth. Vaux et al. found that while Bcl-2 and c-myc overexpression in B-cells from Eμ-myc transgenic mice could form colonies in soft agar, these colonies were not viable when transferred to liquid culture (Vaux et al, 1988). Moreover, when Screaton et al. plated L6 cells transfected with both Bcl-2 and v-myc on soft agar they did not see colony formation above what cells expressing v-myc alone accomplished (which in itself was not significant) (Screaton et al., 1997).
In the tumourigenicity assay, we did not see any tumour formation after 80 days. These results are not consistent with what has been reported by others. When c-myc and Bcl-2 were co-overexpressed in a variety of different cell lines and then injected into mice, syngeneic and nude, tumour formation was seen after 7 weeks (Vaux et al., 1988). When v-myc and Bcl-2 were co-overexpressed in L6 cells and injected into nude mice, tumour formation was seen at 10 weeks (Screaton et al., 1997). Furthermore, N-myc/Bcl-2 double transgenic mice developed tumours with a mean latency period of 64 days, while c-myc/Bcl-2 double transgenics formed tumours after a latency of 3 weeks or 5-6 weeks, depending on the study (Marin et al., 1995; Zörnig et al., 1995; Strasser et al., 1996). The discrepancy between our results and those of others with respect to tumour formation in nude mice may be explained using the same rationalization used for the cloning efficiency assay results. Specifically, Bcl-2 does not totally abrogate c-myc-induced apoptosis. Hence, shortly after the cells were injected into the mice a proportion of them died, thereby reducing the total cell number to a level that could not support tumour formation. Another factor to consider is the latency times observed prior to tumour formation. While it is possible that Bcl-2 and myc are the only two genes contributing to tumour formation in these studies, it is also possible that during the latency period additional genetic alterations are acquired that cooperate with both Bcl-2 and myc. In addition, the oncogenic status of the cell lines used in the other studies is not known for certain. Although one would assume that the only genetic abnormalities present in the cells are the ones that the researchers introduced themselves, it cannot be discounted that there were pre-existing abnormalities. Therefore, additional oncogenes introduced could cooperate with the pre-existing ones resulting in
tumour formation. Finally, it is not known how the expression levels of Bcl-2 and myc in cell lines used in other studies compared to the levels in our own cells. It is conceivable that the levels of Bcl-2, myc or both could be lower in our cell line and below a threshold amount needed for tumour formation.

Although the cloning efficiency, soft agar, and tumourigenicity assays were not capable of detecting oncogenic cooperation between Bcl-2 and c-myc, I inadvertently developed one assay that did. Non-transformed cells maintain a balance between their proliferation and apoptotic rates resulting in homeostasis. One hallmark of transformed cells is a net increase in proliferation resulting from either an increase in cellular proliferation, a decrease in cell death or a combination of both (Hsu et al., 1995). Long-term culture in normal non-selective conditions appears to exploit the imbalance of proliferation and apoptosis in cells overexpressing both Bcl-2 and c-myc. When C5-5/MCAT cells were transduced with the supernatant from GP+E-86/mycGFP cells the level of GFP expression was initially undetectable. Over the next 94 days however, GFP expression became not only detectable but, actually rose consistently while that of the control cells, C5-5/MCAT/GFP, remained stable (Figures 3.8 and 3.9 and TableV). The fact that GFP expression of these cells, C5-5/MCAT/mycGFP bulk #1 and #2, increased over time in normal non-selective culture conditions until 100% of the population expressed GFP, strongly indicates that the c-myc introduced is biologically active and that there may be oncogenic cooperation between Bcl-2 and c-myc. This is due to the fact that the only difference between C5-5/MCAT/myc GFP cells and C5-5/MCAT/GFP cells is overexpression of c-myc.
Taken together, these results strongly suggest that cooperation between Bcl-2 and c-myc is not powerful enough to be detected using the cloning efficiency, soft agar and tumourgenicity assays. However, these transformation assays can be extremely useful in the context of multiple genetic alterations (e.g. OCI LY8 c3) where the cumulative cooperation is detectable at a frequency as low as 1:25,000.

4.5. c-myc-induced apoptosis is not fully blocked by overexpression of Bcl-2

The results obtained from the transformation assays prompted the investigation of the growth characteristics of the Bcl-2/c-myc overexpressing cell lines.

