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ONCOGENE COOPERATION AND CELLULAR DIFFERENTIATION IN THE IN VIVO AND IN VITRO TRANSFORMATION OF MURINE PRE-B CELLS

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ONCOGENE COOPERATION AND CELLULAR DIFFERENTIATION IN THE *IN VIVO* AND *IN VITRO* TRANSFORMATION OF MURINE PRE-B CELLS

By

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ABSTRACT

ONCOGENE COOPERATION AND CELLULAR DIFFERENTIATION IN THE *IN VIVO* AND *IN VITRO* TRANSFORMATION OF MURINE PRE-B CELLS

By

Shu-Chih Chen

An *in vivo* and an *in vitro* model system were used to study oncogene cooperation which presumably occurs in multi-step tumorigenesis. Both model systems involved transforming murine B cells using the Whitlock and Witte culture system in combination with tumor challenge. In the *in vivo* model system, c-*myc* gene activation was found in tumors derived from a v-Ha-*ras* cell line. Two tumors possessed a MoMuLV provirus integration immediately upstream and in a reverse transcriptional orientation to c–*myc*. Elevated expression of c-*myc* was found in these two tumors and another two tumors with no gross gene alteration. This finding parallels the synergy of v–Ha–*ras* and v–*myc* observed in the *in vitro* transformation of murine B lymphoid cells and validates synergy as a model for *in vivo* tumor progression. The insertional activation of the c-*myc* gene by MoMuLV in B cell lymphomas is novel. The flanking region, *bonc*-1, of one of these non-c-*myc* tumor-specific viral integration sites was characterized. Sequence homology to this locus was found in other mammals, and chicken.

In the *in vitro* model system, IL-7, a pre-B cell growth factor, was found to be incapable of cooperating with the v-Ha-*ras* oncogene in inducing a fully transformed phenotype in murine B cells. A discrepancy between the oncogene cooperation in a co-infection procedure and a sequential addition procedure was found. The clonal nature of the cell lines generated from the coinfection procedure suggests the selection of additional oncogenic events. The oncogenic potential of IL-7 expression, in itself, and those of other genes are probably best assessed in well-characterized individual cell lines.

A "lineage switching" from v-Ha-*ras* transformed pre-B cells to "macrophage-like" cells was also found in this study. These pre-B cells have gained the capacity to effectively present antigen in an MHC-restricted fashion. These cells have also rearranged their kappa light chain immunoglobulin locus, suggesting that macrophage differentiation and immunoglobulin rearrangement are not mutually exclusive processes. The existence of both lymphoid and myeloid characteristics in a cell suggests greater plasticity in hematopoietic lineage commitment than conventionally thought to be the case.

TO MY MOM AND DAD

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Last, but not least, I want to thank my parents Mr. S.C. Chen and Mrs. H.F. Tsai, my sister H.H. Chen, my brothers Y.S. Chen and Y.Y. Chen for their endless love and support. Thanks are to all my friends, especially H.-C. Li, in East Lansing, too. With them, life has been delightful for the past five years.

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Introduction

The focus of my thesis has been the identification of putative oncogenes that can cooperate with v-Ha-*ras* in tumor progression or in the *in vitro* transformation of murine B lymphoid cells. Two model systems involving the use of a long term bone marrow culture (Whitlock and Witte, 1982) were used in my studies.

The first model system is derived from the study of Schwartz and coworkers (1986a). Using a murine long term bone marrow culture system, Schwartz et al. (1986a) were able to show that v-Ha-*ras* and v-*myc* have a synergistic effect in transforming murine pre-B cells. In their study, pre-B cell lines carrying both oncogenes were capable of causing lymphoma in syngeneic mice at high frequency and with a short latency, whereas pre-B cell lines carrying v-Ha-*ras* alone only gave rise to tumors occasionally and with a prolonged latency. The latter result indicates that v-Ha-*ras* is not sufficient to cause pre-B cell lymphomas and other secondary events are required to facilitate tumor formation. It is this finding that prompted us to search for these secondary events by the following approach. First, Whitlock-Witte high density bone marrow was infected with a Moloney murine leukemia virus (MoMuLV) based vector carrying a v-Ha-*ras* oncogene with MoMuLV as a helper virus. Usually pre-B cell cells with a single specific v-Ha-*ras* integration were established. This *in vitro* transformation step presumably facilitates multi-step tumorigenesis and provides a molecular marker for further studies. Secondly, these pre-B cell lines were subjected to tumor

challenges, and they gave rise to tumors occasionally. This step provided the opportunity to accumulate other mutations which may cooperate with v-Ha-*ras in vivo* in tumorigenesis. The involvement of secondary events was examined by southern and northern hybridization analyses with various probes of known oncogenes and growth-related genes. Nearly thirty DNA probes (as listed in the Appendix B) of oncogenes, tumor suppressor genes, growth factor genes, or the flanking region of frequent viral integration sites were used to screen for gene rearrangements.

The second model system involved the introduction of v-Ha-*ras* with or without other oncogenes or genes with oncogenic potential into Whitlock and Witte high density bone marrow culture. In this model system, the transforming effect of each gene was studied directly. This model system involves less labor than the first model, although it suffers the shortcoming of all *in vitro* transforming systems, the lack of interactions with the full range of *in vivo* cell types. It is worth mentioning that the transformation potential of any oncogene identified from the first model can be easily tested in this system. A pre-B cell growth factor, interleukin-7 (IL-7), was chosen to study.

In the first chapter of this thesis, I will summarize briefly the methodologies that have been used to identify putative oncogenes that may be involved in tumorigenesis, and to test the oncogenic potential of these putative oncogenes. This is followed by a description of the known involvement of oncogenes and other molecular events in human B cell neoplasia as well as B cell tumors of other animals.

The results of studies on oncogene cooperativity between v-Ha-*ras* and other oncogenes in the *in vivo* and *in vitro* transformation of murine B cells will be presented in Chapters 2, 3, and 4 in this thesis. Chapter 2 details a c-*myc* activation found in the *in vivo* tumor progression of some of the B cell tumors obtained from the first model. This work has been published and will be presented as a manuscript. Data that were not shown in the manuscript because of space limitations will be included in the Appendix B.

Chapter 3 describes the isolation and characterization of a viral integration flanking region common to tumor cell lines. That locus may encode a putative oncogene. This work involves a collaboration with Marge Strobel and Nancy Jenkins (NCI) who did the chromosomal mapping, and will be presented as a manuscript and submitted for publication at a later date.

Chapter 4 reports the effects of IL-7 in cooperation with v-Ha-*ras* in transforming mouse pre-B cells with the second model system. It will be also presented as a manuscript and will soon be submitted to Molecular and Cellular Biology.

In examining the transformed phenotypes of pre-B tumor cell lines, at least two independent tumor cell lines showed an interesting lineage switch phenomenon, in which macrophage specific characteristics were found. Since lineage infidelity has also been described in a minority of hematological malignancies (McCulloch, 1983), we decided to further characterize these two lines with the hope that some correlations can be drawn to the clinical cases, and to provide some insights on lineage determination in hematopoiesis. This work includes molecular, cytological, and functional analysis of the lineage switched

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lines. I had made the discovery of the morphological changes of these tumor cell lines and subsequently subcloned one of these tumor cell lines, the T4 cell lines. I also proved that the T4 subclones were indeed descendants of their parental pre-B cell line, the R2 cell line, and did some of the cytochemical and immunocytological staining analyses on the T4 subclones. The demonstration of macrophage specific gene expression of T4 cells and kappa chain rearrangement of T4 subclones were also performed by me. Other portions of this work were contributed by the other authors listed on this manuscript. This work is in press in Developmental Immunology. I will present it as the submitted manuscript in Appendix A.

At the end, a section of summary and discussion has been included to cover points that can be integrated from all of the above work but were not specified in each individual chapter.

Reference:

McCulloch E.A. (1983). Stem cells in normal and leukemic hemopoeisis. Blood 62, 1-13.

Schwartz R.C., L.W. Stanton, S.C. Riley, K.B. Marcu, and O.N. Witte. (1986a). Synergism of v-*myc* and v-Ha-*ras* in the *in vitro* neoplastic progression of murine lymphoid cells. Mol. Cell. Biol. 6, 3221-3231.

Whitlock C.A., O.N. Witte. (1982). Long-term culture of B lymphocytes and their precursors from murine bone marrow. Proc. Natl. Acad. Sci. USA 79, 3608-3612.

Chapter 1
Literature review

In this chapter, evidence in support of the multi-step nature of tumorigenesis will be described first. It is followed by a brief overview of methodologies used in the identification of oncogenes and the evaluation of their oncogenic potential. Comparison of the use of *in vivo* versus *in vitro* model systems, and animal versus human systems is included in this section. Then, I will discuss in detail the literature on oncogenes known to be involved in the formation of B lymphoid tumors. The discussion will encompass oncogene activation observed in tumors of human and other animals, as well as in *in vitro* transformation of B cells.

1. Tumorigenesis is a multiple-step process

Cancer cells differ from their normal counterparts in that they no longer respond to normal growth controlling mechanisms. Since the proliferation and differentiation of somatic cells in higher organisms are regulated by multiple controls, cancer cells probably are the end result of multiple changes which may take years to develop. Several lines of evidence confirmed that the natural history of spontaneously occurring human and animal cancers is usually a multi-step process.

First, statistical analyses have shown a rapid increase in the incidence of cancer with age among most of the important human cancers (Fisher and Holloman, 1951; Armitage and Doll, 1954; Dix, 1989). Results of these analyses suggest that multiple events may accumulate with time, and thus are responsible for the increased cancer rates in old age. Peto et al., (1975) further demonstrated

that the intrinsic effects of ageing such as failing immunological surveillance or age-related hormonal changes were not required to explain the vastly increased incidence of cancer in old age, and that age was a reflection of the duration of exposure to carcinogenic agents. They used mice at 10, 25, 40, and 55 weeks of age in a skin carcinogenesis experiment with benzpyrene. The rate of malignant epithelial tumors in each group increased steeply with time and the increase was independent of age at the start of exposure.

Second, studies of chemical carcinogenesis have defined stages into which carcinogenesis may be divided: initiation, promotion and progression (Diamond et al., 1980; Pitot, 1990). Each stage is induced independently by a different class of chemical agents.

Third, histological evidence can somewhat arbitrarily be used to divide the neoplastic development into three phases: initiating, intermediate, and advanced (Foulds, 1975). The initiating phase is either clinically "silent" or manifested only by apparently trivial and dubiously neoplastic lesions such as hyperplasia. The intermediate stage is characterized by the emergence of "precancerous" or "premalignant" lesions which may progress into the advanced stage, or persist indolently for a long time with minimal growth and no qualitative change, or regress completely. The last stage is characterized by the presence of malignant carcinomas or sarcomas. The phenomenon of tumor regression suggests the requirement of multiple factors for preneoplastic cells to develop a fully malignant phenotype.

Fourth, cytogenetic studies of tumors reveal a multiplicity of chromosomal abnormalities in all human cancers (Mitelman, 1991; Solomon et al., 1991).

Examination of colorectal carcinoma (Fearon and Vogelstein, 1990) and neuroblastoma (Knudson and Meadows, 1980) indicate that additional cytogenetic lesions are associated with the advanced stage of these malignancies.

Finally, the most direct evidence comes from the discovery of chicken RNA tumor viruses, AMV-E26, MH2, and AEV-ES4 strains, which carry dual oncogenes (*ets-myb*, *myc-mil*, and *erb*-A-*erb*-B, respectively). The full oncogenic potential of these viruses requires both oncogenes (Cole et al., 1983 and Jansen et al., 1983 for MH2; Leprince et al., 1983, Nunn et al., 1983 and Kan et al., 1983 for E26; Frykberg et al., 1983 for ES-4). Viruses possessing only one of these oncogenes shows more limited tissue specificity and longer latency for tumor development. Similar oncogene cooperation in tumor development has also been demonstrated in DNA transfection experiments. Land and coworkers (1983a; 1983b) showed that *myc* and *ras* could fully transform primary rat embryo fibroblasts when transfected together but not singly. Schwartz et al. (1986a) further extended the synergistic effect of the *myc* and *ras* oncogene to the transformation of murine B cells. Other examples of oncogene cooperation involved in tumorigenesis have recently been summarized by Hunter (1991).

In conclusion, through decades of studies, researchers have been able to correlate the multifactorial characteristics of neoplasia from the histological and cellular levels to the molecular level. Identification of genetic lesions and their effects in tumorigenesis not only benefits our understanding of the growth and differentiation of normal and cancerous cells, but also provides insights on prevention or therapy of cancer.

2.1 The search for genes that are involved in tumorigenesis

Studies of mechanisms of chemical carcinogenesis (Pitot, 1990; Balmain and Brown, 1988) and heritability of cancers (Ponder, 1990) have strongly suggested that the lesions of tumors reside in the genetic material. The search for putative oncogenes in a genome size of 3×10^9 base pair containing approximately 10^4 to 10^5 genes was greatly simplified by the discovery of viral oncogenes in animal RNA tumor viruses. The first RNA tumor virus was isolated by Rous (1910), but it was not until the development of molecular biology that the identification of the v-*src* oncogene became possible (Stehelin et al., 1976a). Twenty six viral oncogenes have been identified from animal RNA tumor viruses (Varmus, 1989), and shown to exhibit sequence similarity with cellular genes (later termed protooncogenes) by hybridization and sequencing analyses. Since the cellular protooncogenes are also found to be conserved among different species, it stresses the important roles that these genes may play in regulating growth and differentiation. Thus, alteration or destruction of these genes may contribute to tumor progression.

The list of protooncogenes has been expanded by four major approaches (i) gene transfer, (ii) analysis of known chromosomal translocations or amplifications, (iii) mapping of viral integration sites in virally induced tumors, (iv) sequence homology to the known oncogenes. Some of these genes were repeatedly identified by different methods.

2.1.1 Identification of cellular oncogenes by DNA transfer experiments

The most common method of gene transfer has utilized DNA transfection

and focus formation (Graham and van der Eb, 1973; Shih et al., 1979). DNA is isolated from tumor cells and introduced into recipient cells, usually an immortalized mouse fibroblast line, NIH3T3. NIH3T3 cells have two major advantages other than their easy cultivation: (i) they are flat, contact-inhibited cells that form a monolayer culture, and (ii) their DNA can be distinguished in the hybridization experiments from the DNAs of other species, thus allowing identification of donor DNA. The first property allows one to examine the transfected cultures for the appearance of morphologically altered (transformed) foci due to the expression of an introduced oncogene. Examples of cellular oncogenes identified by this procedure are N-*ras* from neuroblastoma (Shimizu et al., 1983), *trk* from colon carcinoma (Martin-Zanaca et al., 1986), and others as listed in Table 1.1.

Oncogenes of the *ras* family predominate among those found (in about 20% of human tumors tested)(Der et al., 1982; Parada et al., 1982; Santos et al., 1982), whereas cellular analogues of other viral oncogenes are less frequently detected by this method. This could be due, at least in part, to the ability of NIH3T3 cells to respond morphologically to a given oncogene product, to the need for the gene to be genetically dominant, and to the need for the gene to be sufficient for the expression and the maintenance of the transformed state. In other words, NIH3T3 cells may be the wrong lineage or species to respond to the effects of certain oncogenes. In addition, NIH3T3 cells may have a preneoplastic phenotype as they already have the ability to grow continuously in culture. Thus, they may be more susceptible to genes whose effects are manifest during the later stages of tumor progression.

Table 1.1 Oncogenes identified by DNA transfer experiment

Gene	Source[c]	Function	Reference
N-ras	Neuroblastoma	G-protein-like	Shimizu et al., 1983
neu	Rat neuroglioblastoma	EGF receptor like	Shih et al., 1981 Bargmann et al., 1986
mas	Epidermoid carcinoma	Angiotensin receptor	Young et al., 1986
hst	Kaposi's sarcoma	FGF family member	Delli Bovi et al., 1987 Taira et al., 1987
trk	Colon carcinoma	Receptor-like	Martin-Zanaca et al., 1986
met	Osteosarcoma cell line	Receptor-like	Cooper et al., 1984a
ret	T cell lymphoma	Receptor-like	Takahashi et al., 1985
dbl[a]	Diffuse B cell lymphoma	Cytoskeletal matrix associated phosphoprotein	Eva and Aaronson, 1985 Graziani et al., 1989
mcf.2[a]	Mammary carcinoma cell line	as that of dbl, a	Fasano et al., 1984
lca	Hepatocellular carcinoma	b	Ochiya et al., 1986
mel	Melanoma cell line	b	Padua et al., 1984
raf	Stomach cancer	Serine/threonine kinase	Shimizu et al., 1985
ros	Mammary carcinoma cell line	Receptor-like	Birchmeier et al., 1985

a: these two genes were found to be two different activated versions of the same protooncogene (Noguchi et al., 1988)
b: not yet defined
c: human tumors if not specified

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In another aspect, oncogenes that are isolated from RNA tumor viruses carrying a single oncogene probably contain multiple mutations within each oncogene and, therefore, are capable of inducing tumors as a single gene. Thus, the failure of detecting cellular counterparts of other viral oncogenes by this method may simply be attributed to the fact that the cellular counterparts did not contain multiple mutations to induce focus formation of NIH3T3 cells.

2.1.2 Characterization of viral flanking regions

Studies of RNA tumor viruses such as Avian leukosis virus (ALV), Moloney murine leukemia virus (MoMuLV), and Feline leukemia virus (FLV) that do not carry oncogenes reveal another mode of transformation. Insertion of the viral genome into a cellular genome may activate adjacent cellular genes through enhancer or promoter sequences of the viral LTR (Hayward et al., 1981; Fung et al., 1981; for a review see Nusse, 1986a). Therefore, cloning of flanking regions of frequent viral integration sites may allow the identification of putative oncogenes. A variety of genes and "loci" have been isolated by this method. Protooncogenes which contain homology to known viral oncogenes and other known cellular genes are shown in Table 1.2, and "loci" that do not show homology to known viral oncogenes and other known cellular genes are shown in Table 1.3. These "loci" may correspond to novel oncogenes or may be linked to protooncogenes at a distance.

Among the "loci" listed in Table 1.3, *int*-1, *int*-2, and *pim*-1 have been shown to contain detectable transcriptional activity and to encode proteins with distinct functions (Nusse et al., 1984; Dickson et al., 1984; Cuypers et al., 1984; Selten et

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Table 1.2 Cellular genes activated by proviral insertion

Gene	Source	Virus	Reference
c-*myc*	Chicken bursal lymphoma Mouse T cell lymphoma Cat T cell lymphoma	ALV Soule-MuLV FeLV	Hayward et al., 1981[a] Adams et al., 1982[a] Neil et al., 1984[a]
N-*myc*	Mouse T cell lymphoma	Moloney MuLV	van Lohuizen et al., 1989a
c-*erb*B	Chicken erythroleukemia	ALV	Fung et al., 1983
c-*myb*	Mouse lymphosarcoma	defective MoMuLV	Shen-Ong et al., 1984
c-Ki-*ras*	Mouse myeloid cell line	Friend MuLV	George et al., 1986
c-Ha-*ras*	Mouse T cell leukemia	Moloney MuLV	Ihle et al., 1989
IL2	Ape T cell lymphoma cell line	GaLV	Chen et al., 1985
IL-3	Mouse myelomonocytic leukemia	IAP	Ymer et al., 1985
CSF-1	Mouse monocyte tumor	endogenous ecotropic provirus	Baumbach et al., 1988
GM-CSF	Mouse promyelocytic cell line	IAP,R-MuLV,F-SFFV	Stocking et al., 1988
c-*mos*	Mouse plasmacytoma line	IAP	Canaani et al., 1983
p53	Mouse erythroleukemic cell line	Friend MuLV	Hicks and Mowat, 1988

abbreviation:

MuLV: murine leukemia virus
IAP: intracisternal A particle
R-MuLV: Rauscher murine leukemia virus
F-SFFV: Friend spleen focus forming virus
GaLV: Gibbon Leukemia Virus
[a]: only the earliest reference is shown
FeLV: Feline leukemia Virus
ALV: Avian Leukosis Virus

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Table 1.3: Viral flanking regions without viral oncogene analogues.

Name	Source	Virus	mRNA	Reference
Evi-1	AKXD myeloid tumor	Cas-Br-M MuLV		Mucenski et al.,1988a
		MCF	yes	Morishita et al., 1988
Ahi-1	NIH/Swiss or SIM.S pre-B cell lymphoma	Moloney MuLV	nd	Poirier et al., 1988
Fis-1	AKR lymphoma or BXD-2 leukemia	Friend MuLV	nd	Silver and Kozak, 1986
Dsi-1	Fisher rat thymoma	Moloney MuLV	nd	Vijaya et al., 1987
Pim-2	Balb/c or C57BL10 lymphoma	Moloney MuLV	nd	Breuer et al., 1989a
Pim-1	Balb/c or AKR T lymphoma	MCF	yes	Cuypers et al., 1984
Int-1	C3H Mammary carcinoma	MMTV	yes	Nusse and Varmus, 1982
Int-2	C3H Mammary carcinoma	MMTV	yes	Dickson et al., 1984
Int-41	mouse mammary and kidney adenocarcinoma	MMTV	yes	Garcia et al., 1986
Mlvi-1[a] (*Pvt*-1)	rat thymoma	Moloney MuLV	nd	Tsichlis et al., 1983a
Mlvi-2	rat thymoma	Moloney MuLV	nd	Tsichlis et al., 1984
Mlvi-3	rat thymoma	Moloney MuLV	nd	Tsichlis et al., 1985a
Mlvi-4	rat thymoma	Moloney MuLV	nd	Lazo et al., 1990
Gin-1	mouse thymoma	Moloney MuLV	nd	Villemur et al., 1987
Spi-1	mouse erythroleukemia	SFFV	yes	Moreau-Gachelin et al.,1988
Bic	Avain B cell lymphoma	ALV	yes	Clurman and Hayward, 1989

Abbreviation:

MuLV and ALV: same as those in Table 1.2
MMTV: Mouse mammary tumor virus
MCF: mink cell focus forming virus
Cas-Br-M MuLV: Casitas Brain Mousetropic MuLV
SFFV: spleen focus forming virus
[a]: also known as *mis*-1

al., 1986; Nusse, 1988), and to exhibit transforming ability in transgenic mice (Adams and Cory, 1991) or the *in vitro* transformation of an epithelial cell line (int-1 only; Brown et al., 1986). On the other hand, many of them do not have detectable transcriptional activity. Since the enhancer element of the viral LTR may function over a long distance, it is possible that certain putative oncogenes may be located distal to the breakpoints of viral integration sites. Techniques such as chromosomal walking, jumping and YAC cloning may facilitate the identification of such genes. Additionally, exon trapping (Duyk et al., 1990; Buckler et al., 1991), a method used to identify any exon sequence from cloned genomic DNA may also be used to facilitate the identification of any transcription unit. Once a transcription activity is associated with a "locus, it is, however, necessary to test for its transformation potential. Either the *in vitro* focus formation assay (see DNA transfer experiment) or the transgenic animal model (see below) can be used for that purpose.

The spectrum of oncogenes that can be identified by this method relies on the randomness of viral integration. Although retrovirus integration has been shown to have preferred target sites (Shih et al., 1988), these preferred sites comprise only 20% of all the viral integration sites. Thus, this preference does not seriously affect the variety of oncogenes that may be identified by this method. In addition, the nature of this preference is unknown. Perhaps it resides in the active transcription of preferred regions. The fact that both cellular counterparts of viral oncogenes and novel oncogenes are isolated by this method makes it a feasible way of searching for a diverse class of genes that are involved in the regulation of growth and differentiation. It has clearly allowed identification of a wider variety of

oncogenes than transfection and focus formation experiments.

Interestingly, no human oncogene identified thus far is activated or inactivated by viral integration. However, the discovery of multiple copies of endogenous virus-related sequences in human cells suggests that gene activation through this mode may still be possible (Bonner et al., 1982; Callahan et al., 1985).

2.1.3 Molecular Characterization of chromosomal aberrations

Chromosomal aberrations have long been associated with human cancers. Karyotype analyses of metaphase chromosomes has found some abnormalities specific for distinct tumor types (for a recent review, Mitelman, 1991). The discovery of the Philadelphia (Ph1) chromosome in bone marrow cells of patients with chronic myelogenous leukemia (CML) actually laid the foundation for the association of specific chromosomal anomalies with a particular neoplasm (Nowell and Hungerford, 1960). It was not until recently, however, that the molecular basis of these cytogenetic lesions has been elucidated as a result of the improvement of both cytogenetic and molecular cloning methods. Methotrexate treatment (aminopterin) (Yunis, 1976; Hagemeijer et al., 1979) is to block DNA synthesis pathways is blocked so that cells can be collected at the S phase of a cell cycle. The block is then released by adding thymidine to allow DNA synthesis using the salvage pathway. A large number of cells enter mitosis synchronously following these treatments. These synchronized cells are then treated briefly with colchicine followed by standard banding procedures (Chaudhuri et al., 1971; Caspersson et al., 1970; Hsu, 1974). Chromosomes obtained by this procedure are less condensed, allowing 1200 bands to be identified. The increased banding

resolution not only increases the accuracy of identification of chromosomes, but also leads to the recognition of many karyotypic changes and the association of these changes with particular neoplasias. Cloning of genes located in translocation junctions or other chromosomal aberrations has been done by conventional cloning methods, i.e. use of probes for genetic markers near the locus of interest, and chromosomal walking using a genomic library until the authentic gene is identified by *in situ* hybridizations or southern blot analyses.

The introduction of microdissection and microcloning should advance this process. Microdissection was first used by Scalenghe et al.(1981) to clone DNA from Drosophila polytene chromosomes. It was later used in the human genome mapping project (Bates et al., 1986; Kaiser et al., 1987), and to clone genes involved in human genetic diseases (Lüdecke et al., 1989; Kondo et al., 1984). In this technique, a very small segment of the chromosome of interest can be dissected with needles from banded chromosomes by using an electronically controlled micromanipulator. Use of this method in combination with microcloning in which the technique of polymerase chain reaction (PCR) was incorporated (Edström et al., 1986) can reduce the number of genomic clones required to screen for the locus of interest compared to that in conventional cloning procedures. Adapting these approaches may greatly facilitate the cloning of genes that reside at the site of chromosomal lesions.

A recent review by Solomon et al. (1991) summarizes genes identified from chromosomal aberration sites (Table 1.4). Many of them are either analogues of viral oncogenes, or genes involved in the cell cycle or differentiation. More

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Gene

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Bc-1
Bc-2
Bc-3
IL-3
cyt1
TC-S
Rbm
Rbm
Tam
Hox
Pim
Btg-
Bcr-A

Pml-R

Dek-C

E2A-P

Rel-N

Abne

CLL
AML a
CML
AML
AML

Table 1.4 Genes identified from chromosomal aberrations

Gene	Source	Protein type	Reference
chromosomal amplification			
N-*myc*	Neuroblastoma	Nuclear protein	Schwab et al., 1983
L-*myc*	Small-cell lung cancer	Nuclear protein	Little et al., 1983

chromosomal translocation or inversion (adapted from Table 1 in Solomon et al. (1991))

Gene	Disease	Rearrangement	Protein type
myc	Burkitt lymphoma	t(8;14)(q24;q32) t(2;8)(p11;q24) t(8;22)(q24;q11)	HLH domain
	T-ALL	t(8;14)(q24;q11)	
Bcl-1	B-CLL	t(11;14)(q13;q32)	G1 cyclin-like
Bcl-2	Follicular lymphoma	t(14;18)(q32;q21)	Inner mitochondria membrane
Bcl-3	B-CLL	t(14;19)(q32;q13)	CDC 10 motif
IL-3	Pre-B ALL	t(5;14)(q31;q32)	Growth factor
*Lyl*1	T-ALL	t(7;19)(q35;p13)	HLH domain
Tcl-5	T-All	t(1;14)(p32)(q11)	HLH domain
*Rbnt*1	T-ALL	t(11;14)(p15;q11)	LIM domain
*Rbnt*2	T-ALL	t(11;14)(p13;q11)	LIM domain
*Tan*1	T-ALL	t(7;9)(q35;q34)	Notch homolog
*Hox*11	T-ALL	t(10;14)(q24;q11)	Homeo domain
Pth	Parathyroid adenoma	inv(11)(p15;q12)	Deregulate *myc*
Btg-1	B-CLL	t(8;12)(q24;q22)	Deregulate *myc*
Bcr-Abl	CML, B-ALL	t(9;22)(q34;q11)	*Bcr*, Gap for $p21^{ras}$ *Abl*, Tyrosine kinase
Pml-Rara	APL	t(15;17)(q22;q11-12)	*Pml*, Zinc finger *Rara*, Zinc finger
Dek-Can	AML-M2,-M4	t(6;9)(p23;q34)	*Dek*, nuclear protein *Can*, cytoplasmic protein
E2A-Pbx	Pre-B ALL	t(1;19)(q23;p13)	*E2A*, HLH domain *Pbx*, homeodomain
Rel-Nrg	NHL	ins(2;2)(p13;p11-14)	*Rel*, NF-$_k$B family *Nrg*, no homology

Abbreviation:

CLL: chronic lymphocytic leukemia
ALL: acute lymphocytic leukemia
CML: chronic myelogenous leukemia
AML: acute myelogenous leukemia
NHL: non Hodgkin's lymphoma

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significantly, tumor suppressor genes (such as RB and WT-1; Marshall, 1991, recently reviewed by Weinberg, 1991) and genes associated with apoptosis (such as *bcl*-2) were also discovered. Alteration of the genomic structure of these genes may deregulate their expression and participate in the transformation of cells, and consequently lead to tumor formation.

2.1.4 Identification of new oncogenes by homologous sequences

Another approach not commonly used, but potentially useful, is to search for sequences homologous to known oncogenes assuming the conservation of important regulatory genes. Given the fact that all the genes in the *src* family (Hunter and Cooper, 1985) and *ras* family (Barbacid, 1987) share significant homologies among certain genetic domains (such as the effector domain of *ras* genes and the carboxy-terminal region of *src*-related genes), hybridization probes for those regions may identify new members of these gene families. The *lck* oncogene (Marth et al., 1985) was first isolated from a murine T cell lymphoma by using an oligonucleotide probe for the conserved major tyrosine phosphorylation site.

Other proliferation and differentiation related genes may also be identified from cells on the basis of reversion from the transformed phenotype. Such cells have been generated either by cell-fusion with normal cells (Harris, 1988) or DNA transfection. An example of a gene identified by the latter method is the Krev-1 gene, which is responsible for the revertant phenotype of Kirsten sarcoma virus-transformed NIH3T3 cells (Kitayama et al., 1989; Noda et al., 1989).

The oncogenic potential of the putative oncogenes identified from the above

procedures should be tested to verify their active roles in oncogenesis. The following sections describe general approaches used for this purpose.

2.2 Experimental models for transformation and tumor progression

2.2.1 Use of animal versus human models

Animal model systems are obviously required for experimental *in vivo* carcinogenesis studies, since it is ethically unacceptable to use human subjects. The compatibility of results from animal models with those obtained in humans has been supported by several observations. First, the conservation of oncogenes among species suggests their common properties and regulation (Varmus, 1989). Additionally, human oncogenes are able to transform mouse fibroblasts in DNA transfection experiments (see above) and induce tumors in transgenic mice (Palmiter and Brinster, 1986). Second, growth factors and growth regulatory mechanisms do not appear to be species specific since transplanted tumors from a great range of species can grow in immunodeficient, athymic (nude) mice while normal tissue can not (Stiles and Kawahara, 1978). These results suggest the universality of the genetic events causing neoplasia, and the validity of animal models in studying human carcinogenesis. The fact that laboratory animals can be environmentally and genetically controlled makes them an even better system to work with.

On the other hand, the use of human tissues and cells does offer some unique advantages. First, some rare forms of human cancer reflect inherited, predisposing conditions. Their genetic basis and perhaps common pathways to carcinogenesis may be understood through the study of nontumorous cells from

affected individuals (Ponder, 1990). Moreover, the fact that human cells are genetically more stable *in vitro* than most rodent cells (DiPaolo, 1983) makes them especially suitable for studying the multiple steps of tumorigenesis. Lastly. the findings from studies of human cells complement and validate the results derived from studies of laboratory animals.

2.2.2 Use of *in vivo* versus *in vitro* systems

The role of the host, not only in controlling, but also in facilitating the development and spread of cancer has already been established (for a review, see Alexander, 1987). These host factors interact with the genetic lesions that reside in the tumor cells themselves. The *in vivo* system therefore has an essential role in cancer studies because the integral multi-systemic interactions of the organism remain intact.

The disadvantage of using *in vivo* models for studies of tumor progression is its inconvenience for dissecting events within the progression. Most often an endpoint must be chosen for analysis and intervening events must be implied. An *in vitro* model using cell or tissue culture, however, provides opportunities to dissect the processes controlling growth, differentiation, neoplastic transformation and tumor progression of cells, and to describe their mechanisms in biochemical or molecular terms.

2.2.3 Model systems used to test the oncogenic potential of oncogenes

One commonly used *in vitro* culture system, NIH3T3 cells, although allowing the detection of certain oncogenes, presents limited sensitivity since this method tends to identify oncogenes in the *ras* gene family as discussed previously.

Various tissue culture systems including those of epithelial and hematopoietic origins (Gabrielson and Harris, 1985; Rheinwald and Green, 1975; Deeh, 1985; Lechner

^T21%* 'eck, 1985; Dexter et al., 1977; Whitlock and Witte, 1982) were established and have allowed the study of gene interactions within differentiated cell types. Among them, the long-term B cell culture system of Whitlock and Witte (1982) is exclusively used in our studies. This culture system can provide not only a wide range of targets for transformation within the B cell developmental series, but also allows the culture of cells that are not transformed or of an intermediate transformed phenotype. It thus provides an excellent tool to test the transforming potential of oncogenes in the B cell lineage, and to elucidate the mechanisms of transformation on the molecular level.

In vitro human B cell lines such as the lymphoblastoid cell lines (LCLs) are commonly used for the same purpose. These cell lines are derived from the infection of human peripheral blood with Epstein-Barr virus (EBV) (for review see Nilsson and Klein, 1982). Examples of the uses of these lines in testing oncogenic potential and oncogene cooperativity of putative B cell oncogenes are described in human B cell neoplasia (see below).

Transgenic mice can also be used to assess the transforming potential of various oncogenes (for review see Palmiter and Brinster, 1986; Adams and Cory, 1991). Using tissue specific regulatory elements, one can examine the oncogenic potential of the gene of choice in a particular tissue. For example, the enhancer sequences of immunoglobulin genes (Eμ) have been used to test oncogenes such as c-*myc* and *bcl*-2 in the oncogenesis of B-cell neoplasia (Adams et al., 1985;

McDonnell et al., 1989). In addition to its advantages as an *in vivo* system (as described previously), it also provides the only means for studies of tissue specific oncogenesis where an *in vitro* culture system is lacking.

2.2.4 Model systems used to study tumor progression

Two of the most established genetic systems, the mouse skin carcinoma (for a review see Balmain and Brown, 1988) and human colon carcinoma (Fearon and Vogelstein, 1990), share the advantage of ease in access to and recognition of tumor samples at different stages of progression. In contrast, tumors of lymphoid and other organs lack a good animal model to study molecular changes during the tumor progression period, mainly due to the difficulty of identifying preneoplastic cells and obtaining cells at varying stages of neoplastic development in a single host (i.e., the collection of neoplastic samples of these tissue types often requires termination of the donor animal). Even if the experimental animals were blindly sacrificed at different time points to collect tumors at different stages of neoplastic development, one still encounters the problem of insufficient numbers of cells to perform molecular analysis. *In vitro* cultures would be required to expand these isolates and such *in vitro* tissue culture systems are lacking for many of these tissue types. Although the results generated from the above *in vivo* systems may be applicable to tumors of other origins, evidence of the association of certain oncogenes with tumors of particular tissue types from studies of transgenic mice and inherited cancers (Adams and Cory, 1991; Marshall, 1991; Lanes et al., 1981 & 1982; Cooper and Neiman, 1980; Padhy et al., 1982) have suggested the need for tissue specific model systems. Although the use of

transgenic mice has proven to be a useful *in vivo* model to test the ability of an oncogene to initiate tumor formation, little knowledge is gained about the secondary events that have contributed to the progression of tumors. Tests of oncogene cooperativity either involve the construction of transgenic mice with multiple oncogenes or performing crosses with transgenic mice carrying different oncogenes (Adams and Cory, 1991). These procedures may involve intensive labor. Moreover, the cooperative effect is unavoidably under the influence of unnatural environments because cells of all tissue types may express the activated oncogenes. The use of tissue-specific enhancer sequences may avoid this problem; however, these sequences are not available for all tissue types. One approach used to study oncogene cooperation in the tumor progression of transgenic mice is to infect transgenic mice with retroviruses to facilitate tumor progression (van Lohuizen et al., 1991).

The Whitlock and Witte culture system (1982) allows certain advantages in the study of oncogene cooperativity. By comparing the transformed phenotypes of B cells generated after the introduction of two or more oncogenes of interest into this culture to those of B cells generated from cultures with a single oncogene, one can determine whether one oncogene can complement another in transforming B cells. This procedure is relatively easy as compared to that of transgenic mice. It also provides a faster way of testing whether there are temporal effects on the acquisition of genetic alterations in tumor progression.

In summary, using the methodologies described above, extensive studies have been performed to uncover the basis of tumorigenesis. Among them, hematopoietic neoplasias are the best characterized tumors at the molecular

genetic level due to their frequent occurrence in animal models and their high mitotic index, allowing for cytogenetic analyses (see above). Many alterations of protooncogenes have been specifically associated with different subtypes of leukemias/lymphomas of different species, and are summarized elsewhere (Schwartz and Witte, 1988; Solomon et al., 1991; Sawyers et al.,1991). Here, I will describe exclusively the molecular characterization of B cell lymphomas/leukemias of human, mouse, and avian origin, since this has been the focus of my thesis.

3. Multiple genes or factors that are involved in B cell lymphomas/leukemias of human, mouse, and avian

3.1 Human B cell neoplasia

3.1.1 Burkitt's lymphoma

Burkitt's lymphoma (BL) is the best characterized human B cell tumor. It was first described by Burkitt (1958) as a distinct clinicopathological entity occurring with high frequency in the jaws of children from Central Africa. Non-endemic (or sporadic) BL is also found in areas outside of Africa but the rate of incidence is low (O'Conor et al., 1985; Philip et al., 1982). BL is a malignant lymphoma comprising a monomorphic outgrowth of B lymphocytes. The stage of maturation of BL cells varies among individual tumors, from a near pre-B phenotype (Preud'homme et al., 1975) to a more mature phenotype with expression of predominantly IgM (Klein et al., 1967), and occasionally IgD, IgG, or even IgA (Preud'homme et al., 1985). However, the cells never fully differentiate to a plasmacytic phenotype.

The etiology of BL involves at least two steps. Infection with Epstein-Barr Virus (EBV) appears to be the primary event in the development of African Burkitt's

lymphoma from both genetic (Geser et al., 1983) and epidemiological evidence (de-The' et al., 1978; Geser et al., 1982). Geser and coworkers found that 96% of African Burkitt's lymphomas carry multiple copies of the EBV genome in all their cells. Both groups showed that a high multiplicity EBV infection early in the life of African children contributes to the tumor development. Raab-Traub and Flynn (1986) and Neri et al. (1991) have studied the termini of EBV episome genomes in both endemic BL primary tumors and cell lines. The linear EBV DNA has variable numbers of direct tandem 500 bp repeats at each terminus. Restriction endonuclease analyses have indicated that the termini are uniformly clonal in all cases. This result again strongly suggests that EBV infection has preceded, and thus, most likely contributed to clonal expansion in these malignancies. Although the lack of viral markers in 4% of the endemic BL and approximately 85% of the non-endemic BL cases (Lenoir et al., 1984a) may argue the against a necessary causal relationship between EBV and BL, the fact that EBV can transform human peripheral B cells into established cell lines (Takada and Osato, 1979; Robinson and Smith, 1981) and that EBV can induce B cell lymphomas in marmosets (zur Hausen, 1980) implies an important role for EBV in BL.

The immortalizing mechanism of EBV has been suggested to be mediated through autocrine stimulation by the finding of the release of a B-cell growth factor (BCGF) from Burkitt lymphoma cells and EBV infected cell lines (Gordon et al., 1984). The precise consequences of this autocrine stimulation are unknown. However, two EBV-encoded proteins are found to be involved in the process of oncogenesis. The EBNA2 gene product, a nuclear protein, is required for immortalizing B cells since EBV deleted for this gene fails to do so (Menezes et al.,

1975; Delius and Bornkamm, 1978; Bornkamm et al., 1980; Heller et al., 1981). EBNA2 can also transiently stimulate cellular DNA synthesis upon transfection into B cells (Volsky et al., 1984). Another EBV encoded protein, LMP1 (a latent membrane protein), can transform established rodent cells (Wang et al., 1985; Baichwald and Sugden, 1988). The linkage between the release of the BCGF and the biochemical effects of these two proteins are becoming clear through the following studies. EBNA2 is a multiple-function protein, and may act similarly to T antigen of SV40 virus. EBNA2 was found to induce CD21, CD23, and LMP1 expression upon introducing this gene into cell lines which are infected with EBV strains, deletion mutants that do not encode EBNA2 protein (Wang et al., 1990; Abott et al., 1990). CD21 is a surface protein involved in human B cell differentiation (Tedder et al., 1984). CD23 is a membrane-bound B cell activation marker, and acts as an autocrine BCGF for normal and transformed B cells when shed (Swendeman and Thorley-Lawson, 1987). LMP1 was found to cooperatively induce CD23 expression, and to induce the expression of several cellular adhesion molecules (Wang et al., 1990). The increased expression of the cellular adhesion molecules may explain the phenotypic changes of LMP1 transfected cells including growth in large tight cell clumps (Wang et al., 1988). Taken together, these findings suggest that EBNA2 may immortalize B lymphoid cells by a autocrine mechanism through induction of CD23. This autocrine stimulation is augmented through the cooperation with LMP1, which may act via cellular second messengers. Whether EBNA2 acts directly or through other cellular intermediates in the upregulation of CD23 transcription remains to be determined.

The second step in the development of Burkitt's lymphoma may involve

deregulation of the c-*myc* gene through chromosomal translocations of chromosome 8 on which it residues. The almost universal presence of chromosomal translocations between chromosome 8 and one of three other chromosomes (chromosome 14, 2 and 22) is another characteristic of Burkitt lymphoma. Only one exception to this has been reported (Zech et al., 1976). Translocation t(8;14)(q24;q32) was found in approximately 80% of cases of Burkitt lymphoma (Croce and Nowell, 1986), while the remainder of these tumors carry t(2;8)(p11;q24) or t(8;22)(q24;q11) translocations. It is striking that each of these translocations involves the cytogenetic location of one of the immunoglobulin (Ig) loci. The heavy-chain gene is located at chromosome 14q32 (Croce et al., 1979); the kappa and the lambda genes are at 2p11 (Malcolm et al., 1982) and 22q11 (Erikson et al., 1981), respectively. This may suggest a molecular relationship between the rearrangement of Ig genes and oncogenesis, perhaps through a aberrant recombination between two chromosomes. The identification of the crossover point of the translocation on chromosome 8 was facilitated by the discovery in murine plasmacytomas of a similar translocations (see below).

The involvement of the c-*myc* gene in these chromosomal translocations was suggested by the finding of c-*myc* gene activation in an abortive immunoglobulin gene recombination in a mouse plasmacytoma (Shen-Ong et al., 1982). Additionally, the c-*myc* gene was mapped to chromosome 8q24 using human/rodent somatic cell hybrids by Dalla Favera et al. (1982). Finally, southern blot analyses showed that the c-*myc* gene was rearranged in approximately 50% of Burkitt's lymphomas examined (Bernard et al., 1983; Dalla Favera et al., 1983; Taub et al., 1982). Molecular cloning ultimately enabled the detailed analysis of the

translocation break points and demonstrated that the translocations join sequences from the Ig genes to regions surrounding c-*myc* (reviewed by Haluska et al., 1987).