The growth curve assay performed in the presence of 15% BCS revealed essentially no differences in growth rates between the parental (C5-5/MCAT), vector control (C5-5/MCAT/GFPc7-3) and Bcl-2/c-myc (C5-5/MCAT/mycGFPc2-3 and c2-4) cell lines. The Bcl-2/c-myc lines reached lower saturation densities compared with the parental and vector control lines, but they did not display enhanced growth characteristics, such as reduced doubling time (Figure 3.11). Percent viability calculations did reveal however, that after day 7, the two Bcl-2/c-myc lines had markedly increased numbers of dead cells compared to both control lines (Figure 3.13 and Table IX). Therefore, although Bcl-2/c-myc cells had comparable numbers of viable cells they had far more dead cells (compared to controls) which resulted in a reduced percent viability. Cell cycle analysis revealed that although both Bcl-2/c-myc lines displayed slightly increased levels of "S phase" cells, they also possessed markedly higher apoptotic
populations (Figure 3.15). These results indicate that although the Bcl-2/c-myc cell lines did not demonstrate an increased overall growth potential, compared to parental and vector controls, this was due to an apparent balance between an increased proliferation rate and an increase in apoptosis. The proliferation rates, suggested by the proportion of cells in S phase, were slightly higher in the Bcl-2/c-myc cells compared to the control cells. However, the large degree of apoptosis in both of the Bcl-2/c-myc cell lines probably nullified the increase in proliferation, resulting in no noticeable growth advantages such as reduced doubling times, increased percent viability or increased saturation densities.

Interestingly, the growth curve assay performed in 4% BCS did unveil differences in growth characteristics between the Bcl-2/c-myc and control cell lines. Cell counts taken on days 4 - 6 demonstrated an increased growth rate in the Bcl-2/c-myc cells, especially the c2-4 line with a doubling time of 18 hrs, compared with both control lines (doubling time of C5-5/MCAT = 36 hrs, C5-5/MCAT/GFPc7-3 = 34.5 hrs). Although Bcl-2/c-myc cells cultured in 4% BCS did show an increased growth potential, it was only evident for 3 days (until day 6) and then the cell numbers quickly decreased (Figure 3.12). As seen with cells cultured in 15% BCS, percent viability calculations demonstrated that after day 7, the Bcl-2/c-myc cell lines had a much larger number of non-viable cells, as determined by trypan blue exclusion, compared with both control lines (Figure 3.14 and Table X). Once again, the decrease in percent viability observed in the Bcl-2/c-myc cells was not due to a reduction in the number of viable cells, but due to an increase in the number of dead cells (compared to both control lines). As seen in the cell cycle assay, performed on cells grown in the presence of 15% BCS, Bcl-2/c-myc cells cultured in the presence of 4% BCS also had a slight
increase in the proportion of "S phase" cells and a considerably higher amount of apoptotic cells when compared with both control lines (Figure 3.16). Taken together, these results suggest that although the Bcl-2/c-myc cell lines cultured in 4% BCS had a slight growth advantage, compared to parental and vector control cell lines, it would have been far more significant if the Bcl-2/c-myc lines had had lower degrees of apoptosis. If Bcl-2 had completely abrogated c-myc-induced apoptosis, I would have seen a striking increase in the growth rate of the Bcl-2/c-myc lines compared with the control lines.

Interestingly, when levels of proliferation (as measured by the proportion of cells in "S phase") and apoptosis in Bcl-2/c-myc cells are compared between said lines cultured in 15% BCS vs. 4% BCS, those cultured in the reduced serum condition showed higher levels of proliferation and lower levels of apoptosis. Perhaps this explains why a growth advantage was seen in Bcl-2/c-myc cells cultured in 4% BCS but not 15% BCS. A possible explanation for this observed phenomenon is that reduced serum conditions may initially increase endogenous levels of Bcl-2 and c-myc expression resulting in increased proliferation and decreased cell death. This initial increase in endogenous Bcl-2 and c-myc levels would not be seen in cells cultured in 15% BCS because it is not a limiting condition.

The result of the growth curve assay does not appear to correlate with that of other groups, which tend to see larger growth advantages in cells expressing Bcl-2 and c-myc (Vaux et al., 1988; Screaton et al., 1997). However, the results of the cell cycle assays are in agreement with reports of others. Although most groups report that overexpression of Bcl-2 completely suppresses c-myc-induced apoptosis, while not interfering with its mitogenic effect, some of their data does
not support that conclusion. Wagner et al. cultured Rat 1a fibroblasts in 0% serum media for 48 hrs and then stained the DNA with PI. Cell cycle analysis revealed the following apoptosis levels: parental Rat 1a cells = 12.8%, Rat 1a myc/neo = 47% and Rat 1a myc/bcl-2 = 27.1%. Although Bcl-2 decreased the amount of myc-induced apoptosis (from 47% to 27.1%) it did not completely block it since the level of apoptosis in the myc/bcl-2 cells is still significantly higher than in the parental control (12.8%) (Wagner et al., 1993). This result of "partial blockage" of c-myc-induced apoptosis by Bcl-2 is essentially what I saw in the model developed.