The result of all three translocations is the constitutive expression of the c-*myc* gene. In contrast to the high levels of c-*myc* expression associated with the viral transformation of avian B-cell lymphomas (see below) and human tumor cell lines that contain amplified c-*myc* genes (Collins and Groudine, 1982; Dalla Favera et al., 1982), high levels of c-*myc* expression are not found consistently in all Burkitt's lymphoma cell lines. A variety of mechanisms have been proposed to account for the *myc*/Ig juxtaposition in its contribution to tumorigenesis (review by Klein and Klein, 1985). These include abnormally high transcription rates, abnormal transcription size, changed promoter usage, mutations, and lack of transcriptional pausing at exon 1 of c-*myc* gene. None of the above mechanisms seems to be applicable universally to all or even most of the tumors. The current idea is that c-*myc* is deregulated as a consequence of *cis*-acting sequences associated with the constitutively active Ig region. The result of such deregulation would be to keep the cells in continuous division through a loss of c-*myc*'s normal regulation.

The oncogenic potential of c-*myc* genes in Burkitt's lymphoma was tested by Lombardi et al. (1987). They introduced an expression vector containing constitutively expressed c-*myc* into EBV-infected human B lymphoblastoid cell lines (LCLs)(Nilsson and Klein, 1982). The resulting *myc*-transfected LCLs displayed a reduced serum requirement for growth, an increased soft-agar cloning efficiency, and an increased tumorigenicity in nude mice as compared to those of vector-only-

transfected LCLs. Their results indicate a contribution of the c-*myc* gene in the tumorigenesis of Burkitt's lymphoma, and a cooperation between two putative immortalizing functions. However, the inability of *myc*-transfected LCLs to form colonies as efficiently as BL cell lines, and their low tumorigenicity have suggested that additional events are required for a fully tumorigenic phenotype.

In addition to the common events of EBV infection and c-*myc* translocation, p53 mutations were found associated with 9/27 biopsies of BLs and 17/27 BL cell lines (Gaidano et al., 1991). The p53 gene encodes a 53-kDa nuclear phosphoprotein that may be involved in the negative regulation of cell growth (Lamb and Crawford, 1986; for review see Iman and Harris, 1991). Additionally, p53 may differ from prototypical tumor suppressor genes in that at least some p53 mutant alleles can behave as dominant oncogenes by transforming target cells *in vitro* and causing tumorigenesis in transgenic mice even in the presence of the normal allele (Halevy et al., 1990; Lavigueur et al., 1989). Gaidano and coworkers' results suggest both loss of function and dominant negative mutations are present in BLs.N-*ras* activation was found in a sporadic Burkitt's lymphomas (Murray et al., 1983; Lenoir et al., 1984b). The effect of the *ras* oncogene in the tumorigenesis of Burkitt's lymphoma has been studied by Seremetis et al. (1989). Introduction of activated N-*ras* or H-*ras* oncogenes into EBV immortalized LCLs has led to malignant transformation of these cells. However, their results show that these *ras* genes are also capable of inducing terminal differentiation of LCLs into plasma cells, and therefore, may have implications in the pathogenesis of terminally differentiated B-lymphoid malignancy such as multiple myeloma rather than in Burkitt's lymphoma.

3.1.2 Other human B-cell leukemias and lymphomas

Most of the human B-cell leukemia or lymphoma associated oncogenes that will be described here were identified by molecular cloning of frequent or nonrandom translocation sites, and are recently reviewed by Solomon et al. (1991). It should be noted that unlike the tight association of the c-*myc* gene with both Burkitt's lymphoma and murine plasmacytoma, some of the genes described in this section are not invariably found to be activated in all tumors of a particular type. Their impact on the progression of particular neoplasia requires further investigation.

B-cell chronic lymphocytic leukemia

Four translocations t(11,14)(q13,q32), t(14,19) (q32,q13), t(8,12)(q24,q22), and t(18;22) have been found in B cell chronic lymphocytic leukemia (B-CLL). The t(11,14)(q13,q32) translocation also occurs in some diffuse small-cell lymphocytic leukemias and diffuse large-cell lymphomas (Yunis, 1983), and multiple myelomas (van der Berghe et al., 1984). The chromosome 11 breakpoints in two CLL patients have been shown to occur only seven nucleotides away from each other, whereas the breakpoint in a diffuse B-cell lymphoma is approximately 0.9 kb distant from those characterized in CLL (Tsujimoto et al., 1984a, 1985a). The break point cluster region of this translocation has been denoted as *bcl*-1. No transcription unit has been detected in this region other than the closely linked PRAD1 gene (Lammie et al., 1991), a gene which was found to be a putative oncogene in parathyroid adenoma (Arnold et al., 1989; Friedman et al., 1990; Rosenberg et al., 1991). PRAD1 encodes a G1 cyclin-like protein (Motokura et al., 1991). Cyclins can form a complex with and activate $p34^{cdc2}$ protein kinase,

thereby regulating progress through the cell cycle (for review see Nurse, 1990). PRAD1 mRNA is expressed in many tissues and is highly conserved in bovine and murine tissues (Rosenberg et al., 1991). PRAD1 has been implicated in non-parathyroid neoplasia, squamous cell and mammary carcinomas, and is invariably amplified and overexpressed in these tumors (Lammie et al., 1991). No direct demonstration of the altered expression of PRAD1 has been reported in B-CLL as this thesis is being written. Presumably, the disruption of the cell cycle after alteration of this gene may contribute to the course of B-CLL as in the other tumors.

The t(14,19)(q23,q13) translocation involves a deregulation of the *bcl*-3 gene (McKeithan et al., 1987; Ohno et al., 1990). As a result of this translocation, chromosome 19 sequences including the *bcl*-3 gene are juxtaposed to the 5' end of the α1 switch region of the IgH gene on chromosome 14 in a head to head manner. The *bcl*-3 transcription unit is not disrupted by the translocation, and a more than 3.5 fold increase of the mRNA level was found in total RNA from the peripheral blood of two CLL patients with the t(14;19) translocation as compared to RNA from a patient with the prolymphocytic variant of CLL, which does not contain this translocation. The *bcl*-3 gene encodes seven tandem copies of the cdc10 motif, a proline rich N-terminal, and a proline-serine rich C-terminal (Ohno et al., 1990). The cdc10 motif was previously identified in yeast genes that regulate events at the start of the cell cycle (for review see Simanis et al., 1987) and in invertebrate transmembrane proteins involved in cell differentiation pathways (Austin and Kimble, 1987; Seydoux and Greenwald, 1989; Sternberg and Horvitz, 1989). It is not clear which of these two classes of proteins that the *bcl*-3 gene

resembles, but it is clear that it is not a transmembrane protein. The proline rich region has been shown to have transcription activating properties (Mermod et al., 1989). Thus, it is plausible that the *bcl*-3 gene could be a transcriptional activating factor. The involvement of the *bcl*-3 gene in B-CLL was later analyzed in a large series of patients by Raghoebier et al. (1991). Unexpectedly, none of the forty four B-CLL studied had a rearrangement within 15 kb of the *bcl*-3 locus. However, mutations in the *bcl*-3 gene undetectable in their assays may exist. Whether the *bcl*-3 gene contributes to the oncogenesis of B-CLL awaits further investigation.

The t(8,12)(q24,q22) translocation links c-*myc* not with an Ig enhancer but rather with a locus termed BTG1 on chromosome 12 that presumably deregulates *myc* (Rimokh et al., 1991). The breakpoint is located in the 3' end of the *myc* locus, and increased c-*myc* expression has been found. Sequences cloned from the breakpoint recognize a 1.8 kb transcript in the CLL cells and in tissues of lymphoid origin. In addition, this chromosome 12 coding sequence is conserved in evolution and a transcript of similar size is present in murine tissues. However, little is known about the function of this putative gene. Whether or not this gene activation represents a general feature for the B-CLL is also unknown.

In addition to the possible involvement of the PRAD1, *bcl*-3, and c-*myc* genes in this malignancy, *bcl*-2 was found rearranged in 3/34 B-CLL through a variant translocation t(18;22). In this translocation, the *bcl*-2 gene is juxtaposed to the Igλ or κ genes in a head to head configuration (Adachi et al., 1990). *Bcl-2* was also found rearranged in 3/44 B-CLL by Raghoebier and coworkers (1991). The role of *bcl*-2 in tumorigenesis will be discussed in the following section.

The relatively low frequency of any individual oncogene activation in B-CLL

may relate to the accuracy of clinical diagnosis, and the broad range of cell types involved in this malignancy. For example, CLL may sometimes be difficult to distinguish from non-Hodgkin's lymphoma (Bennett et al., 1989; Deegan, 1989). Systemic analyses on a large scale of samples may be required for a better understanding of the molecular basis of this malignancy.

Follicular lymphoma

The t(14,18)(q32,q21) translocation which occurs in 85% of follicular lymphomas was first described by Fukuhara et al. (1979). The interchromosomal junction has been cloned from several follicular lymphomas (Tsujimoto et al, 1985b; Cleary and Sklar, 1985). The recombination region of chromosome 18, *bcl*-2, is rearranged into the heavy chain enhancer region on chromosome 14 resulting in deregulation of *bcl*-2 expression (Tsujimoto et al., 1984b, 1985c; Cleary and Sklar, 1985; Bakhshi et al., 1985). The normal *bcl*-2 gene is quiescent is resting B cells, expressed in proliferating B cells, and downregulated in differentiated cells (Graninger et al., 1987; Reed et al., 1987). Inappropriately high levels of *bcl*-2-immunoglobulin chimeric RNA are present in t(14;18) follicular lymphoma considering their mature B-cell stage (Seto et al., 1988). This indicates that the translocated *bcl*-2 allele has escaped normal control mechanisms. Nucleotide sequence analysis and biochemical studies from one group suggest that *bcl*-2 is a GTP-binding protein located on the cytoplasmic surface of cell membranes (Haldar et al., 1989), but a second group has localized it to the inner mitochondrial membrane (Hockenbery et al., 1990). *Bcl*-2 has been shown to prolong cell survival by blocking programmed cell death (Nuñez et al., 1990). A similar effect may thus contribute to the formation of follicular lymphomas. Activated *bcl*-2 genes

proved capable of transforming or enhancing the survival of a cultured human B-cell line (Tsujimoto, 1989) or NIH3T3 cells (Reed et al., 1988). It is also capable of inducing follicular hyperplasia in transgenic mice (McDonnell et al., 1989) which progresses to a malignant diffuse large-cell lymphoma after a long latency (McDonnell and Korsmeyer, 1991). The long latency, progression from polyclonal to monoclonal disease, and histological conversion, are all suggestive of second alterations. C-*myc* activation is suggested to be a candidate oncogene both for its occurrence of rearrangement in the diffuse lymphoma, and its well known involvement in B cell neoplasia. In fact, the cooperativity of the c-*myc* oncogene and *bcl*-2 in tumorigenesis has been shown by Vaux et al. (1988). The authors found the *bcl*-2 gene can promote the proliferation of bone marrow cells of Eμ-*myc* transgenic mice, some of which become tumorigenic. Another oncogene, c-Ha-*ras*, was found to complement *bcl*-2 in malignant transformation of rat embryo fibroblasts (Reed et al., 1990).

Pre-B cell acute lymphocytic leukemia

Two translocations t(1,19)(q23,p13) and t(5,14)(q31,q32) have been found in pre-B cell acute lymphocytic leukemias (pre-B ALL). The t(1,19)(q23,p13) translocation was described as a common feature for pre-B ALLs (Carroll et al. 1984; Michael et al., 1984; Williams et al., 1984). A fusion protein, E2A-PBX, results from this translocation which links the E2A gene on chromosome 19 to the homeobox containing PBX gene on chromosome 1 (Kamps et al., 1990; Nourse et al., 1990). The E2A gene encodes for two similar immunoglobulin enhancer binding factors, each with a 5' effector domain and a 3' DNA binding domain (Murre et al., 1989). The translocation switches the DNA binding domain of the

E2A transcription factor with that of PBX, thus placing those genes usually regulated by PBX under the trans-activational control of E2A. Because PBX is not normally transcribed in pre-B cells, the translocation results in ectopic expression of the PBX DNA binding domain and therefore implicates the fusion protein in the tumorigenesis of pre-B cells.

The other translocation, t(5,14)(q31,q32) was identified by Grimaldi and Meeker (1989). At the breakpoint of this translocation, interleukin-3 (IL-3) on chromosome 5 is positioned next to the heavy chain enhancer region on chromosome 14 (Meeker et al., 1990). This has led to the hypothesis that the overproduction of IL-3 may result in an autocrine loop that favors leukemogenesis (Meeker et al., 1990).

3.2 Murine plasmacytoma

Murine plasmacytoma is induced by the application of a mineral oil such as pristane into the intraperitoneal cavity of a mouse (with a restriction to Balb/c or NZB mice) (Potter and Boyce, 1962; Anderson and Potter, 1969). Balb/c mice develop plasmacytoma with a latency period of 6-12 months after the injection of mineral oil. The long latency has suggested a multi-step nature for the formation of this tumor. Similar to BL, two common characteristics have been associated with almost all plasmacytomas.

First, the involvement of a paracrine stimulation is critical for the formation of plasmacytomas. Prior to formation of a plasmacytoma, induction of chronic granuloma tissues that consist primarily of macrophages and neutrophils is found. Granuloma formation has been shown to play an important role both in the

development (Potter and MacCardle, 1964) and in the maintenance (Cancro and Potter, 1976) of the primary plasmacytoma. A growth factor (25-kD) that is expressed by a macrophage cell line was found to stimulate the growth of plasmacytoma cells *in vitro* (Nordan and Potter, 1986). This growth factor is likely to be interleukin-6 (IL-6) as judged by its size and biological activity. IL-6, a pleiotropic cytokine, induces the terminal differentiation of B cells into antibody-secreting cells, and also acts on a variety of other cell types (Kishimoto and Hirano, 1988). Pristane treatment presumably results in the secretion of this growth factor from the chronic granuloma tissue. The involvement of IL-6 in the *in vivo* growth of plasmacytomas is further implied by the following finding. Overexpression of IL-6 in transgenic mice, although not sufficient to cause the development of mouse plasmacytomas, induces a massive plasmacytosis in thymus, lymph node, spleen, and other tissues (Suematsu et al., 1989). Transfection with the IL-6 gene into IL-6-dependent plasmacytoma cells has been shown to increase their tumorigenicity (Vink et al., 1990). The involvement of growth factor stimulation is similar to that seen in Burkitt's lymphoma by EBV with the exception of its being paracrine instead of autocrine and its being induced chemically rather than through viral infection.

Chromosomal translocation has also been found in almost all plasmacytomas (Yosida et al., 1970; and references listed below).

Two types of translocations were first described by Shepard and coworkers (1974a, 1974b, 1978) in transplanted plasmacytoma lines. One consists of a reciprocal translocation between chromosomes 12 and 15, and was later termed

the "typical" translocation by Ohno et al. (1979) because of its higher occurrence. The other is a reciprocal translocation between chromosomes 6 and 15, and was named the "variant" translocation by the same group because of its relatively lower occurrence. Both translocations are associated with Ig gene loci; the "typical" one usually involves the alpha switch region of the heavy chain gene (Kirsch et al., 1981; Adams et al., 1982; Harris et al., 1982), whereas the "variant" one involves the kappa light chain gene (van Ness et al., 1983; Webb et al., 1983; Cory et al., 1985). Analyses of the breakpoint on chromosome 15 in these translocations have revealed the involvement of the c-*myc* gene (Shen-Ong et al., 1982; Adams et al., 1983; Marcu et al., 1983). The consequence of the translocation has been proposed to be similar to that of Burkitt's lymphoma, i.e., the deregulation of c-*myc* gene expression (Adams et al., 1983; Bernard et al., 1983).

Other secondary events have been observed in some but not all of plasmacytomas. In a few plasmacytomas, activation of the c-*mos* gene by insertion of an intracisternal A particle element was found in addition to c-*myc* activation (Rechavi et al., 1982; Canaani et al., 1983; Gattoni-Celli et al., 1983). The v-*mos* protein exhibit activities of both serine/threonine autophosphorylation (Maxwell and Arlinghaus, 1985) and ATP-dependent DNA binding (Seth et al., 1987) *in vitro*. Two different biological activities have been associated with the *mos* protein: inducing monocyte differentiation into macrophages (Kurata et al., 1989) and transforming both NIH3T3 cells and normal kidney cells into malignant cells (Kurata et al., 1987). The biochemical basis of these two different potentialities is not clear. Its cytoplasmic location and kinase activity suggest a role in a signaling pathway. Alteration of a signaling pathway may contribute to the progression of

malignancy.

Perlmutter and associates (1984) found a t(6,10) recombination besides t(12,15) in the NS-1 murine plasmacytoma line and another plasmacytoma. Transcription from this locus was detected at a high level, and homologous sequences could be detected in mouse, rabbit, and human. Therefore, it is possible that t(6,10) may encode a gene that plays a role in the course of B cell tumor progression.

Lastly, chromosome 11 trisomy is another frequent secondary alteration in murine plasmacytomas (Ohno et al., 1984). Several oncogenes (Rel, ErbA, ErbB, p53), and cytokine genes (G-CSF, GM-CSF, IL-3, IL-4, IL-5) have been mapped to chromosome 11 (Buchberg et al., 1989; Wilson et al., 1990). Among these genes, IL-4 is a factor involved mainly in the activation of resting B cells, and IL-5 is a factor for the growth and maturation of activated B cells (Kishimoto, 1985). An increased dosage of these two genes may contribute to the formation of plasmacytoma, and perhaps act cooperatively with IL-6.

The involvement of multiple oncogenes in the development of murine plasmacytoma is not only suggested by the above findings, but also by the rapid induction of murine plasmacytoma with viral oncogenes. Infection of mice with Abelson murine leukemia virus after application of pristane greatly reduces the latency of tumor induction (Ohno et al., 1984). These tumors not only express v-*abl*, but show the same translocations activating c-*myc* that are observed in tumors induced by pristane alone. Infection with a recombinant retrovirus expressing an avian v-*myc* after pristane application also accelerated plasmacytoma formation (Potter et al., 1986, 1987). In this case, expression of v-*myc* seems to replace the

requirement for c-*myc* translocation. As with Burkitt's lymphoma, no single gene is sufficient for plasmacytoma formation.

3.3 Avian lymphoid leukosis

Lymphoid leukosis, a B-cell lymphoma induced by avian leukosis viruses (ALVs), progresses through a series of clinically distinct stages (Cooper et al., 1968). Three stages have been identified in ALV-induced lymphoid leukosis (Cooper et al., 1968; Baba and Humphries, 1985). The earliest detectable lesion is the transformed follicle, a hyperplastic bursal follicle in which the normal follicular architecture is obscured by an abnormal proliferation of lymphoblasts. The lymphoblasts are confined to their follicle of origin during this early stage. Up to 100 transformed follicles may be present in a single bursa, but most of these regress during bursal involution. Then, one or sometimes more than one of the transformed follicles gives rise to a bursal nodule, which is readily identifiable at necropsy. Ultimately, tumor cells from the bursal nodule disseminate to other tissues, resulting in widespread metastasis in the liver, kidney, and spleen. The instance of regression after the first stage suggests the requirement of additional events for further progression.

Molecular analyses of ALV-induced lymphomas show that the majority of them contain proviral integrations within or near the c-*myc* gene resulting in deregulated expression of c-*myc* (Hayward et al., 1981; Neel et al., 1981; Payne et al., 1982). The insertional activation of c-*myc* appears to be an early event in lymphomagenesis according to studies of Neiman et al. (1985), but additional proto-oncogene activations are required for progression to the late stages of the

disease. Neiman and coworkers have found that HB-1, a v-*myc*-containing virus, induces transformed follicles when virus-infected cells are used to reconstitute a chemically ablated bursa (Neiman et al., 1985; Thompson et al., 1987). Like ALV-induced transformed follicles, only a small portion of these progress to become lymphomas. These results again indicate that additional genetic events beyond c-*myc* induction may be required for late stages of tumor progression. Clurman and Hayward (1988) have used a double-infection protocol to facilitate the occurrence of multiple insertional activation in order to identify those genetic events that may function in cooperation with c-*myc* to induce late stages of progression in ALV-induced lymphomas. In their study, c-*myc* rearrangement was found in 70% of lymphomas (both primary and metastatic) identified 3 to 4 months after hatching. Additionally, a frequent viral integration locus, c-*bic*, was found in 14% of the primary lymphomas, and 50% of the metastatic lymphomas. These results confirm that the c-*myc* gene is frequently a target for insertional activation in ALV-induced lymphomas. They also indicate that c-*bic* may act synergistically with c-*myc* during lymphomagenesis. The increased frequency of viral integration at c-*bic* in metastatic tumors suggests that c-*bic* is involved in late stages of tumor progression.

In summary, studies on oncogene alteration in B-cell lymphomas/leukemias of three species have revealed the mutli-factorial nature of tumor formation. These gene alterations caused by either insertional mutagenesis or chromosomal translocations show different degrees of association with B-cell lymphomas/leukemias. The most commonly seen gene altered, c-*myc*, shows a more broad range of association with tumors of different cell types, whereas other

genes altered, such as *bcl*-2 and IL-6, show a more restricted linkage to lymphoid tumors. The *bcl*-2 gene alteration has been implicated in lymphoid tumors of multiple developmental stages such as follicular lymphoma, diffuse small and large cell lymphoma, and chronic lymphocytic leukemia. The IL-6 gene alteration, however, couples specifically to plasmacytoma. The significance of these differences is not yet known. Maybe only mature cells have IL-6 receptors. A systemic analysis with various probes on a greater number of tumor samples will be required to obtain a clear sense of the tissue and developmental specificity of these oncogene activations.

These affected genes found in B cell neoplasias encode a variety of proteins including growth factors (e.g., IL-6), membrane associated proteins (e.g., N-*ras*, *bcl*-2), signal transduction proteins (e.g., c-*mos*), and many cell cycle or differentiation-related regulatory proteins (e.g., c-*myc*, *bcl*-1, *bcl*-3 etc). Since these proteins are presumably involved in the regulation of cell growth and differentiation, deregulation of these genes should be important in the oncogenesis of B-cell lymphomas/leukemias. Although their roles in these malignant tumors seem obvious, a direct test of their oncogenic potential should be provided to ultimately distinguish their causative effects in tumorigenesis from their simply being the result of malignancy. Furthermore, analyses of end point specimens, such as malignant tumors, do not provide direct information on the effects of differentiation upon oncogene activation or the nature of genes that may be activated in lymphomas/leukemias in addition to those in an obvious translocation. To address these questions, a few model systems, including the use of *in vitro* culture systems, have been used. Results of these studies are summarized in the

following section.

3.4 *In vitro* transformation of murine B cells

A number of oncogenes or growth factors have been found to induce transformation of B lymphoid cells *in vitro*. These may include (i) oncogenes of the *ras* oncogene family which code for protein with GDP-binding activity (for review see Barbacid, 1987), (ii) oncogenes of the *src* oncogene family which code for tyrosine-specific protein kinases (for review see Hunter and Cooper,1985), (iii) the v-*fms* oncogene whose cellular counterpart encodes a colony stimulating factor-1 receptor, and (iv) the interleukin-7 (IL-7) gene which codes for a pre-B cell growth factor gene (Namen et al., 1988).

V-*abl* is the first viral oncogene that is capable of transforming murine pre-B cells *in vitro* (Sklar et al., 1974; Rosenberg et al., 1975; Rosenberg and Baltimore, 1976). Infection of cell cultures derived from murine fetal liver, bone marrow or spleen, but not thymus with Abelson murine leukemia virus (A-MuLV) carrying the v-*abl* oncogene give rise to permanently growing, neoplastic cell lines. These *in vitro* isolated cell lines are similar morphologically to A-MuLV-induced tumor cells display a characteristic of B cells, and have been found to be preferentially pre-B cells (Siden et al., 1979). Another oncogene of the *src* gene family, v-*fes*, also exhibits oncogenic potential on murine pre-B cells *in vitro* (Pierce and Aaronson, 1983). In this study, bone marrow cells were infected with a v-*fes* containing retrovirus (Snyder-Theilen feline sarcoma virus, ST-FeLV) and selected for their ability to grow on soft agar, a characteristic of transformed cells. In both cases, A-MuLV or ST-FeLV infection alone appears to be sufficient to induce aggressive

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tumors in syngeneic mice with a 1-3 weeks latency. However, results from Whitlock and Witte (1981) using a fresh bone marrow cell suspension as target for the A-MuLV infection and a feeder layer to support the growth of the initial infected population, show that the A-MuLV infected cells are initially poorly oncogenic and that they become progressively tumorigenic and growth independent only after progression on normal adherent bone marrow feeder layers. This latter result suggests that a minor subpopulation capable of unrestricted growth is present at the initiation of the culture and gradually becomes the predominant population through a slight growth advantage. Alternatively, the A-MuLV-infected cells may undergo secondary changes which further alter their growth properties. Using clonal cell lines isolated from the A-MuLV infected cells, Whitlock et al. (1983b) have shown that the progression of these clonal cell lines to a more malignant growth phenotype occurs with no changes in viral related properties. The expression of cellular genes appears to alter the growth properties of lymphoid cells after their initial transformation by A-MuLV. The discrepancy between Rosenberg's and Whitlock's results may residue in the properties of cells used for the initial infection, and the stringency of the growth parameters used to determine transformation. Since the cells used by Rosenberg's group have been cultured *in vitro* for a period of time, cells with a slight growth advantage may be preselected for viral infection. The lack of a feeder layer again selects for cells with a more transformed phenotype. A similar explanation may be applied to the results from Pierce and Aaronson (1983), in which the soft agar assay may select for events additional to the ST-FeLV infection. In conclusion, these results suggest that v-*abl* or v-*fes* may, by itself, be sufficient to initiate transformation of B cells but may

require additional events, such as the activation of cellular oncogenes, for expression of the fully transformed state.

The involvement of the v-Ha-*ras* and v-*bas* oncogenes in the *in vitro* transformation of lymphoid cells was demonstrated by Pierce and Aaronson (1982; 1984) using a similar approach as that described for the v-*fes* oncogene (see above). The Harvey murine sarcoma virus carrying a v-Ha-*ras* oncogene, and the Balb-murine sarcoma virus carrying a v-*bas* oncogene were used instead. The resulting transformed cell lines were as tumorigenic as those infected by A-MuLV. Cells transformed by v-Ha-*ras* or v-*bas* displayed characteristics of immature lymphoid cells: high terminal deoxynucleotidyl transferase (Tdt) activity, the presence of Fc receptor, and mercaptoethanol dependence for growth. The lack of μ chain expression, and of Thy-1 antigen on these transformed cells suggested that these cells might be at an earlier stage of differentiation than pre-B or pre-T lymphoid cells. Holmes et al. (1986) further confirmed the role of oncogenes in the *ras* gene family in the *in vitro* transformation of B cells. They demonstrated that transformed cell lines obtained from cells infected with viruses containing v-Ha-*ras*, v-*bas*, and v-K-*ras* display characteristics of pro-B and pre-B cells. Their finding suggest that a wide range of oncogene can induce B cell transformation *in vitro*.

The difference in the target cells for the *ras* oncogene family in the results of Holmes et al. and, Pierce and Aaronson may be a reflection of a continuous differentiation, which may occur under the influence of the microenvironment of a particular experimental setup, of a lymphoid progenitor (or a hematopoietic progenitor in a broader aspect). This hypothesis is supported by the isolation of

macrophage-like cell lines from pre-B cell lines containing the v-Ha-*ras* oncogene obtained in the same study (Holmes et al., 1986). This lineage-switch phenomenon is also found in pre-B cells transformed by the v-*fms* oncogene (Borzillo et al., 1990; see below), and the v-*raf* oncogene (Klinken et al., 1988), as well as in our study (Appendix A).

Another disparity is that viruses carrying v-Ha-*ras*, v-*bas*, and v-Ki-*ras* can also induce sarcomas and erythroleukemias in susceptible animals (Peters et al., 1974; Harvey, 1964; Scher et al., 1975; Hankins and Scolnick, 1981) and transform myeloid cells *in vitro* (Pierce and Aaronson, 1985), whereas A-MuLV seems to have a restricted target cell for transformation. This tissue-preference of tumorigenesis of A-MuLV appears to be directly associated with the v-*abl* oncogene from the studies of transgenic mice in which tumors of a B lymphoid origin are found predominately in transgenic mice carrying the v-*abl* oncogene (for review, see Adams and Cory, 1991). These results suggest that the tyrosine kinase encoded by the v-*abl* oncogene may belong to a pre-B cell specific signal transduction pathway, whereas the G-protein activity encoded by the *ras* gene family may represent a common signal pathway shared by several cell types.

Borzillo and Sherr (1989) have shown that the v-*fms* gene is capable of inducing transformation of pre-B cells. As with v-*abl* transformants, only the late-passage cultures give rise to factor-independent variants that proliferate in the absence of feeder layers, and become tumorigenic in syngeneic mice. The v-*fms* oncogene encodes an analog of CSF-1R that retains a functional ligand-binding domain but acts constitutively as a tyrosine kinase because of mutations. The mechanism of this mononuclear-macrophage-lineage related receptor functioning

in pre-B cells is not well known. The fact that some of the v-*fms* transformed pre-B cell line may undergo a lineage switch to macrophages when transferred from RPMI1640 to Iscove modified Dulbecco medium suggests that v-*fms* or its cellular analogue may be a common intermediate in the signal transduction pathways of both the lymphoid and myeloid differentiation (Borzillo et al., 1990).

In addition to the above oncogene, a pre-B cell growth factor, IL-7 also shows transforming potential on pre-B cells (Young et al., 1991; Overell et al., 1991). However, the low cloning frequency of IL-7 transformants and the long latency for tumorigenesis suggest that additional events are required in generating a fully transformed phenotype.

In summary, a variety of oncogenes, growth factors or receptors have been implicated in the *in vitro* transformation of B cells as in the *in vivo* tumorigenesis of B cell tumors from different species. Consistent with the multi-step tumorigenesis dogma, none of them are tumorigenic by themselves, and additional events must exist. These results validate the use of the *in vitro* model to examine the events that underlie tumor progression.

In my thesis, oncogene cooperation was used as a model to define the multi-factorial molecular events in the tumor progression of murine pre-B cells. The advantage of using oncogene cooperativity as a model to dissect the events contributing to tumor progression is the enhanced likelihood of identifying genes that by themselves are not capable of demonstrable transformation in growth factor independence assays, soft agar assays, and tumor challenges. However, genes with a subtle or negligible phenotype in the above assays may be able to transform B cells in cooperation with other oncogenes in a synergistic manner, for example

with v-*myc* or v-Ha-*ras*, and thus are potentially involved in the course of tumor progression. In setting up a system to examine cooperative transformation of B lymphoid cells, v-*myc* was the first candidate because c-*myc* activation was found in many of the B cell malignancies described above. Unfortunately, the v-*myc* gene was not able by itself to transforme primary bone marrow cells in the Whitlock and Witte culture system and may be lethal (data not shown, Stevenson and Volsky, 1986). We therefore chose v-Ha-*ras*, partly because v-Ha-*ras* has been found to transform murine B cells into an intermediate transformed phenotype (Schwartz et al., 1986a; Holmes et al., 1986), and partly because *ras* gene aberration was found in some B cell neoplasias (see previous section). The two model systems used in my studies are described in the "Introduction".

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Chapter 2

Vol. 178, No. 3, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
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Tumorigenesis of a V-Ha-Ras-Expressing Pre-B Cell Line Selects for C-Myc Activation

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Seven tumors independently derived from a v-Ha-ras-expressing pre-B cell line were examined to determine the oncogene activations cooperating with v-Ha-ras in in vivo tumor progression. The pre-B cell line was generated by infection with Moloney murine leukemia virus (MoMuLV) and a MoMuLV-derived recombinant expressing v-Ha-ras. Two of seven tumors possessed a MoMuLV integration immediately upstream and in reverse transcriptional orientation to c-myc. This correlated with a 3-fold increased level of c-myc mRNA. Two other tumors displayed elevated c-myc mRNA levels, although the mechanism of enhanced expression was unclear. Thus the tumor progression of a v-Ha-ras-expressing murine pre-B cell line selects for the activation of c-myc.

It is generally accepted that tumorigenesis proceeds through multiple events involving the altered expression or structure of specific oncogenes that function in the regulation of cellular growth. The requirement of two cooperating oncogenes for the in vitro neoplastic transformation of many primary cells may be a reflection of this process. Several examples of oncogene cooperativity in lymphoid transformation have been described in experimental systems. Infection by Abelson murine leukemia virus accelerates the induction of plasmacytomas by pristane (1). These tumors not only express v-abl, but possess the same c-myc translocations observed in tumors induced by pristane alone. Cooperativity between v-raf and v-myc has been observed in the induction of both B lymphomas (2) and plasmacytomas (3). A synergy of v-Ha-ras and v-myc has been observed in the in vitro transformation of pre-B lymphoid cells (4). Either v-Ha-ras or v-raf can cooperate with activated myc in the transformation of Eμ-myc transgenic pre-B lymphoid cells (5,6). While in vitro manipulations have revealed pairs of oncogenes capable of eliciting full neoplastic transformation, there have been few tests of what oncogenes might be selected in vivo to achieve transformation given the prior expression of a single oncogene that is incapable by itself of eliciting transformation. The retroviral activation of c-fms has been observed in a monocytic tumor

induced by a c-myc-expressing retrovirus (7). Ras mutations were observed in some lymphomas generated in Eμ-myc transgenic mice (8).

In earlier studies, we found that v-Ha-ras-expressing pre-B lymphoid cells displayed an intermediate transformed phenotype and were infrequently tumorigenic (4). The irregular occurrence of tumors derived from these cells suggested continued neoplastic progression in vivo, or selection in vivo for pre-existing subpopulations containing additional neoplastic mutations. Since v-myc was found to cooperate with v-Ha-ras in the in vitro transformation of pre-B lymphoid cells, we decided to investigate the status of c-myc in tumors derived from a v-Ha-ras-expressing pre-B cell line.

MATERIALS AND METHODS

Cell culture. R2, a v-Ha-ras-transformed murine pre-B cell line, and tumors derived from R2 are described in Schwartz et al. (4). Tumor cell lines were produced from explanted tumors by dispersal onto feeder cultures of adherent bone marrow cells (9). Cell lines were cultured over feeder cells in RPMI 1640 with 5% fetal calf serum and 5 x 10^{-5} M 2-mercaptoethanol.

Nucleic acid analyses. Cytoplasmic RNA was isolated by a sodium dodecyl sulfate-urea procedure as described by Schwartz et al. (10). Poly A^+ RNA was selected by oligo-dT cellulose chromatography. RNA was denatured, electrophoresed in a formaldehyde-1% agarose gel and transferred to Nytran (Schleicher and Schuell). High molecular weight DNA was isolated from nuclei collected in the preceding RNA isolation procedure as described in Schwartz et al. (4). DNA was digested with restriction enzymes as noted, electrophoresed through agarose and transferred to Nytran.

Hybridization probes were prepared by nick translation through the incorporation of [α-^{32}P]dATP (3000 Ci/mmol; ICN). The v-Ha-ras probe was a 0.46 kb EcoRI fragment corresponding to v-Ha-ras encoding sequences (11). The env probe was a 0.8 kb BamHI fragment from the env region of Friend murine leukemia virus (12). The c-myc probes were the 4.7 kb HindIII fragment of murine c-myc encompassing exons 1, 2 and 3 (Figures 3 and 6) and the 0.8 kb SmaI-SacI fragment encompassing 5' upstream flanking sequences and part of exon 1 (Figures 4 and 5). The rat glyceraldehyde-3-phosphate dehydrogenase (rGAPDH) probe was a cloned 1.3 kb cDNA (13). All hybridizations were performed under aqueous conditions in 5 x SSC at 65°C and washed to a stringency of 0.1 x SSC at 65°C.

RESULTS

Seven tumors independently derived from a v-Ha-ras-expressing pre-B cell line were studied in regard to their retroviral integration sites and status of c-myc structure and expression. These tumors were derived from the clonal R2 cell line that was generated by infection of fresh murine bone marrow with a recombinant v-Ha-ras-expressing retrovirus and MoMuLV (4). This cell line is dependent upon a bone marrow-derived cellular feeder layer for growth and gives rise to tumors infrequently.

The tumors are derived from R2. In order to verify that the tumors were derived from R2, sites of integration of the v-Ha-ras-expressing retrovirus were compared between that cell line and the tumors. Southern hybridization of EcoRI-digested DNA with a v-Ha-ras probe showed that the tumors contained

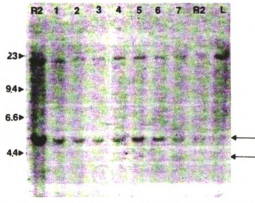
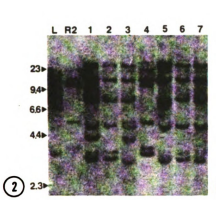


FIGURE 1. Viral *ras* integrations. Southern blot analysis of DNAs from liver (L), R2, and seven tumors (1-7). DNA was digested with EcoRI and 10μg of each sample was electrophoresed through 0.8% agarose. The blot was probed for v-Ha-*ras*. Size markers are the positions of an ethidium bromide-stained Hind III digest of bacteriophage λ and are denoted in kilobases.

the same 5.3 kb proviral integration fragment as R2 (Figure 1). In addition to the 5.3 kb fragment, there is a 23 kb fragment representing the endogenous c-Ha-*ras* gene in all the DNAs, and an additional 4.2 kb fragment in tumor 7. This 4.2 kb fragment represents an additional v-Ha-*ras* proviral insertion site (data not shown).

The tumors are clonal derivatives of R2. If the tumors resulted from genetic lesions subsequent to the introduction of v-Ha-*ras*, then they would be expected to be clonal outgrowths from the parental R2 cell line. R2 was generated by infection with a recombinant v-Ha-*ras*-expressing retrovirus and MoMuLV. Since proviral integrations can occur subsequent to the original integration event, it is reasonable to expect clonal outgrowths to contain unique sites of proviral integration in addition to any common sites derived from the parent. Examination of integrations of the v-Ha-*ras* retrovirus revealed only one new site in tumor 7 (Figure 1). We then examined the sites of MoMuLV integration in R2 and the tumors by Southern hybridization of BglII-digested DNA, using a probe for the ecotropic MuLV *env* gene. All of the tumors possessed *env*-hybridizing BglII fragments unique to the tumors and not present in R2 (Figure 2). Conversely, some fragments present in R2 were absent in the tumor cell lines. Fragments unique to the tumors represent either new proviral integrations or rearrangements of pre-existing integrations. The tumors (with the exception of tumor 4) clearly possess *env*-hybridizing BglII fragments more similar to each other than to R2, although individual tumors also possess unique BglII fragments. These data suggest that the tumors (with the exception of tumor 4) are clonal outgrowths derived from a single subclone of R2. Tumor 4 appears more closely related to the original R2 isolate.



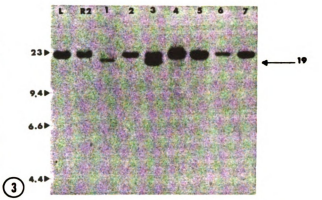


FIGURE 2. MoMuLV integrations. Southern blot analysis of DNAs from BALB/c mouse liver (L), R2, and seven tumors (1-7). DNA was digested with BglII and 10μg of each sample was electrophoresed through 0.8% agarose. The blot was probed for murine ecotropic env sequences. Size markers are the positions of an ethidium bromide-stained HindIII digest of bacteriophage λ and are denoted in kilobases.

FIGURE 3. Rearrangement of c-myc in tumors 1 and 3. Southern blot analysis of DNAs from liver (L), R2 and seven tumors (1-7). DNA was digested with EcoRI and 10μg of each sample was eletrophoresed through 0.8% agarose. Size markers to the left of photographs are the positions of an ethidium bromide-stained HindIII digest of bacteriophage λ and are denoted in kilobases.

Two tumors possess a c-myc allele rearranged by integration of MoMuLV. Having demonstrated that v-myc can cooperate with v-Ha-ras in the in vitro neoplastic progression of pre-B lymphoid cells (4), we investigated the status of the c-myc locus in the tumors. Southern hybridization of EcoRI-digested DNAs with a c-myc probe revealed that tumors 1 and 3 possessed a myc-hybridizing fragment of 19kb in addition to the normal 23kb fragment (Fig. 3).

Since the tumors displayed several proviral integrations, we investigated whether the rearrangement of c-myc in tumors 1 and 3 was caused by integration of MoMuLV. Assuming a proviral integration site near c-myc, and with complete restriction maps of both MoMuLV and c-myc, we could predict the sizes of restriction fragments that would be revealed by a c-myc probe in a Southern analysis (Fig. 4). Assuming that an intact MoMuLV provirus had integrated adjacent to exon 1 of c-myc in a reverse transcriptional orientation (the 5' long terminal repeat (LTR) juxtaposed to exon 1), a series of expected sizes were calculated (Table 1). We performed double digestions with XbaI/XhoI, SacI/XhoI and KpnI/XhoI to examine the presence of a MoMuLV LTR pattern of restriction sites in the vicinity of c-myc (Fig. 4). The data generated by digestions with restriction enzymes specific for LTR sites were in excellent agreement with the predicted sizes (Fig. 4 and Table 1). These data suggested integration of a MoMuLV genome within 100bp of the 5'-terminus of c-myc. In order to further verify the presence of MoMuLV near c-myc in tumors 1 and 3, BglII-digested DNA was analyzed by Southern blot with a probe for the MuLV env gene and a probe for c-myc that included exon 1 and 5'

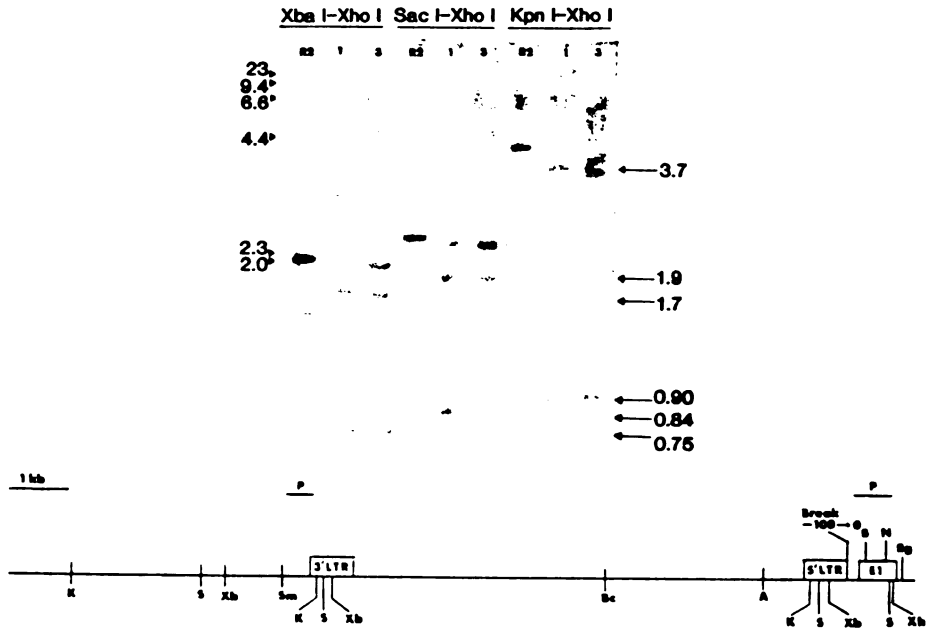


FIGURE 4. MoMuLV integration near c-myc. Southern blot analysis of DNAs from R2 and tumors 1 and 3. DNA was digested and 10μg of each sample was electrophoresed through 1.6% agarose. Size markers to the left are the positions of an ethidium bromide-stained HindIII digest of bacteriophage λ and are denoted in kilobases. The positions of rearranged c-myc fragments are marked to the right of photographs and denoted in kilobases. DNA was doubly digested with either XbaI and XhoI, or SacI and XhoI, or KpnI and XhoI. The blot was probed with a SmaI-SacI fragment encompassing upstream flanking sequences and part of the first exon of murine c-myc. A schematic of the murine c-myc locus with MoMuLV integrated upstream in a reverse transcriptional orientation is included. The following restriction sites are marked: KpnI (K), SacI (S), XbaI (Xb), SmaI (Sm), BclI (Bc), AatII (A), NotI (N), XhoI (Xh) and BglII (Bg). The probe is delineated by a line labeled "P" above the map. Note that probe "P" is interrupted by MoMuLV integration.

flanking sequences extending to a SmaI site about 500bp upstream of c-myc (Fig. 5). Tumors 1 and 3 displayed two identical rearranged c-myc-hybridizing fragments, one of which (see arrowheads) co-hybridized with the envelope

Table 1. Restriction fragments in the 5' c-myc region of tumors 1 and 3

Fragment	Expected Size	Observed Size
Exon 1/LTR:		
XhoI-XbaI	809bp	750bp
SacI-SacI	919	840
XhoI-KpnI	992	900
Upstream Region/LTR:		
XbaI-XbaI	1797	1700
SacI-SacI	1954	1870
KpnI-KpnI	4000[a]	3700

[a] Estimated value since the region between the upstream KpnI site and c-myc is not completely sequenced.