There have been some reports, using a variety of models, that have shown that Bcl-2 cannot block myc-induced apoptosis, albeit not as many that say it does. Trudel et al. show c-myc-induced apoptosis is Bcl-2 (and p53) independent in polycystic kidney disease. They demonstrate that overexpression of both Bcl-2 and c-myc in double transgenic mice produce a similar phenotype, with a high apoptotic rate, compared with c-myc single transgenics, indicating that c-myc can bypass Bcl-2 in vivo (Trudel et al., 1997). Another group demonstrated that overexpression of Bcl-2 and Bcl-xL in IL-3-dependent 32D cells could protect the cells from apoptosis after IL-3 withdrawal or drug exposure. However, in some cases, this protective effect could be overridden by overexpression of myc oncoproteins, especially N-myc and L-myc, but including c-myc (Nesbit et al., 1998).

A combination of results from other research groups may provide a potential explanation as to why myc-induced apoptosis is not, under all circumstances, blocked by overexpression of Bcl-2. Chen et al. found that expression of the Deleted in Colorectal Cancer gene (DCC) in tumour cells
activates caspase-3 and results in apoptosis. DCC-induced apoptosis was not abrogated when Bcl-2 was overexpressed in these cells (Chen et al., 1999). Another study provides evidence that, like DCC, c-myc activates caspase-3. Kangas et al. reports that when c-myc is overexpressed Rat 1a cells they see cleavage of poly(ADP-ribose) polymerase (PARP), which is known to be cleaved by caspase-3. In addition, it is reported that c-myc overexpression results in caspase-3 being processed from its inactive to active form (Kagaya et al., 1997; Kangas et al., 1998). Perhaps, as in the DCC study, apoptosis induced by activation of caspase-3 is independent and not preventable by overexpression of Bcl-2. Evidence supporting this theory comes from reports of caspase-3 possessing the ability to cleave Bcl-2. One group found that apoptosis in M-07e cells was associated with a gradual cleavage of Bcl-2 into a 22 kDa fragment. When the rate of Bcl-2 cleavage was increased (with the addition of a reversible ubiquitin-proteasome inhibitor) the rate of apoptosis was also found to increase. It was also found that cleavage of Bcl-2 could be blocked with the addition of a caspase-3 specific inhibitor (Zhang et al., 1999). These results taken together indicate that caspase-3 (or other downstream caspases) was responsible for the cleavage of Bcl-2 which, in turn, was responsible for apoptosis. Fujita and Tsuruo also demonstrated that caspase-3 cleaves Bcl-2. They used VP-16 (an anti-cancer drug) to induce apoptosis in U937 cells. Their results show that caspase-3 was activated in the U937 cells after VP-16 treatment and that caspase-3 was responsible for the direct cleavage of Bcl-2 protein. In fact, overexpression of the cleaved Bcl-2 fragment increased the sensitivity of the cells to VP-16 and actually promoted cell death (Fujita and Tsuruo, 1998). This phenomenon was also seen in another experimental system in which it was found that deletion of
Bcl-2’s NH₂-terminal BH4 homology domain, which is required for its anti-apoptotic activity, by caspase-3 not only inactivates the anti-apoptotic function of Bcl-2 but also releases a pro-apoptotic fragment. It was also shown that cleavage of Bcl-2 by caspases activates the pro-apoptotic activity of the BH3 domain (Cheng et al., 1997).

Taken together, these results suggest that it is possible that overexpression of c-myc activates caspase-3 which, in turn, cleaves Bcl-2. Cleavage of Bcl-2 either voids its anti-apoptotic function or may, in fact, convert it from an anti-apoptotic to a pro-apoptotic molecule resulting in accelerated cell death.

4.6. Proposed model of oncogenic cooperation between Bcl-2 and c-myc

During the course of these experiments, I have observed different behaviours of Bcl-2/myc cells depending on which system they are placed in, mainly: 1) in normal culture conditions where serum is not limiting and 2) in the cloning efficiency assay in which serum is limiting.

When the Bcl-2/myc cells are placed in normal culture conditions, where the media is replenished on a regular basis, c-myc induces slightly increased proliferation and apoptosis rates compared to control cells. Bcl-2 is likely capable of partially blocking this level of c-myc-induced apoptosis while not interfering with its mitogenic function. Therefore, what is seen over an extended period of time is an outgrowth of these cells that eventually takeover the entire population. Even though there is still a small amount of apoptosis, the increase in proliferation is enough to favour a net increase in growth rate.
However, when Bcl-2/myc cells are placed in a system in which low serum is a limiting factor, c-myc induces much larger increases in both proliferation and apoptosis. Interestingly, it seems that this level of apoptosis overrides Bcl-2's anti-apoptotic function. So, even though c-myc induces a large increase in both apoptosis and proliferation, this amount of apoptosis cannot be blocked and the end result is a net decrease in growth rate with the majority of cells eventually dying. This explains why I did not see an outgrowth of Bcl-2/myc cells in the cloning efficiency assay. Perhaps there is a threshold amount of apoptosis that Bcl-2 is capable of blocking and anything above this threshold amount becomes independent of Bcl-2. In limiting serum conditions, c-myc might send an “apoptotic signal” to the cell that bypasses the usual mitochondria/cytochrome c/caspase pathway that Bcl-2 is capable of blocking. Perhaps c-myc sends a signal that activates caspase-3 directly, which would bypass cytochrome c release, the step at which Bcl-2 is thought to act. Once caspase-3 is activated, it in turn can cleave Bcl-2, abolishing its anti-apoptotic function and potentially converting it into a pro-apoptotic molecule. In either case, without Bcl-2 to block c-myc-induced apoptosis, these cells are committed to die.