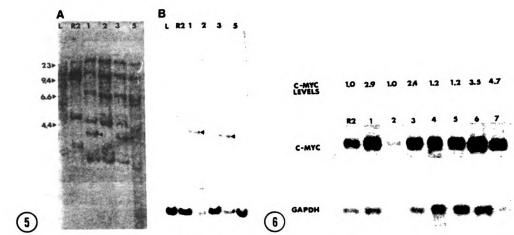


FIGURE 5. Env and c-myc co-hybridize. Southern blot analyses of DNAs from liver (L), R2 and tumors 1, 2, 3 and 5. DNA was digested with BglII and 10μg of each sample was electrophoresed through 0.8% agarose. Size markers are the positions of an ethidium bromide-stained HindIII digest of bacteriophage λ and are denoted in kilobases. Arrowheads mark co-hybridization. (A) The blot was probed for murine ecotropic virus env sequences. (B) The blot was probed for c-myc with a SmaI-SacI fragment encompassing upstream flanking sequences and part of exon 1.

FIGURE 6. Elevated c-myc mRNA levels. Northern blot analyses of polyA$^+$ RNA from R2 and seven tumor cell lines (1-7). Each sample of RNA was the polyA$^+$ fraction selected from 150μg of total cytoplasmic RNA. The blot was probed for both c-myc and rGAPDH. Relative levels of c-myc mRNA were determined by densitometry and normalized to rGAPDH.

region probe. An LTR probe detected fragments that co-migrate with both rearranged c-myc-hybridizing fragments indicating integration of both viral LTRs (data not shown). Clearly, tumors 1 and 3 had suffered MoMuLV integration in close proximity to c-myc.

MoMuLV integration causes increased transcription of c-myc. Having observed proviral insertion near c-myc in two tumors, we decided to test whether expression of c-myc mRNA was altered in these or any of the other tumors. Cytoplasmic poly A$^+$ RNAs of R2 and the seven tumor cell lines were prepared from actively growing cells and examined by Northern hybridization (Fig. 6). The same blot was hybridized successively with c-myc and rGAPDH probes. Hybridization to the rGAPDH probe provided a control for gel loading. Densitometry revealed that tumors 1 and 3 had 3-fold elevated levels of c-myc mRNA. Tumors 6 and 7, which displayed no gross abnormalities in c-myc, had 4-fold elevated levels of c-myc mRNA. The c-myc mRNAs were all 2.4kb in length, suggesting normal promoter usage and an unaltered RNA structure.

DISCUSSION

The data presented here demonstrate selection for c-myc activation in the lymphomagenesis of a v-Ha-ras-transformed murine pre-B lymphoid cell line. Four of seven tumors examined had 3 to 4-fold elevated levels of c-myc mRNA. In the case of tumors 1 and 3, MoMuLV had integrated immediately 5' to c-myc

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in a reverse transcriptional orientation, suggesting enhancer activation. The nature of the events leading to elevated levels of c-myc mRNA in the other two tumors is unclear.

The activation of c-myc by retroviral integration is the prominent feature of lymphomas induced by avian leukosis virus (14,15). MoMuLV activation of c-myc is also a frequent event in T lymphomas induced by that virus (16,17). This report of MoMuLV activation of c-myc in a murine B lymphoma is novel. The structure of the MoMuLV integration in tumors 1 and 3 is reminiscent of retroviral integrations observed in murine T lymphomas (16-19). The provirus is located upstream of c-myc in a reverse transcriptional orientation. The modest 3-fold increase in the steady state level of c-myc mRNA is similar to that observed by others in T lymphomas (18,20,21).

We have examined v-Ha-ras-induced B lymphomas for rearrangements at other frequent MoMuLV integration sites found in T lymphomas. Neither Mlvi-1 (22), Mlvi-2 (23), Mlvi-3 (24), Mlvi-4 (25), nor pim-1 (26) were found to be rearranged (data not shown). We have not yet examined a large enough panel of tumors to assess the importance of these frequent integration sites in B cell lymphomagenesis, but examination of MoMuLV integration sites may be fruitful in this system. The seven tumors studied possess MoMuLV integration-related restriction fragments unique to the tumors and absent from R2, the parental v-Ha-ras-expressing cell line. This indicates that the tumors are clonal outgrowths presumably having advanced in tumor progression due to mutagenic events. The patterns of viral integration in six of the tumors are more similar to each other than to R2. This suggests that a single subclone of R2 was predisposed toward tumorigenesis. The presence of MoMuLV integration near c-myc in two of these six tumors indicates that retroviral activation of c-myc is at least the third event in the progression of these tumors. This suggests that the activation of oncogenes other than ras and myc may be important for tumorigenesis. Our finding of common MoMuLV integration sites in addition to two instances of integration near c-myc presents the possibility that sites of MoMuLV integration may identify additional genetic elements that can cooperate with v-Ha-ras in the tumor progression of B lymphoid cells.

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Chapter 3

Cloning and characterization of a viral flanking region common to B lymphoid tumors derived from a v-Ha-*ras* infected pre-B cell line

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Abstract:

A viral flanking region, designated as *bonc*-1, common to B lymphoid tumors derived from a v-Ha-*ras* oncogene-transformed cell line, R2 (Chen et al., 1991) was cloned and characterized. This locus may be associated with the growth advantage of tumors derived from the R2 cell line. We were unable to detect any transcriptional activity over a 20 kb region in the vicinity of the *bonc*-1 locus. However, sequence homology within *bonc*-1 was found to the genome of other mammals, and chicken. The high evolutionary conservation within the *bonc*-1 region and the presence of several open reading frames suggest that one or more sequences of function importance exist therein. *Bonc*-1 was mapped to mouse chromosome 19, closely linked to the *lpr* locus, and distinct from another frequent viral integration site, *gin*-1, on the same chromosome. The *lpr* locus is linked to a recessive lymphoproliferation trait. Whether *bonc*-1 is identical to *lpr* awaits further investigation. The fact that it is close to the *lpr* locus provides a use for this locus in the search for genes responsible for the *lpr* lymphoproliferation trait.

Introduction:

Oncogenic transformation by nonacute transforming retroviruses such as avian leukosis virus (ALV), mouse mammary tumor virus (MMTV), and murine leukemia virus (MLV) depends on the activation of cellular protooncogenes by viral integration. Studies of frequent viral integration sites in animal tumors have been fruitful both in identifying new gene loci that could be involved in tumorigenesis, and in reconfirming the transformation potential of known protooncogenes (for review see Nusse, 1986a). Examples of the first case are *pim*-1 (Cuypers et al., 1984), *int*-2, (Nusse and Varmus, 1982) and *int*-2 (Dickson et al., 1984). *Pim*-1 was identified as a common region of insertion for mink cell focus-forming (MCF) virus or other MuLV in T cell lymphomas of several mouse strains (Cuypers et al., 1984; Selten et al., 1985). *Pim*-1 encodes a serine kinase (Selten et al., 1986), and may thus participate in cell signaling. Studies of transgenic mice carrying the *pim*-1 gene have verified its role in T lymphoid neoplasia (van Lohuizen et al., 1989b; Breuer et al., 1989a; Breuer et al., 1991). Increased expression of *int*-1 and *int*-2 as a result of MMTV insertion is thought to contribute to the formation of mouse mammary tumors through an autocrine loop (Aaronson, 1991). Both *int*-1 and *int*-2 are expressed temporally in a very restricted pattern during early development of the mouse, and the protein products show the characteristics of genes encoding secreted factors (for review see Nusse, 1988). Studies of transgenic mice bearing activated *int*-1 (Tsukamoto et al., 1988) or *int*-2 (Muller et al., 1990) confirm their oncogenic properties. Such animals develop mammary hyperplasia, and those with an *int*-1 transgene gradually develop mammary carcinomas with age. A wide range of cellular oncogenes activated by viral integration has been

found in a variety of tumors and these integration events have been implicated in the malignant phenotypes of the tumors. These include IL-3 (Morishita et al., 1988), GM-CSF (Stocking et al., 1988), CSF-1 (Baumbach et al., 1988), c-Ha-*ras* (Ihle et al., 1989), c-*erb* B (Fung et al., 1983), c-*myb* (Shen-Ong et al., 1984), N-*myc* (van Lohuizen et al., 1989a), p53 (Hicks and Mowat, 1988) and, most frequently, c-*myc* (Hayward et al., 1981; Neel et al., 1981; Payne et al., 1982; Westaway et al., 1984; Swift et al., 1985; Corcoran et al., 1984; Selten et al, 1984; Li et al., 1984; Steffen, 1984; O'Donnell et al., 1985; Neil et al., 1984).

Here we describe a locus that we have designated as *bonc*-1. *Bonc*-1 is one of several common integration sites found in 6 of 7 Balb/c B lymphoid tumor cell lines (Chen et al., 1991) (Chapter 2). These 7 tumor cell lines were derived from independent tumors which were collected after tumor challenge of Balb/c mice with a cell line (R2) established after infecting Balb/c bone marrow cells with a retroviral vector carrying the v-Ha-*ras* oncogene and helper Moloney murine leukemia virus (MoMuLV) (Schwartz et al., 1986a). The long latency and infrequent occurrence of tumor development within these animals led us to hypothesize the involvement of secondary events in the tumorigenesis of these 7 cell lines (Chen et al., 1991). The association of gene activation through viral integration to these secondary events was suggested by the observation of common viral integration sites in 6 of these 7 tumor cell lines (Chen et al., 1991). This result indicates the outgrowth of a transformed subclone from the original R2 cell population during the period of tumor progression. Studies of the viral flanking regions may reveal the molecular basis of the growth advantage possessed by this subclone, and, furthermore, the putative roles of the flanking regions in oncogenesis of B lymphoid

tumors.

Materials and Methods:

Mice. Balb/c.lpr mice were a gift from The Jackson Laboratories (Ban Harbor, Maine).

Cell culture. R2 and tumor cell lines were maintained on cellular feeder cultures as described in Chen et al. (1991). Only short term cultures of two weeks or less were used for molecular analyses to avoid accumulation of genetic alterations *in vitro*.

Nucleic acid isolation. High-molecular-weight DNA was isolated from nuclei collected as described by Schwartz et al. (1986a). Cytoplasmic RNA was isolated by a sodium dodecyl sulfate (SDS)-urea procedure as described by Schwartz et al. (1981). Poly A^+ RNA was selected by oligo-dT cellulose chromatography (Aviv and Leder, 1975). Testicle RNA was prepared by a LiCl/urea method with a motor driven polytron homogenizer (Auffray and Rougeon, 1980).

Molecular cloning. DNA from tumor 5 (T5) was partially digested with *Sau*3A and partially end-filled with dGTP and dATP, and then cloned into a *Xho*I half-site LambdaGEM-11 vector (Promega). The ligated DNA was packaged using a Packagene kit(Promega). The packaged DNA was titered and plated onto LB plates using LE392 (Promega) as host. The library was screened by a *env* or LTR enhancer-specific probe as described (Chen et al., 1991). Positive clones were purified and used to prepare phage DNA (Sambrook et al., 1989). Viral flanking regions were identified by hybridization and restriction mapping analyses. DNA fragments which contain viral flanking regions and with appropriate restriction site

ends were then subcloned into pBluescript IIKS+ (Stratagene). A *Pst*I DNA fragment of *bonc*-1 was used to "walk" through a NIH3T3 phage genomic library (a gift from Dr. DeWitt at Michigan State University). This library was generated in Lambda Fix™ II (Stratagene). Clones 11 and 32 derived from this library were isolated by similar approaches to those described above. Three *Sac*I DNA fragments of clone 11 and clone 32, respectively, were also subcloned into the pBluescript KSII+ vector for fine structure restriction mapping. Sequences unique to clone 11 and clone 32 were identified by hybridization of DNA blots used in the restriction mapping with a labelled liver DNA probe. Since 1/3 of total genomic DNA consists of repetitive sequences, restriction fragments that hybridize strongly to labelled liver DNA contain repetitive sequences unlikely to encode gene products. Therefore, sequences of these restriction fragments were excluded in northern analyses for detecting mRNA expression of *bonc*-1. Sequences unique to *bonc*-1 included the retire insert fragment of clone 11 except the two *Xba*I fragments in the very ends of the insert fragment, and included the 2.2 kb *Sac*I fragment in the right side of the clone 32 insert fragment as shown in Figure 2.

Blots and Hybridization analyses. Southern and Northern blot hybridization procedures were performed as described by Chen et al. (1991) except that a oligomer random priming kit (USB) was occasionally used as the probe labelling method. Plaque lifts were prepared as described in Sambrook et al. (1989). All washing conditions were performed to a stringency of 0.1 x SSPE at 65° C except in the case of the zoo blot where conditions are indicated in the figure legend. The rat glyceraldehyde-3-phosphate dehydrogenase (rGAPDH) and *env* probes were as described (Chen et al., 1991). The C3P2 probe was a 1.3 kb *Pst*I fragment

from the *bonc*-1 locus (this paper).

Restriction mapping. Restriction enzyme sites in the multiple cloning regions of the various vectors were used as reference points for mapping. Double digestion with these enzymes and sites in the cloned DNA were performed to construct the restriction map of *bonc*-1.

DNA sequencing. The C3P2 fragment was subcloned into Mp19 for single-strand-sequencing. Subclones which produced either (+) or (-) strand of the recombinant M13 phage DNAa were obtained. DNA sequences were determined by the dideoxy sequencing procedure (Sanger et al., 1977) using a sequencing kit from USB. The predicted sequence was analyzed with the sequence analysis software package of the University of Wisconsin Genetics Computer Group (Devereux et al., 1986) or Genepro4.2 (Riverside Scientific, Seattle).

Results:

Cloning of a tumor specific viral flanking region: *bonc*-1

A genomic library, lambda T5, was constructed from DNA of tumor cell line 5 (T5) to isolate tumor specific viral flanking regions. T5 was chosen because it apparently contains the highest number of viral integration sites as judged by the number of hybridized fragments on a southern blot hybridization analysis with a virus-specific probe for the envelope gene of ecotropic viruses (Chen et al., 1991). Four positive clones (A-D) were originally identified from 2 x 10^5 recombinant phages. Viral flanking regions of these clones were subcloned by first identifying the integration junction fragment and then isolating the non-viral subfragment from the junction fragment. Subsequent analyses of viral flanking sequences of individual clones revealed that clones A and B were derived from a viral integration site that was already present in the R2 parental cell line (data not shown); clone D consisted of the viral flanking region of an endogenous virus (data not shown); and clone C contained the flanking region of a tumor-specific virus integration site (Figure 1). Southern blot hybridization of *Eco*RI-digested DNA with a probe derived from the flanking region of clone C (C3P2) demonstrated that six of the seven tumor cell lines (lanes 3, 4, 5, 7, 8, and 9) contained a 20 kb fragment in addition to the 11 kb germ-line fragment, whereas the R2 cell line and the tumor 4 (T4) contained only the same germ-line fragment (lanes 2 and 6) as that in liver (lane 1). The size difference (9 kb) of these two fragments was about the size of a MoMuLV provirus (8.8 kb). Since *Eco*RI does not cut within the MoMuLV sequence, the 20 kb fragment probably resulted from an integration of an intact virus. This locus was designated as *bonc*-1, a putative B cell oncogene.

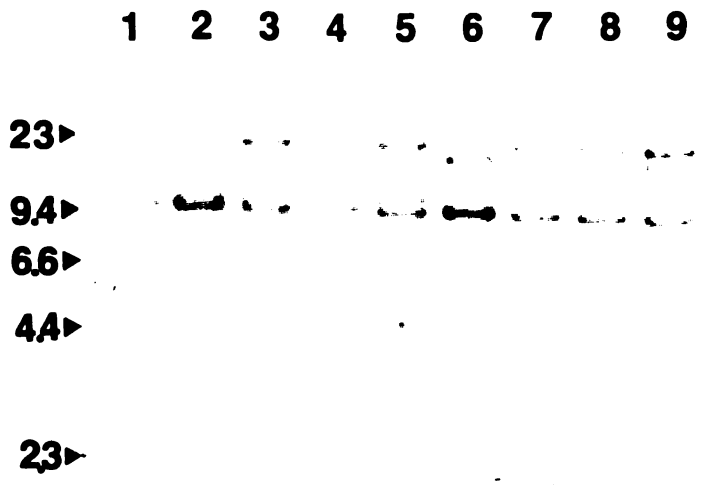

Figure 1. Tumor specific viral integration in the *bonc*-1 locus. Southern blot analysis of DNAs from liver (lane 1), R2 (lane 2) and tumor cells (lanes 3-9). DNA was digested with *Eco*RI and 10 μg of each sample was electrophoresed through 0.8 % agarose. The blot was probed for *bonc*-1. Size markers are the positions of an ethidium bromide-stained *Hind*III digest of bacteriophage λ, and are denoted in kilobases.

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Expression of *bonc*-1 was examined by northern blots of poly A^+ RNA isolated from 400 micrograms of cytoplasmic RNA to ensure the detection of low levels of expression. No mRNA was detectable in R2 or the tumor cell lines using the 1.3 kb C3P2 fragment as a probe (data not shown).

Since the viral LTR may function over a relatively long distance, the putative activated gene may be located distal to the integration junction region. Therefore, we decided to clone the distal regions of the flanking sites by chromosomal walking. We failed to obtain positive clones from the Lambda T5 library, when we rescreened this library with the C3P2 probe. A NIH3T3 library was used instead since NIH Swiss mice are closely related genetically to Balb/c mice. Two different clones were isolated. Clone 11 consisted of an 18.2 kb genomic fragment and clone 32 consisted of a 14.5 kb genomic fragment. Restriction mapping analyses revealed that the genomic fragments of these two overlapping clones covered a 20.5 kb region at the *bonc*-1 locus, with about 10.5 kb flanking sequence at each side of the viral integration (Figure 2). Three *Sac*I fragments derived from clone 11 and clone 32 were subsequently cloned into plasmid vectors to perform finer restriction mapping. The results obtained from restriction mapping these subclones and the original clones correlated well, and yielded the map illustrated in Figure 2.

Northern analysis was repeated with probes to the unique regions within the 20.5 kb region (Figure 2E). RNAs from different tissues were included for the detection of transcripts. Still, no mRNA was detected.

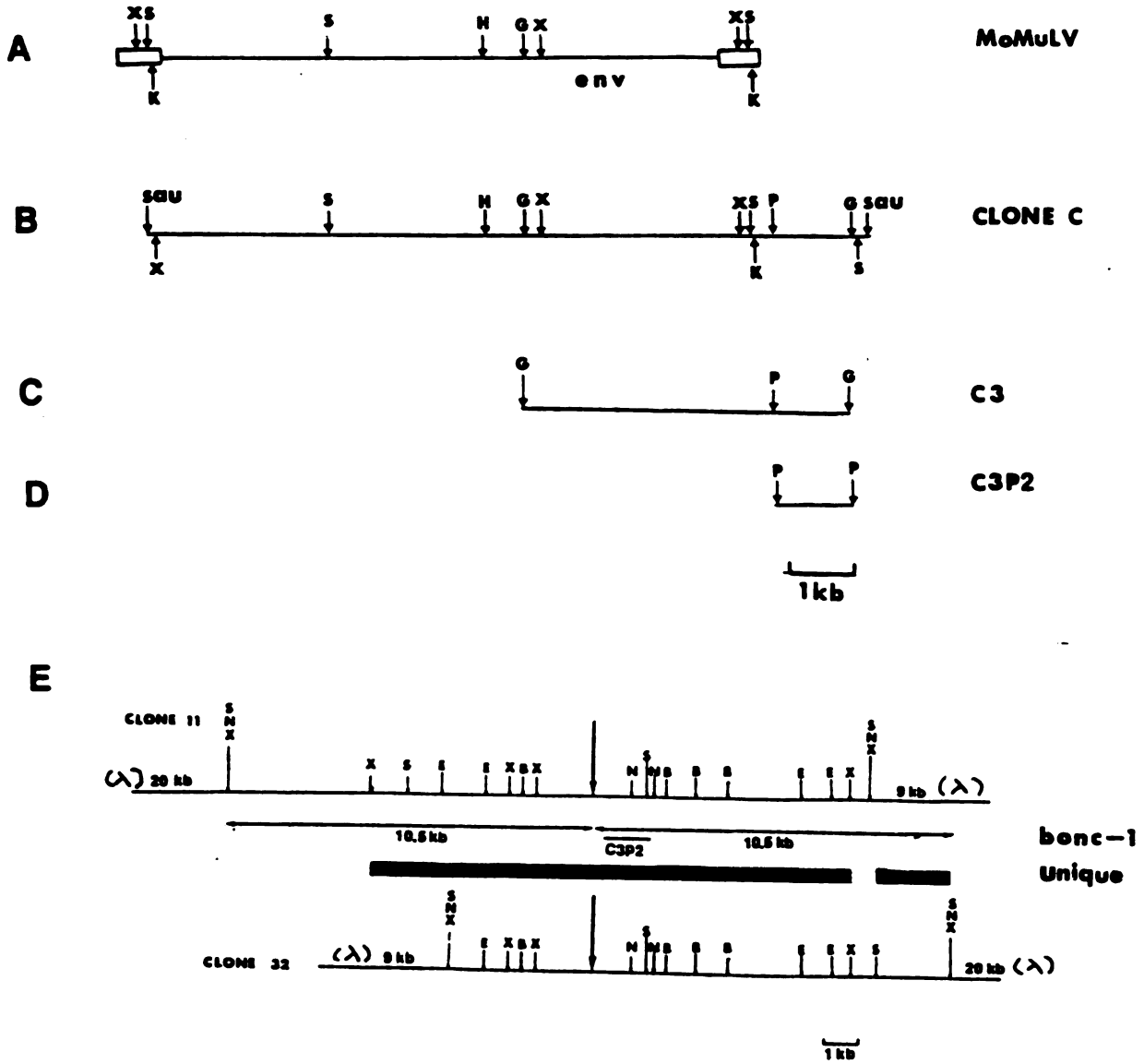

Figure 2. Cloning strategy and restriction endonuclease mapping of the *bonc*-1 locus. (A) Map of MoMuLV. (B) Map of the clone C isolated from the lambda T5 library. (C) Map of the C3 fragment. This *env*-containing *Bgl*II DNA fragment was subcloned for the isolation of the flanking cellular region. (D) The *Pst*I fragment (C3P2) containing the flanking cellular region was subcloned for use as a hybridization probe. (E) Map of genomic DNA from the *bonc*-1 locus. The big arrow represents the site of viral integration in Tumor 5. Restriction fragments containing sequences unique to this region are indicated by filled boxes. Restriction endonucleases: B, *Bam*HI; E, *Eco*RI; G, *Bgl*II; H, *Hind*III; K, *Kpn*I; N, *Not*I; P, *Pst*I; Sau, *Sau*3A; S, *Sac*I; X, *Xba*I.

Bonc-1 sequence was conserved among some species

A "zoo" blot containing DNAs from chicken, rat, human, hamster, bovine, and goat was used to examine the sequence conservation of the *bonc*-1 locus. Surprisingly, we could detect discrete bands by hybridization with C3P2 in all of the species after washing to a stringency of 0.2 x SSPE at 65° C (Figure 3) despite the fact that no mRNA was detected in this region.

Sequence analyses of *bonc*-1

The sequence conservation suggested that *bonc*-1 might contain important gene sequences. We therefore sequenced the C3P2 fragment. Figure 4 shows the DNA sequence of the entire 1327 bp C3P2 fragment, with some uncertainty in nucleotide numbers 444 and 446. This uncertainty is due to the strong pause of the sequence reaction at this region. Several methods including the use of ITP and temperature modification in the sequencing reactions have been used to resolve this sequence ambiguity without success. Sequences of the C3P2 fragment were compared to those in the GeneBank and EMBL databases. No homology was found (data not shown). When the sequence was examined for the presence of possible open reading frames (ORFs), several possible ones were identified (Figure 5). The longest ORF, from nucleotide 565 to 957, encoded 131 amino acids. When the protein sequences of these putative ORFs were examined for the presence of known functional domains (motifs) such as leucine-zipper, no known protein motifs were found (data not shown).

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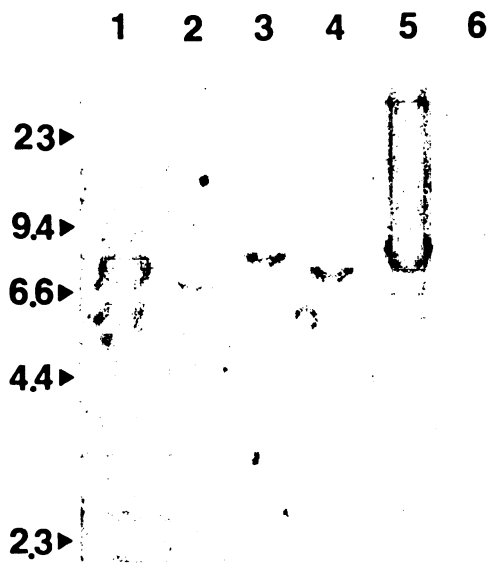

Figure 3. *bonc*-1 is evolutionary conserved. Southern blot analysis of DNAs from human (lane 1), hamster (lane 2), bovine (lane 3), goat (lane 4), chicken (lane 5), and rat (lane 6). DNA was digested with *Eco*RI and 10 μg of each sample was electrophoresed through 0.8 % agarose. The blot was probed for *bonc*-1. Size markers are the positions of an ethidium bromide-stained *Hind*III digest of bacteriophage λ, and are denoted in kilobases.

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CGCCGCGCGAGCTCGCCCAGACGGGGGCCCGCCCAGCCTGTCAGCGCGGCGGGAG 110

GCCGGCGGTGGCCGCTGCTGCCCCCTTTCTGCTCTGCCTCAGCTTCCTGCAGCCC 165

ACGCCACGCCCACCGCGCGCCGAGCCCGTCTCCGCCCTCCAGGGGCCGCACGCCG 220

CCTCCGCTCGGCCCCGCGACGCCGGCTCAGCTGCCCTGCTCGGCGGGCTCCGGCG 285

CGGTGCAGCTTCGGGAAAGCGGCCCCGAGCGGCGGACGGGGCGTGGGCAAGCCGG 330

CGGGGGTTGGCGGGGGGGCGGAGGCGGCACGCCCCACTGCGCCTGCGCGCCCGCG 395

GCCGCTCTGCGGGCTGGGCCGGGACGGGGAGGCGGCCGCGGGCTCCGGGGAAGCG 440

GAGNCNCGCGTGGAGATTCCCGGGGCAGCCCCCGCGAGAGCGCGCGAGGAGGAGG 495

AGGAGCGGGGCGGGTGCGGGTTGGCGCAGCGTGCTTGCGGCCTCGGCTCGCGGAG 550

GAGACGGCTGGGAATGAGTCAGCCCGGCCGGGAAGGCCCCGCTGCGTCCGAGCTG 605

ATAGGATTGGCGGCGCTGCCTCCGCGGAGACTTTCCCCTTCCTGCTTTCGTTTCC 660

ACAGGCGGCTCCCTGCCCTAAGCGCTCGGCCCAGGGGACGTGGCACCGTGGACCG 715

GGCGCTGAGACCCAAGTACCTGACTTCAGTTAACCACCACTTCTGCGAAGGGACC 770

GCTTGGAAGAAGAAACGACGTATAGGGTCCCAAAGAGCCACGTTCATTCATTCAA 825

GAATTTGTTCCCTGCTAGGCAAGGGCTCCCAGGAGAGGAGACGAAGGCAGCACCC 880

ATTCTGCATGTGCTATTCTCATAGAAGCCAGAAAAGAGGCTTTAAATCTGCACTG 935

CTCTTTGTGTGTGATTGCCAGTAACGGGTACACACACACACACAAAGGTGAGTGG 990

AGATGTTATTTTGAGATGACCAGAAAAGACCTCTTAGAGTGGCATTTGAGCAGAA 1045

ATGTGAAGGATGAGAACGATCCAGTCCTGGAACTATCTGGTCAAGAAAGAAGCTA 1100

ACAATGAAAAGGATTCTGAAAAGGGAATGTGTTTTAAGGACCAAGAAAGAACCCT 1155

CAATGGAAGACAGTAAAAGGAAAGGGGCTAAATAAGAAGACAAAGAAATAACTGG 1210

GGTCATTCTGTATAGGGCTTCTTATTTTTTCTCTTAGCCCCAGGTTTGTCAACCG 1265

AAATGAAGCATTCACAACACCCACGTCCCCTTATATACGTGACGGATGCTGTTAT 1320

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Figure 4. DNA sequences of the C3P2 fragment. X indicates undetermined nucleotides.

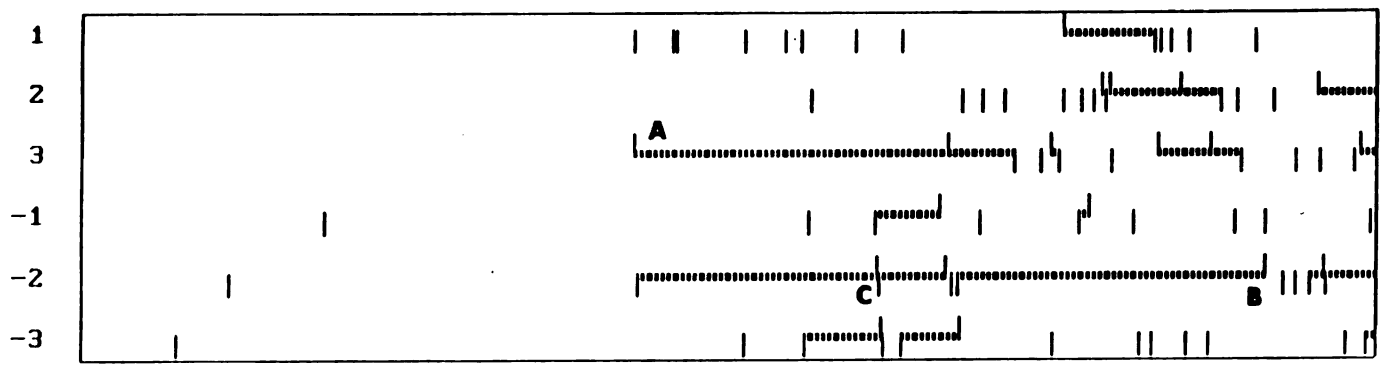

Figure 5. Putative open reading frames in the sequence of the C3P2 fragment. Open reading frames were predicted by the Genepro4.2 computer program. 1, 2, 3, -1, -2, and -3 represent the six possible reading frames. The solid boxes indicate sequences within an open reading frame. The vertical bars above the solid box represent initiation codons, whereas those below the solid box represent termination codons. A, B ,and C are the three longest open reading frames among found. The open reading frame A consists of 131 amino acid residues (565-957 nucleotide). The open reading frame B consists of 105 amino acid residues (1214-899 nucleotide). The open reading frame C consists of 82 amino acid residues (815-569 nucleotide).

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Bonc-1 was mapped to chromosome 19 by RFLP analyses

In collaboration with Marge Strobel, Neal Copeland and Nancy Jenkins (NCI, Frederick), the *bonc*-1 locus was mapped to mouse chromosome 19 by restriction fragment length polymorphism (RFLP) analyses. *Bonc*-1 was mapped to a region about 16.0 ± 2.7 cM from *Pomc*-2 and 2.3 ± 1.6 cM from *Cyp2c*. *Pomc*-2 is about 6.0 ± 1.7 cM from *Ly*-1, and *Cyp2c* is about 2.3 ± 1.3 cM from *Tdt*. Therefore, *bonc*-1 was 4.7 ± 3.0 cM from *Tdt*, and 22.0 ± 4.5 cM from *Ly*-1 (Figure 6). Two other loci were also mapped near this region: *lpr*, a genetic locus linked to a recessive lymphoproliferation trait (Lyon and Scarle, 1989), and a frequent virus integration site, *gin*-1 (Villemur et al., 1987). RFLP analyses showed that *gin*-1 is not identical to *bonc*-1 (Figure 6). Watanabe et al. (1990) have mapped *lpr* to 6.1 cM from *Tdt* and 19.3 cM from *Ly*-44, a locus physically mapped to chromosome 11q12-13 which is very close to *Ly*-1 in chromosome 11q13.

Due to the close linkage of *bonc*-1 to *lpr* and their common association with lymphoid disorders, we examined the possibility of both loci being identical by an RFLP analysis. DNA of Balb/c.lpr mice and Balb/c were digested with 7 different restriction enzymes, and subjected to a southern hybridization with the C3P2 probe. No DNA rearrangements were found in DNA of Balb/c.lpr mice (data not shown).

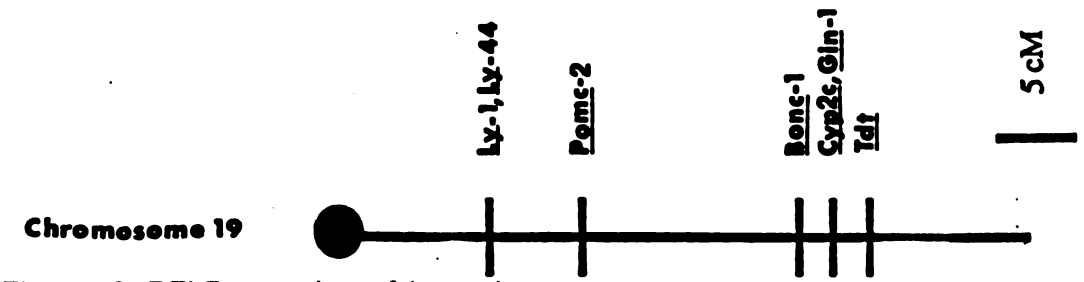

Figure 6. RFLP mapping of *bonc*-1.

Discussion:

A tumor-specific viral integration site (*bonc*-1) which may be associated with the growth advantage of tumors derived from the R2 cell line was isolated. Sequence homology to other species was found within this locus, significance of which remains unknown. Similar results have been observed at other loci: *evi*-1, which is a frequent viral integration site found in AKXD murine myeloid tumors (Mucenski et al., 1988a); and *dsi*-1, which is also a frequent viral integration site identified from Fisher rat thymomas (Vijaya et al., 1987). *Evi*-1 was later found to encode a zinc finger protein (Morishita et al., 1988), whereas no transcripts have been associated with *dsi*-1 thus far.

Although no mRNA was found over a region extending 10 kb to each side of the site of viral integration, we cannot rule out the possibility of the activation of a gene beyond this 20 kb region. Long distance activation of the *myc* protooncogenes has been reported by Lazo et al. (1990). They found that provirus integrations 30 kb 3' of c-*myc* (*mlvi*-4) and 270 kb 3' of c-*myc* (*mlvi*-1) enhanced c-*myc* expression in rat T lymphomas. Further chromosomal walking may resolve this question. It is also possible that the tumor-specific viral integration at this locus is coincidental with another true activation event. For example, there are still three or four tumor-specific viral integration sites remaining to be analyzed. Nevertheless, the conservation of the *bonc*-1 locus across species and the presence of open reading frames leaves open the possibility that it may represent a gene expressed at low levels, restricted to a tissue type, or restricted temporally.

Bonc-1 was mapped to mouse chromosome 19 near a previously described

frequent viral integration site, *gin*-1 (Villemur et al., 1987), and the *lpr* locus which is linked to a recessive lymphoproliferation trait (Lyon and Scarle, 1989). Results from RFLP analyses suggest that *bonc*-1 and *gin*-1 are not identical. Our results from RFLP analyses on the DNA of Balb/c and Balb/c.lpr mice did not suggest or exclude the possibility of *bonc*-1 and *lpr* being identical. However, because *lpr* is linked to a recessive trait and we only detected the gene rearrangement of *bonc*-1 on one allele, they are likely to be non-identical. The fact that it is close to the *lpr* locus provides a use for this locus in the search for genes responsible for the *lpr* lymphoproliferation trait.

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Chapter 4

IL-7 expression in a v-Ha-*ras* transformed pre-B cell line is not sufficient for tumorigenicity: differing assessments in clonal versus heterogeneous populations

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Abstract:

A recombinant retrovirus expressing IL-7 was superinfected into a murine pre-B cell line previously infected with a retrovirus expressing v-Ha-*ras*. Populations of cells polyclonal for integration of the IL-7 virus and independent of IL-7 for growth were generated. IL-7 expression caused only a minimal enhancement of tumorigenicity. Surprisingly, the introduction of v–*myc* expression also had only a minimal effect. In contrast, co-infection of murine bone marrow with retroviruses expressing IL-7 and v-Ha-*ras* generated clonal B lymphoid outgrowths that were tumorigenic. We propose that although IL-7 and v-Ha-*ras* may contribute to tumor progression, their introduction into a heterogeneous population of bone marrow cells allows the selection of additional oncogenic events occurring independently in that population. The oncogenic potential of IL-7 expression, in itself, and deregulated expression of other genes is probably best assessed in well-characterized individual cell lines.

Introduction:

The neoplastic transformation of hematopoietic cells is generally accepted to be a multi-step process (Schwartz and Witte, 1988). This process can involve the overexpression of hematopoietic growth factors, as well as events involving other classes of oncogenes (Sawyers et al., 1991). For example, a translocation activating IL-3 expression has been observed in a form of human acute pre-B leukemia (Meeker et al., 1990). Overexpression of IL-3 by itself has been shown to cause a myeloproliferative disorder rather than leukemia (Chang et al., 1989; Wong et al., 1989) suggesting that its role in leukemogenesis requires cooperation with other events. IL-7, a cytokine for pre-B cells (Namen et al., 1988) and T cells (Morrissey et al., 1989), has recently been examined for its role in the transformation of pre-B cells. Young et al. (1991) found that hyperexpression of IL-7 in a pre-B cell line was neither necessary nor sufficient for neoplastic transformation. Although the cell line's dependence upon exogenous IL-7 was alleviated, its growth in soft-agar medium was not enhanced and only one of six IL-7-expressing derivatives of the cell line was tumorigenic. In contrast, Overell et al. (1991) found that a pre-B cell line made growth factor-independent by hyperexpression of IL-7 was tumorigenic. However, their data indicated that events in addition to IL-7 expression were required for factor independence. These seemingly contradictory findings leave unresolved the potential for autocrine IL-7 expression to contribute to the tumor progression of pre-B cells. On the other hand, both studies suggest that hyperexpression of IL-7 may have a role in the cooperative transformation of pre-B cells with other oncogenes.

In previous studies, we found that v-Ha-*ras*-expressing pre-B lymphoid cells

displayed an intermediate transformed phenotype and were infrequently tumorigenic (Schwartz et al., 1986 a, b). The tumors that did arise were not dependent upon IL-7 for growth, unlike the pre-B cells from which they arose (unpublished observations, Chen and Schwartz). This led us to investigate whether high level autocrine expression of IL-7 would be sufficient for the tumorigenicity of a v-Ha-*ras*-expressing pre-B cell line. Since we had previously demonstrated the cooperativity of v-*myc* and v-Ha-*ras* in pre-B cell transformation (Schwartz et al., 1986 a, b) and a role for *myc* activation in the tumor progression of a v-Ha-*ras*-expressing pre-B cell line (Chen et al., 1991), we directly compared the oncogenic potential of IL-7 and v-*myc* in a v-Ha-*ras*-expressing pre-B cell line. While expression of IL-7 may partially alleviate dependence on exogenous growth factors for growth, it is clearly not sufficient for tumorigenesis. Curiously, we have found a disparity for both IL-7 and v-*myc* between the consequences of introducing their expression into a v-Ha-*ras*-expressing cell line and the simultaneous introduction of these oncogenes by co-infection of primary bone marrow cells. Pre-B cell lines derived by co-infection of bone marrow were fully tumorigenic (see results reported here and Schwartz et al., 1986a), while lines derived by sequential addition to a cell line were not. Neither IL-7 nor v-*myc* in combination with v-Ha-*ras* are truly sufficient in and of themselves for cooperative transformation of pre-B cells.

Results:

Superinfection of a v-Ha-*ras*-expressing pre-B cell line

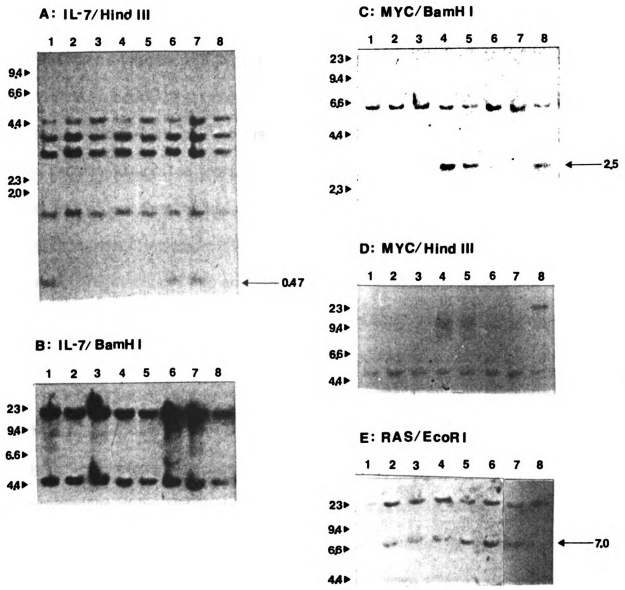
In order to test the oncogenic potential of IL-7 expression in cooperation with v-Ha-*ras* expression, we generated a v-Ha-*ras*-expressing cell line that could easily be superinfected by a retrovirus carrying the gene for IL-7. Fresh murine bone marrow was infected with a helper-free stock of SV(X)-Ha-*ras*, a v-Ha-*ras*-expressing retrovirus (Schwartz et al., 1986a), and the bone marrow was placed under long-term B cell culture conditions (Whitlock and Witte, 1982). A clonal pre-B cell outgrowth designated ψ2R4 resulted from this infection. This cell line expresses v-Ha-*ras* from a single proviral integration site and was found to be dependent on a cellular feeder layer or IL-7 for growth, unable to grow in soft agar medium, and not to be tumorigenic (data not shown). ψ2R4 was subjected to three different retroviral infections:

1. An IL-7-expressing retrovirus, IL-7(SH) (Young et al., 1991) and Moloney murine leukemia virus (MoMuLV).
2. A v-*myc*-expressing retrovirus, MMCV-*neo* (Wagner et al., 1985) and MoMuLV.
3. MoMuLV.