In conclusion, I have demonstrated that there is cooperation between Bcl-2 and c-myc that results in subtly increased growth rates. However, this cooperation is not enough to be detected by the transformation assays developed. What is required in this system, for detection of full transformation, is an additional genetic alteration that can either a) block c-myc-induced apoptosis without affecting its mitogenic function, b) induce proliferation
without a concomitant induction of apoptosis or c) confer anchorage-independent growth.

4.7. Conclusions

In conclusion, I have developed a novel system for detecting oncogenic cooperation in lymphoma. The two transformation assays, cloning efficiency and soft agar, are capable of detecting oncogenic cooperation at a sensitivity level as low as 1:25,000. However, the amount of cooperation that has to be present for detection of transformation remains in question. Transformation could be detected when a cell line with 3 (possibly more) genetic alterations was used but not when a line with only 2 alterations was used as our transformed control. Although the cell line overexpressing both Bcl-2 and c-myc demonstrated increased growth capabilities, the oncogenic cooperation present was not enough to transform the cells in our model system. Therefore, although c-myc overexpression is a common secondary event in the development of B-cell lymphoma it may not be enough to cause its progression. Additional genetic lesions that can cooperate with Bcl-2 and c-myc overexpression likely need to be acquired for full transformation of B-cells and progression of B-cell lymphoma. Due to the fact that increased apoptosis in the cell lines appeared to be the limiting factor, perhaps a gene whose product has control over apoptosis, such as p53, needs to be mutated in conjunction with Bcl-2 and c-myc overexpression for full transformation to occur.

In addition, I have developed a two new cell lines, C5-5/MCAT and C5-5/MCAT/mycGFP, that could prove to be exceptionally valuable in studying the
effects of known oncogenes or in detecting novel oncogenes that cooperate with Bcl-2 or Bcl-2 and c-myc. Expression of MCAT-1 on these cells provides a very safe, easy and efficient system for gene transfer. Due to the fact that these cells are human and possess a known number of oncogenic alterations (overexpression of Bcl-2 and overexpression of Bcl-2 and c-myc, respectively) they provide a physiological system for studying the effects of human oncogenes in various experimental conditions (versus a murine system, e.g. NIH 3T3).

Discovering novel oncogenes that play a role in the development and progression of B-cell lymphoma is crucial. Understanding the molecular mechanisms of the lymphoma will allow for new therapies to be developed that are based on the biological action of the neoplasm itself.

4.8. Future directions

The experimental system we developed could be very useful in studying oncogenic cooperation in the pathogenesis of human B-cell lymphoma. Although I did not detect cooperation between Bcl-2 and c-myc using our system, I now have a cell line that possesses two oncogenic abnormalities that have been shown to be very important in human B-cell lymphoma. This cell line, C5-5/MCAT/mycGFPc2-4, and the transformation assays, can now be used to detect novel oncogenes that are involved in the progression of B-cell transformation. Due to the fact that these cells express MCAT-1 they are permissive to infection with murine retrovirus, therefore a cDNA expression library derived from a B-cell lymphoma could be cloned into a murine retroviral vector and transfected into a ecotropic packaging cell line, such as GP+E-86. The
resulting supernatant would be used to transduce the C5-5/MCAT/mycGFPc2-4 cell line safely and efficiently. This infected population would then be plated in the cloning efficiency and soft agar assays and cells possessing a gene capable of cooperating with Bcl-2 and c-myc could be detected and isolated.

It is known that cells possessing even one oncogenic abnormality are more susceptible to acquiring additional genetic lesions compared with cells that are genetically "normal". Therefore, another method for assessing additional oncogenic events would be to culture C5-5/MCAT/mycGFPc2-4 cells in non-selective media for a long period of time (6 - 12 months) and then use differential display to compare gene expression at time 0 and 12 months later. Considering that only genetic changes that are favourable and advantageous will be selected for over time, the resulting population would possess changes that would presumably cooperate with Bcl-2 and c-myc.
REFERENCES