Three weeks post infection, DNA and RNA were isolated from the infected cultures and analyzed to assess infection and integration of the above mentioned viruses and to verify expression of v-Ha-*ras*, IL-7 and v-*myc* in the infected cells. Southern blots of DNAs isolated from two cultures each of MoMuLV, "IL-7" and "v-*myc*" infected cells were hybridized with probes for IL-7 (Figure 1A and B), v-*myc* (Figure 1C and D) and v-Ha-*ras* (Figure 1E). *Hind*III digestion (Figure 1A)

Figure 1. Proviral integration. DNA (10 μg) was digested with the indicated restriction enzymes, electrophoresed through 0.8% agarose, and transferred to nylon membranes. Lane 1, NIH3T3/IL-7(SH); lane 2, ψ2R4/MoMuLV-1; lane 3, ψ2R4/MoMuLV-2; lane 4, ψ2R4/*Myc*-1; lane 5, ψ2R4/*Myc*-2; lane 6, ψ2R4/IL7–1; lane 7, ψ2R4/IL7–2; lane 8, RM2. Size markers are the positions of an ethidium bromide-stained *Hind*III digest of bacteriophage lambda DNA. (A) *Hind*III-digested DNA was hybridized with an IL–7 probe. The position of the retroviral IL–7 gene is marked at 0.47 kb. (B) *Bam*HI-digested DNA was hybridized with an IL-7 probe. (C) *Bam*HI-digested DNA was hybridized with a v–*myc* probe. The position of the retroviral v–*myc* gene is marked at 2.5 kb. (D) *Hind*III-digested DNA was hybridized with a v-*myc* probe. (E) *Eco*RI-digested DNA was hybridized with a v-Ha-*ras* probe. The position of the v–Ha–*ras* integration-related restriction fragment is marked at 7 kb.

Figure 1. Proviral integrations.



revealed the diagnostic viral 0.47 kb IL-7-specific restriction fragment in both IL-7-infected cultures (lanes 6 and 7), as well as in the NIH3T3 cell line that produced the IL-7 virus (lane 1). The intensity of hybridization to this fragment is as great in the infected cultures as it is in the NIH3T3 virus-producing cell line, suggesting a high proportion of infected cells. The other restriction fragments represent the endogenous IL-7 gene. *Bam*HI digestion, which cleaves once within the IL-7 virus genome, was used to assess the number of viral integrations and the clonality of the IL−7-infected populations. This analysis (Figure 1B) revealed the IL-7-infected cultures to contain multiple viral integration-related restriction fragments of less than haploid abundance (lanes 6 and 7) indicating polyclonal populations of IL-7-infected ψ2R4 pre-B cells. All cells possessed two IL-7-specific restriction fragments of ˜4 kb and ˜18 kb. Rehybridization of the *Bam*HI-digested DNAs with a v-*myc* probe (Figure 1C) revealed a 2.5 kb restriction fragment diagnostic of v-*myc* infection in those infected populations (lanes 4 and 5), as well as in RM2 (lane 8), a v-Ha-*ras*/v-*myc* transformant previously characterized (Schwartz et al., 1986a). The restriction fragment of ˜6 kb represents endogenous c-*myc*. The v-*myc* infected populations were evaluated for viral integration by rehybridization of the *Hind*III-digested DNAs with a probe for v-*myc* (Figure 1D). *Hind*III cleaves the v-*myc* viral genome only once. Similarly to the case of IL-7 infection, the v-*myc*-infected cultures (lanes 4 and 5) were shown to be polyclonal with respect to v-*myc* viral integration with a smear of v-*myc*-specific restriction fragments as opposed to the single viral fragment in the clonal RM2 cell line (lane 8). The ˜5 kb restriction fragment represents endogenous c-*myc*. In order to verify that the outgrowths in the infected cultures were all derived from ψ2R4, the

v-Ha-*ras* viral integration sites were compared among the cultures. An *Eco*RI digestion cleaves once within the viral genome to yield integration site specific fragments. All of the infected cultures contained the same size v-Ha-*ras*-related restriction fragment of ~7 kb confirming their derivation from ψ2R4 (Figure 1E, lanes 2-7). The ~20 kb restriction fragment represents the endogenous c-Ha-*ras* gene.

Cytoplasmic RNAs of the infected ψ2R4 cells were examined by Northern hybridization analysis. The expected genome-length retroviral transcript of ~5.4 kb was observed in all the populations with a probe for v-Ha-*ras* (Figure 2A). Rehybridization with an IL-7 probe revealed the expected 4.0 kb genome-length retroviral transcript in both IL-7-infected populations (Figure 2B, lanes 5 and 6) and a v–*myc* probe showed the expected genomic ~8.0 kb and subgenomic ~5.5 kb RNAs in both v-*myc*-infected populations (Figure 2C, lanes 3 and 4). Rehybridization to a probe for rat glyceraldehyde phosphate dehydrogenase (GAPDH) verified that similar quantities of RNA were analyzed among the various infected populations (Figure 2D). Therefore the levels of mRNA expression for v-Ha-*ras*, v-*myc* and IL-7 were roughly equivalent between populations infected with viruses expressing those genes.

The superinfected ψ2R4 populations consist of pre-B cells

Given that the activity of IL-7 within the B lineage is restricted to pre-B cells, it was important to confirm that the superinfected cells had maintained the pre-B phenotype of ψ2R4. Southern hybridization analysis of *Eco*RI-digested DNAs revealed that all of the populations were identically rearranged in their mu heavy

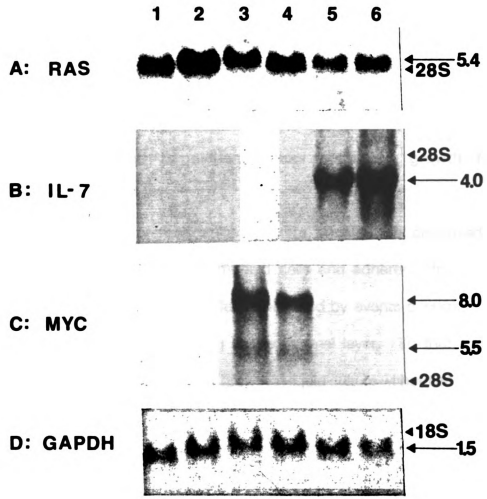


Figure 2. Retroviral transcription. Cytoplasmic RNA (20 μg) was denatured, electrophoresed in a 1% agarose-formaldehyde gel, and transferred to a nylon membrane. Lane 1, ψ2R4/MoMuLV-1; lane 2, ψ2R4/MoMuLV-2; lane 3, ψ2R4/*Myc*-1; lane 4, ψ2R4/*Myc*-2; lane 5, ψ2R4/IL7-1; lane 6, ψ2R4/IL-2. The positions of ethidium bromide-stained 28S and 18S rRNAs are marked. (A) Hybridization with a v-Ha-*ras* probe. The position of the SV(X)-Ha-*ras* genome-length RNA is marked at 5.4 kb. (B) Hybridization with an IL-7 probe. The position of the IL-7(SH) genome-length RNA is marked at 4.0 kb. (C) Hybridization with a v-*myc* probe. The positions of the MMCV-*neo* genome-length and subgenomic v-*myc* RNAs are marked at 8.0 and 5.5 kb, respectively. (D) Hybridization with a GAPDH probe. The position of GAPDH mRNA is marked at 1.5 kb.

chain locus (Figure 3A), again verifying that all of the cells were derived from ψ2R4. Analysis of *Bam*HI-digested DNAs revealed all of the populations to have agermline configuration in the kappa light chain locus (Figure 3B) confirming a pre-B phenotype. This confirmed that the cells should indeed retain responsiveness to IL-7.

IL-7 and v-*myc* infected populations show enhanced growth factor independence

Transformation in a complex culture system, such as that employed here where there are both nonadherent lymphoid cells and adherent stromal cells, requires one to distinguish between effects mediated by events occurring in the lymphoid cells versus those occurring in the stromal layer. To that end, the infected populations were examined for growth in liquid culture without an adherent stromal layer (Table 1). While the MoMuLV-infected controls showed no growth, or rather indolent growth, at plating densities up to $5x10^4$ cells/ml, the IL-7- and the v-*myc*-infected populations displayed a 3 to 8 fold increase in growth over the MoMuLV infected control when seeded at 5×10^4 cells/ml. At 10^5 cells/ml, the differences in growth were not nearly as apparent suggesting the autocrine stimulation of growth factors other than IL–7. IL-7-infected cells do not show enhanced growth in soft agar medium, while v–*myc*-infected cells do (Table 2). When plated in soft agar medium over a cellular feeder layer, populations infected with IL-7 had a plating efficiency of about 0.1% (Table 2). This is actually less than the plating efficiency of the MoMuLV-infected control populations, about 0.2%. In contrast, v-*myc* infection elevated the plating efficiency of ψ2R4 about 5-fold to 1%.

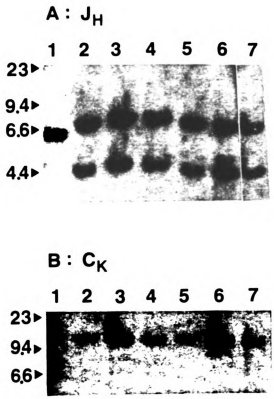


Figure 3. Immune loci define a pre-B phenotype. DNA (10 μg) was digested with the indicated restriction enzymes, electrophoresed through 0.8% agarose, and transferred to nylon membranes. Lane 1, fibroblasts; lane 2, ψ2R4/MoMuLV-1; lane 3, ψ2R4/MoMuLV-2; lane 4, ψ2R4/*Myc*-1; lane 5, ψ2R4/*Myc*-2; lane 6, ψ2R4/IL7-1; lane 7, ψ2R4/IL7-2. Size markers are the positions of an ethidium bromide-stained *Hind*III digest of bacteriophage lambda DNA. (A) *Eco*RI-digested DNA was hybridized with a probe for heavy chain J regions. (B) *Bam*HI-digested DNA was hybridized with a probe for the kappa light chain constant region.

Table 1. Growth without cellular feeder layers.

Cell population	Initial Density[a] 5×10^3/ml	1×10^4/ml	5×10^4/ml	1×10^5/ml
	Density on Day 7			
RM2	$2.2 \pm 0.1(10^6)$	$4.0 \pm 0.2(10^6)$	$6.8 \pm 0.5(10^6)$	$7.4 \pm 1.0(10^6)$
ψ2R4/MoMuLV-1	$< 10^3$	$< 10^3$	$5.3 \pm 0.4(10^5)$	$2.0 \pm 0.0(10^6)$
ψ2R4/MoMuLV-2	$< 10^3$	$1.4 \pm 0.0(10^3)$	$6.0 \pm 0.5(10^5)$	$2.1 \pm 0.3(10^6)$
ψ2R4/*Myc*-1	$1.0 \pm 0.0(10^5)$	$4.5 \pm 0.5(10^5)$	$2.0 \pm 0.0(10^6)$	$3.8 \pm 0.3(10^6)$
ψ2R4/*Myc*-2	$2.8 \pm 2.8(10^3)$	$2.1 \pm 0.7(10^3)$	$1.7 \pm 0.2(10^6)$	$3.6 \pm 0.0(10^6)$
ψ2R4/IL7-1	$< 10^3$	$7.7 \pm 3.5(10^3)$	$2.3 \pm 0.3(10^6)$	$4.2 \pm 0.2(10^6)$
ψ2R4/IL7-2	$1.0 \pm 0.8(10^3)$	$3.8 \pm 0.0(10^3)$	$4.2 \pm 0.0(10^6)$	$5.7 \pm 0.5(10^6)$
RIL7.1	ND[b]	$2.8 \pm 0.0(10^3)$	ND	$2.0 \pm 0.2(10^5)$
RIL7.2	ND	$2.0 \pm 0.2(10^5)$	ND	$1.6 \pm 0.1(10^6)$

[a]Cells were plated onto 6 cm culture dishes in 4 ml media. On day 4, each dish received 2 ml fresh medium. Values are the average (with the range) of duplicate cultures.

[b]Not done.

Table 2. Soft agar growth and tumorigenicity.

Cell population	% Cloning efficiency in soft agar	Animals with tumors/ animals tested	Average days until dead or moribund
RM2	>5	13/14[c]	25
ψ2R4/MoMuLV-1	0.26	0/8[b]	--
ψ2R4/MoMuLV-2	0.20	0/8[b]	--
ψ2R4/*Myc*-1	1.08	1/8[b]	42
ψ2R4/*Myc*-2	0.84	1/8[b]	73
ψ2R4/IL7-1	0.09	3/8[b]	36
ψ2R4/IL7-2	0.10	0/8[b]	--
RIL7.1	ND[a]	3/4[d]	53
RIL7.2	ND	7/8[d]	40

[a]Not done.

[b]These values have no statistically significant differences among them by Fisher's exact test ($p \leq 0.05$).

[c]This value is statistically different from those in [b] by the same test in [b].

[d]These two values are combined, and the resulting value is statistically different from those in [b] by the same test in [b].

A positive control of RM2 cells had a plating efficiency greater than 5%. Curiously, cells produced by the introduction of v-*myc* expression subsequent to v-Ha-*ras* expression have poorer plating efficiencies in soft agar than cells produced byco-infection of primary bone marrow cells (i.e. RM2; Schwartz et al., 1986a)

The IL-7 and v-*myc*-infected populations are not tumorigenic

The abilities of the superinfected ψ2R4 populations to form tumors *in vivo* was tested by intraperitoneal injection of these cells into syngeneic BALB/c mice. While positive control RM2 cells formed tumors quite consistently, both the v-*myc*- and IL–7-infected populations exhibited no statistically significant difference in tumorigenicity from the MoMuLV-infected ψ2R4 cells with Fisher's exact tests (Table 2). These results suggested that neither IL-7 nor v-*myc* were not capable of cooperating with v-Ha-*ras* in the induction of B cell tumors.

Co-infection of primary bone marrow cells with IL-7 and v-Ha-*ras* retrovirus yields clonal outgrowths that are tumorigenic

The surprisingly low tumorigenicity of v-*myc*-infected populations of a v–Ha–*ras*-expressing pre-B cell contrasts sharply with our earlier observation of highly tumorigenic cell lines generated by the co-infection of primary bone marrow cells with v-Ha-*ras* and v-*myc* retroviruses (i.e. RM2; Schwartz et al., 1986a). We therefore decided to examine the effects of co-infecting freshly explanted murine bone marrow cells with v-Ha-*ras* and IL-7 retroviruses. Bone marrow cells from BALB/c mice were infected singly with the IL-7 retrovirus, the v–Ha–*ras* retrovirus or doubly infected with both retroviruses. These cells were then plated in liquid

culture by the procedure of Whitlock and Witte (1982). In 20 co-infections, only two cell lines containing both IL-7 and v–Ha–*ras* retroviral integrations were obtained (RIL7.1 and RIL7.2) after about 2 months in culture. Other outgrowths contained only the v-Ha-*ras* retrovirus. Single infections with the IL-7 retrovirus never resulted in outgrowths containing that virus, while single infections with the v-Ha-*ras* retrovirus consistently produced v-Ha-*ras*-expressing cell lines similar to those previously reported (data not shown; Schwartz et al., 1986a). Southern blot analysis of DNAs isolated from RIL7.1 and RIL7.2 revealed these outgrowths to be clonal, unlike the isolates derived by superinfection of ψ2R4. *Bam*HI digestion (Figure 4A) revealed one IL-7 viral integration-related restriction fragment for RIL7.1 (lane 1) and two integration-related fragments for RIL7.2 (lane 2). DNA from uninfected (lane 3) and infected cells possessed two endogenous IL-7-specific restriction fragments of ~4 kb and ~18 kb. *Eco*RI digestion of the DNAs (Figure 4B) showed one v-Ha-*ras*-specific viral integration-related restriction fragment for RIL7.1 (lane 1) and two integration-related fragments for RIL7.2 (lane 2) in addition to the endogenous c-Ha-*ras* fragment of ~20 kb. Subcloning of RIL7.2 in soft agar medium demonstrated that the multiple viral restriction fragments represent multiple integrations in a single clone rather than several clones (data not shown). Northern analyses detected v-Ha-*ras* mRNA in both RIL7.1 and RIL7.2 (Figure 5A, lanes 1 and 2), but detected IL-7 mRNA only in RIL7.2 (Figure 5B, lane 2). Southern blot and Northern blot analyses showed RIL7.1 to be a B cell rearranged in both its mu and kappa loci, and expressing the kappa locus (data not shown). RIL7.2 was shown to be a pre-B cell with germline configuration of its kappa DNA (data not shown). Perhaps the progression of RIL7.1 to a B cell phenotype

dependent upon factors other than IL-7 for growth eliminated any selective pressure to maintain IL-7 expression in that cell line. RIL7.1 showed only indolent growth when plated without a feeder layer at 10^5 cells/ml (Table 1). Not surprisingly, RIL7.2, which expresses IL-7 mRNA, was growth factor independent (Table 1).

Analysis of the tumorigenicity of RIL7.1 and RIL7.2 revealed these cell lines to be more obviously transformed than populations of ψ2R4 superinfected with the IL-7 retrovirus. Similar to cell lines produced by the co-infection of bone marrow with v-*myc* and v-Ha-*ras* retroviruses (i.e. RM2; Schwartz et al., 1986a), the two cell lines produced by co-infection with IL-7 and v-Ha-*ras* retroviruses were highly tumorigenic (Table 2). RIL7.1 produced tumors in three of four animals challenged and RIL7.2 produced tumors in seven of eight animals.

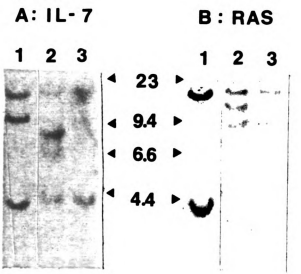


Figure 4. Proviral integration. DNA (10 μg) was digested with the indicated restriction enzymes, electrophoresed through 0.8% agarose, and transferred to nylon membranes. Lane 1, RIL7.1; lane 2, RIL7.2; lane 3, uninfected cells. Size markers are the positions of an ethidium bromide-stained *Hind*III digest of bacteriophage lambda DNA. (A) *Bam*HI-digested DNA was hybridized with an IL-7 probe. (B) *Eco*RI-digested DNA was hybridized with a v-Ha-*ras* probe.

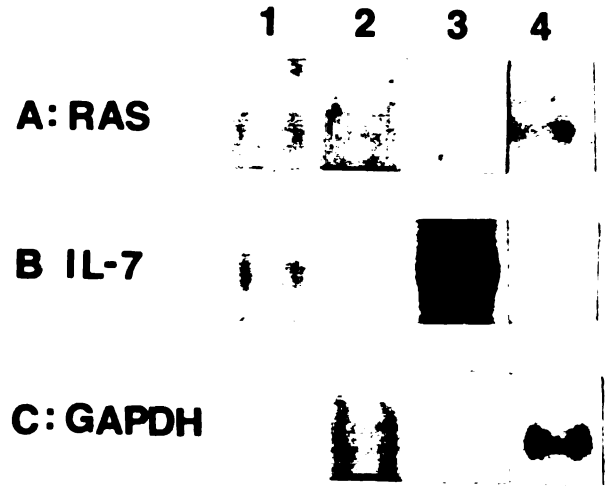


Figure 5. Retroviral transcription. Cytoplasmic RNA (20 μg) was denatured, electrophoresed in a 1% agarose-formaldehyde gel, and transferred to a nylon membrane. Lane 1, RIL7.2; lane 2, RIL7.1; lane 3, NIH3T3/IL-7(SH); lane 4, NIH3T3/SV(X)-Ha-*ras*. (A) Hybridization with a v-Ha-*ras* probe. (B) Hybridization with an IL-7 probe. (C) Hybridization with a GAPDH probe.

Discussion:

The experiments presented here find autocrine expression of IL-7 insufficient to cause tumorigenicity of a v-Ha-*ras*-expressing pre-B cell line. This contrasts with previous studies by Overell et al. (1991) that found a pre-B cell line made growth factor-independent by hyperexpression of IL-7 to be tumorigenic. Our results are more consistent with those of Young et al. (1991) that found a pre-B cell line infected with an IL-7-expressing retrovirus to be converted to tumorigenicity only rarely.

While it may capable of enhancing growth factor independence of a v-Ha-*ras* transformant, IL-7 expression had no direct correspondence to tumorigenicity. The rare tumors recovered had a long latency suggesting that ultimately other events were required for tumorigenicity. The lack of IL-7 dependence in tumors derived from v-Ha-*ras*-transformed pre-B cells is likely to be a consequence of tumor progression rather than its cause.

Kremer et al. (1991) have recently found c-*src* and c-*ras* to be downstream effectors of signal transduction by nerve growth factor and fibroblast growth factor. If the effects of both IL-7 and v-Ha-*ras* expression were upon the same signal transduction pathway, cooperativity in transformation might not be expected. Perhaps c-*ras* is a downstream effector of IL-7, as well as nerve growth factor and fibroblast growth factor.

Overell et al. (1991) found the induction of IL-7 independence in a pre-B cell line to be tumorigenic. However, very few of the cells infected with their IL-7-expressing retrovirus actually attained IL-7 independence. Thus, it seems that in their system, too, IL-7 expression is not sufficient for tumorigenicity. Other

events are required. Overell et al. (1991) do find that IL-7 independence (as opposed to IL-7 expression) does correlate with tumorigenicity and this result is more difficult to reconcile with our findings. This difference probably lies in the intrinsic properties of the individual cell lines used in the several studies. The v-Ha-*ras*-transformant used in our studies may require growth factors in addition to IL-7 for optimal growth. A similar v-Ha-*ras*-expressing pre-B cell line, as well as the cell line used by Young et al. (1991), requires a non-IL-7 factor(s) released by bone marrow stromal cells for optimal growth (Muirhead et al., 1990). The cell line used by Overell et al. (1991) may have more simple growth factor requirements, having been selected for its utility in IL-7 assays.

Comparing IL-7 directly to v-*myc*, we found that neither was able to confer tumorigenicity to ψ2R4, although v-*myc* expression enhanced the ability to grow in soft agar medium. This result was surprising in light of our earlier studies (Schwartz et al., 1986a; 1986b), where the *in vitro* co-infection of bone marrow with retroviruses expressing v-*myc* and v-Ha-*ras* yielded fully tumorigenic cell lines. When we performed co-infections of bone marrow cells with IL-7 and v-Ha-*ras*-expressing retroviruses, tumorigenic cell lines were similarly recovered. The co-infection of primary bone marrow cells seems to yield more transformed cell lines than does sequential addition of the same oncogenes to a cell line. The fact that the outgrowths from co-infection of a heterogeneous population were clonal, while those produced in sequential infection of a cell line were polyclonal may point towards an explanation. The clonal outgrowths of co-infection may represent the dominance of a rare and more highly transformed cell over many other cells carrying one or both oncogenes. The fact that only two of 20 cultures contained

co-infected clones and that their outgrowth required 2 months may reflect the necessity for other oncogenic events. While IL-7 and v-Ha-*ras* expression may contribute to the transformed phenotype, the cooperation of other rare oncogenic events is critical to the tumorigenic phenotype. In the superinfection of ψ2R4 cells, the more homogeneous population offers less opportunity for detecting rare transforming events. In addition, superinfected populations were recovered more rapidly (3 weeks). The sequential introduction of oncogenes into clonal cell lines seems to provide a more accurate assessment of their contribution to cooperative transformation than co-infection of a heterogeneous population.

Materials and methods:

Viruses. IL-7(SH) (Young et al., 1991) is derived from the Moloney murine sarcoma virus vector, pMV6*tKneo* (Kirschmeier et al., 1988). A cDNA for murine IL-7 that lacks translation-inhibiting flanking regions is expressed from the viral long terminal repeat (LTR), as well as the Tn5 *neo* gene (G418-resistant) from the herpesvirus thymidine kinase promoter.

MMCV-*neo* (Wagner et al., 1985) is derived from Moloney murine leukemia virus (MoMuLV) and Harvey murine sarcoma virus. It expresses the v-*myc* gene of retrovirus OK10 from the viral LTR as a subgenomic mRNA and the Tn5 neo gene from the herpesvirus thymidine kinase promoter.

SV(X)-Ha-*ras* (Schwartz et al., 1986a) is derived from the MoMuLV vector, pZIP-NEOSV(X)1 (Cepko et al., 1984). It expresses v-Ha-*ras* from the viral LTR and the Tn5 *neo* gene as a subgenomic mRNA from the viral LTR.

Virus stocks consisted of culture medium from NIH3T3 cell lines that had

been cotransfected with a proviral clone of MoMuLV and IL-7(SH), MMCV-*neo*, or SV(X)-Ha-*ras*. For the helper-free SV(X)-Ha-*ras* stock, culture medium was collected from the ψ2 cell line (Mann et al., 1983) that had been transfected with SV(X)-Ha-*ras*. Virus titers of $2x10^5$ to $8x10^5$ G418-resistant colonies per ml were obtained for IL-7(SH); $2.4x10^6$ for MMCV-*neo*; $1.0x10^5$ to $1.5x10^5$ for SV(X)-Ha-*ras*. Helper-free SV(X)-Ha-*ras* had a titer of $5x10^4$ G418-resistant colonies per ml.

Cell culture and viral infections. Bone marrow from 3- to 5-week old BALB/c mice was cultured by the procedure of Whitlock and Witte (1982) with the addition of a viral infection step. Bone marrow was suspended at $2.0x10^6$ cells per ml in RPMI 1640 medium supplemented with 5% fetal calf serum and $5x10^{-5}$ M 2-mercaptoethanol. To this was added an equal volume of virus stock and Polybrene (Sigma Chemical Co., St Louis, MO) to a final concentration of 8 μg/ml. After 3 hours of incubation at 37° C, the cells were pelleted and resuspended at 10^6 cells per ml in RPMI 1640 supplemented with 5% fetal calf serum and $5x10^{-5}$ M 2 mercaptoethanol. 5 ml of this suspension was plated per 6-cm culture dish.

ψ2R4 cells were cultured in RPMI 1640 supplemented with 5% fetal calf serum and $5x10^{-5}$ M 2-mercaptoethanol over a feeder culture of adherent bone marrow cells (Whitlock et al., 1983). For superinfection, these cells were suspended in the same medium at 10^5 cells per ml and mixed with an equal volume of virus stock and Polybrene as described above.

All cultures were fed twice weekly with RPMI 1640 supplemented with 5% fetal calf serum and $5x10^{-5}$M 2-mercaptoethanol. Once a week approximately 80% of the spent medium was replaced with fresh medium. The cultures were expanded by transfer of nonadherent cells to feeder cultures (Whitlock et al.,

1983). Growth in soft agar medium was performed over a feeder culture as described by Whitlock et al. (1983).

Nucleic acid isolation and analysis. Cytoplasmic RNA was isolated by a sodium dodecyl sulfate-urea procedure as described by Schwartz et al. (1981). High molecular weight DNA was isolated from nuclei collected in the preceding procedure by a method described in Schwartz et al. (1986a).

Restriction enzyme-digested DNAs were electrophoresed through 0.8% agarose. RNAs were electrophoresed through 1% agarose-formaldehyde gels (Rave et al., 1979). Transfer and hybridizations were washed to a stringency of 0.1 x SSPE in 0.1% SDS.

Hybridization probes were prepared by random priming using a kit from United States Biochemical Corp. (Cleveland, OH) with the incorporation of 5'-[α-32P]dATP (3,000 ci/mmol; ICN, Costa Mesa, CA). The IL-7 probe was a 0.5 kb *Pst*I fragment of the murine IL-7 cDNA (Young et al., 1991). The v-Ha-*ras* probe was a 0.46 kb *Eco*RI fragment corresponding to v-Ha-*ras*-encoding sequences of Harvey murine sarcoma virus (Ellis et al., 1980). The v-*myc* probe was a 0.8 kb *Cla*I-*Bam*HI fragment corresponding to 3'-terminal v-*myc*-encoding sequences of retrovirus OK10 (Hayflick et al., 1985). The mu heavy chain probe was a genomic 1.9 kb *Bam*H1-*Eco*RI fragment, which corresponds to the JH2, JH3, and JH4 regions that are 5' to the mu heavy chain constant region gene (Early et al., 1980). The kappa light chain probe was a genomic 0.48 kb *Hpa*I-*Bgl*II fragment extending from a point about 50 bp within the 5'- terminus of the kappa light chain constant region gene to the 3'-terminal poly(A) addition site (Seidman and Leder, 1978). The GAPDH probe was a 1.3 kb cDNA (Fort et al., 1985).

Tumor challenges. Cells were washed twice in RPMI 1640 and were then resuspended in the same at $8x10^6$ cells per ml. BALB/c mice, 3 to 5 weeks old, were injected intraperitoneally with 0.25 ml of the cellular suspension. Animals were observed for a maximum of 73 days post injection. Animals were sacrificed and autopsied when they became moribund or at 10 weeks. Tumors were verified as being derived from the challenging cell lines by Southern blot analysis of retroviral integration sites (data not shown).

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APPENDICES

APPENDIX A

Lineage Switch Macrophages Can Present Antigen[1]

ABSTRACT:

Recent reports of "lineage switching" from a lymphoid to macrophage phenotype have left unresolved the question of whether such cells are functional macrophages or nonfunctional products of differentiation gone awry. This study demonstrates that several "macrophage-like" cell lines derived from v–Ha–*ras*-transformed pre-B cells have gained the capacity to effectively present antigen in an MHC-restricted fashion. Using an assay involving the co-cultivation of putative antigen-presenting cells with chicken ovalbumin (cOVA) and a cOVA-specific T cell hybridoma, "lineage switch" cell lines were found to present antigen as effectively as macrophage-containing peritoneal exudates. Neither the original pre-B cell precursors nor B cell lymphomas derived from them present antigen. Thus we have demonstrated that these "lineage switch" macrophages are capable of antigen presentation, a mature differentiated function.

While gaining macrophage characteristics, these cells have also rearranged their kappa light chain immunoglobulin locus, suggesting that macrophage differentiation and immunoglobulin rearrangement are not mutually exclusive processes. The existence of both lymphoid and myeloid characteristics in a cell fully capable of antigen presentation suggests greater plasticity in hematopoietic lineage commitment than conventionally thought to be the case.

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INTRODUCTION:

The concept that hematopoietic differentiation involves an early and irreversible lineage commitment is brought into question by numerous observations of leukemias and lymphomas that express myeloid or lymphoid markers outside of their respective lineages. The coexpression of differentiation markers has been interpreted as being either an aberrant phenomenon caused by leukemogenesis (McCulloch, 1983) or a reflection of the normal but transient existence of bipotential progenitors in hematopoiesis (Greaves et al., 1986). In particular, the existence of a number of transformed cell lines with both lymphoid and macrophage characteristics has suggested a close relationship between these lineages. Murine macrophage cell lines have been derived from lymphoid tumors and from *in vitro* transformants induced either by murine leukemia viruses or chemical carcinogens (Boyd and Schrader, 1982; Holmes et al., 1986; Hanecak et al., 1989). Three groups have studied systems where a transition from a lymphoid to a macrophage phenotype could be induced. Klinken et al. (1988) demonstrated that B lymphoid cells from transgenic mice that express c–*myc* using the immunoglobulin mu enhancer could be induced to take on macrophage-like characteristics when infected with a retrovirus expressing v–*raf*. Davidson et al. (1988) showed that a v–Ha–*ras* transformed lymphoid cell line could be stimulated by lipopolysaccharide (LPS) to differentiate along either the lymphoid pathway into pre–B-like cells or along the myeloid pathway into macrophage-like cells. Recently, Borzillo et al. (1990) reported the CSF–1 dependent macrophage lineage transition of a pre–B cell line expressing the human CSF–1 receptor.

The macrophage-like cell lines that have been derived from B lymphoid cells

have been classified as macrophage on the basis of their morphology, expression of MAC–1, MAC–2, α-naphthyl acetate esterase and lysozyme, and their ability to phagocytose latex beads. More functional assays for antigen presentation and tumoricidal activity that would establish whether these cells could act *in vivo* similarly to authentic macrophages have not been presented. In this paper, we demonstrate the ability of several macrophage cell lines derived from v–Ha–*ras*-transformed pre–B cells to present antigen to a T helper cell hybridoma.

RESULTS:

A tumor consisting of adherent cells with a macrophage morphology was identified during our studies on the tumor progression of a pre-B lymphoid cell line expressing v-Ha-*ras* (Chen et al., 1991). This tumor, designated tumor 4, was derived from a clonal cell line, designated R2, that was generated by infection of fresh murine bone marrow with a mixture of a v-Ha-*ras*-expressing retrovirus and Moloney murine leukemia virus (MoMuLV) (Schwartz et al., 1986a). The R2 cell line was classified as being a pre-B cell on the basis of several criteria. It possessed a blast cell morphology with a large nucleus and scant cytoplasm. It expressed the B lineage–specific marker, B220 (Coffman and Weissman, 1981). While not expressing detectable immunoglobulin mu chain, R2 showed a rearrangement in the DNA of that locus. The immunoglobulin kappa chain locus was in a germline configuration.

Tumor 4 is Derived from the R2 Cell Line.

In order to ascertain whether we had identified a probable instance of

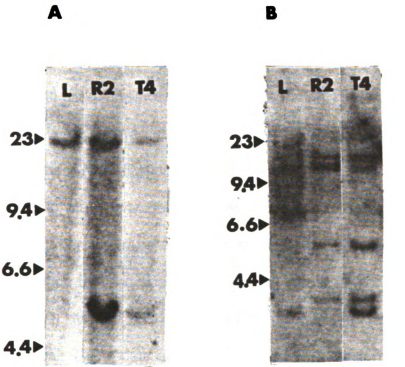

Figure 1. Viral integrations. Southern blot analysis of DNAs from liver (L), R2 and tumor 4 (T4). (A) DNA was digested with *Eco*RI and 10 μg of each sample was electrophoresed through 0.8% agarose. The blot was probed for v–Ha–*ras*. (B) DNA was digested with *Bgl*II. The blot was probed for murine ecotropic env sequences. Size markers are the positions of an ethidium bromide-stained *Hind*III digest of bacteriophage λ and are denoted in kilobases.

lineage switching, it was necessary to demonstrate that tumor 4 was derived from R2. To that end, the sites of integration of the v-Ha-*ras*-expressing retrovirus and MoMuLV were compared between the tumor and the cell line. Southern hybridization analysis of *Eco*RI-digested DNA with a v-Ha-*ras* probe showed that tumor 4 contained the same 5.3 kb proviral integration fragment as R2 (Figure 1A). This proviral integration fragment is defined by a 3' *Eco*RI site internal to the viral genome and a 5' *Eco*RI site peculiar to the site of integration. In addition to the 5.3 kb fragment, there is a 23 kb fragment representing the endogenous c-Ha-*ras* in all the DNAs. Southern hybridization analysis of *Bgl*II-digested DNA, using a probe for the ecotropic MuLV *env* gene, revealed similar MoMuLV integration fragments in R2 and tumor 4 (Figure 1B). The MoMuLV genome possesses a *Bgl*II site within *env*, such that the above hybridization would detect a fragment extending from that *Bgl*II site to a *Bgl*II site in the host cell genome flanking the 3' terminus of the provirus. These data demonstrate that the putative macrophage tumor was derived from the pre-B cell line.

Tumor 4 Cells Possess Macrophage Characteristics.

Tumor 4 was initially suspected to be a macrophage because of the large size of its cells and its adherent growth in cell culture. Microscopic examination of Wright-Giemsa stained cells confirmed their large size and revealed the cells of tumor 4 (Figure 2B) to have a much more extensive and granular cytoplasm than R2 (Figure 2A). An immunoperoxidase detection procedure found tumor 4 cells to have retained some expression of B220, and to have gained expression of high levels of MAC-1 (data not shown). MAC-1 is generally considered to be a marker

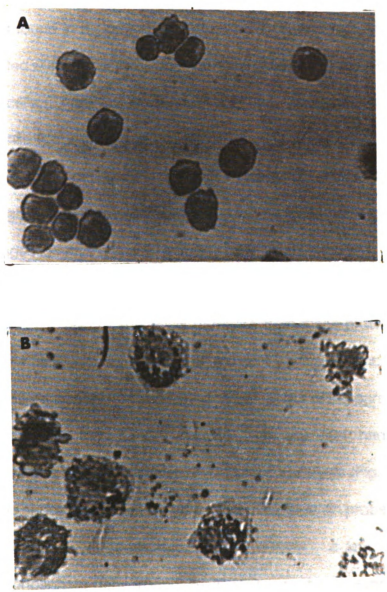

Figure 2. Nonspecific phagocytosis of latex beads. The cells were Wright-Giemsa stained and photographed at 200x magnification. (A) R2; (B) tumor 4.

for cells of the myeloid lineage (Springer et al., 1979). Histochemical proceduresrevealed a high level of α-naphthyl acetate esterase activity in tumor 4 cells, which is not found in R2 cells (data not shown). This is an enzyme activity generally associated with cells of the monocyte-macrophage lineage (Rogers et al., 1980). Tumor 4 cells (Figure 2B) were positive for the nonspecific phagocytosis of latex beads, while R2 cells (Figure 2A) were not. Nonspecific phagocytosis is another marker of the monocyte-macrophage lineage (Raschke et al., 1978). These data strongly suggest a macrophage phenotype for tumor 4 cells.

At late stages of myeloid differentiation, the levels of c-*myc* and c-*myb* mRNA decrease, while the level of c-*fms* mRNA increases (Gonda and Metcalf, 1984; Sheng-Ong et al., 1987). The levels of mRNA from these protooncogenes detected in tumor 4 cells was consistent with tumor 4 having advanced to a late stage of myeloid differentiation. Cytoplasmic poly A+ RNAs of the parental R2 cell line, tumor 4 and six other tumors derived from R2 that had lymphoid characteristics were examined by Northern hybridization analysis (Figure 3). One blot was hybridized successively with c-*myc* and β2-microglobulin probes. Another blot was hybridized successively with c-*myb*, c-*fms* and rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probes. Hybridization to the β2-microglobulin and GAPDH probes provided a control for gel loading. Tumor 4 cells clearly show reduced levels of c-*myc* and c-*myb* expression in comparison to R2 cells and lymphoid tumors. In contrast c-*fms* expression is elevated in tumor 4 cells. A cell line with macrophage characteristics has also been isolated from tumor 5 cells, which show elevated c-*fms* expression (Figure 3).

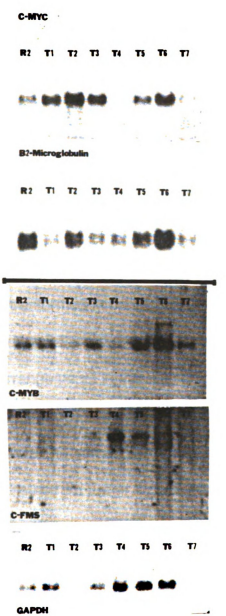

Figure 3. RNA analyses of c–*myc*, c–*myb* and c–*fms*. Northern blot analyses were performed on polyA+ RNA from R2 and seven tumors (T1–T7). Each sample of RNA was the polyA+ fraction selected from 150 μg of total cytoplasmic RNA. One blot (upper panel) was probed successively for both c–*myc* and ß2-microglobulin, while the other blot (lower panel) was probed successively for c–*myb*, c–*fms* and rGAPDH.

Another aspect of macrophage function is the ability to release cytokines in response to LPS stimulation. We examined the presence of IL–1, IL–6 and TNF in the media of cells cultured in the presence or absence of 10 μg/ml of LPS for 24 hours. Cellular proliferation assays for IL–1 and IL–6, and a cytotoxicity assay for TNF revealed varying levels of cytokine release for six subclones of tumor 4 (see below), while the parental R2 pre–B cell line did not elaborate any of these cytokines except low levels of IL-1 (Table 1). The LPS-inducible release of cytokines was again consistent with a macrophage phenotype for tumor 4 cells. Tumor 4 Cells Also Show Differentiated Lymphoid Characteristics. Davidson et. al. (1988) found that a v-Ha-*ras*-tranformed lymphoid cell line could be stimulated to differentiate along either the myeloid or lymphoid pathways. Since tumor 4 cells showed a variety of DNA rearrangements in the kappa light chain locus (data not shown), it was of interest to determine whether the cells that had gone on to rearrange the kappa locus were the same cells that had progressed toward a macrophage phenotype or whether the tumor 4 cells were a mixed population of B cells and macrophages. To that end, tumor 4 cells were plated in soft agar medium and six subclones were recovered. Southern hybridization analysis of *Eco*RI-digested DNA isolated from the subclones showed that they all contained the same 5.3 kb v-Ha-*ras* proviral integration fragment as R2 and tumor 4 cells (data not shown; see Figure 1A). The six subclones of tumor 4 possessed the same myeloid characteristics described above for the uncloned tumor, but varied in their pattern of kappa light chain gene rearrangement. Southern hybridization analysis of *Bam*HI-digested DNAs with a kappa probe revealed that subclones 3 and 5 possessed one germline and one rearranged kappa allele, while subclones

Table 1. LPS-induced cytokine release by tumor 4 macrophage subclones.

	IL-1 (U/ML)		IL-6 (U/ML)		TNF (U/ML)	
	-LPS	+LPS	-LPS	+LPS	-LPS	+LPS
R2	0	2	0	0	0	0
T4.1	0	2	0	1	0	0
T4.2	0	6	0	100	0	40
T4.3	0	6	0	1500	0	85
T4.4	0	19	0	39	0	34
T4.5	1	4	0	0	0	0
T4.6	0	4	0	0	0	0

Table 1. The capacity of cell lines to release the cytokines IL–1, IL–6 and tumor necrosis factor (TNF) was determined by assaying culture supernatants. For this purpose, cell lines were incubated for 24 hours at $2.5x10^5$ cells/ml with 10 μg/ml LPS in RPMI 1640 supplemented with 10% fetal calf serum and $5x10^{-5}$ M 2-mercaptoethanol. Culture supernatants were collected, passed through a 0.2 micron filter and stored at –70° C until assayed. IL–1 activity was assayed by its ability to induce proliferation of D10.G4.1 cells in the presence of Concanavalin A as described by Ayala et al. (1990a). IL–6 activity was determined by its ability to induce the proliferation of the 7TD1 B-cell hybridoma as previously described by Hultner et al. (1989). TNF activity was assessed by its cytotoxicity to WEHI–164 clone 13 cells as previously described by Ayala et al. (1990b). The relative units of cytokine activity were determined by comparison of the activity of dilution series of experimental supernatants to the activities of dilution series of purified human IL–1 (Genzyme), recombinant human IL–6 (Amgen Corp.) or murine TNF–alpha (Amgen Corp.) standards.

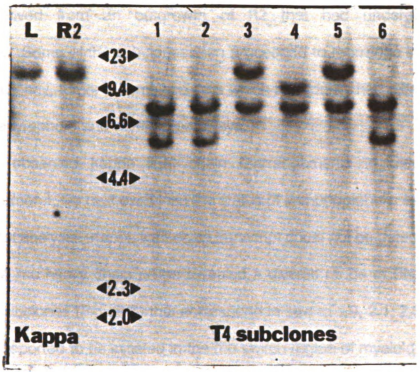

Figure 4. Kappa light chain rearrangements. Southern blot analysis of DNAs from liver (L), R2 and six subclones of tumor 4 (1–6). DNA was digested with *Bam*HI and 10 μg of each sample was electrophoresed through 0.8% agarose. The blot was probed for kappa light chain constant region sequences. Size markers are the positions of an ethidium bromide-stained *Hind*III digest of bacteriophage λ and are denoted in kilobases.

1,2,4 and 6 possessed rearrangements in both alleles (Figure 4). All the subclones possessed a rearranged *Bam*HI fragment of approximately 7 kb. Tumor 4 was apparently derived from an outgrowth of R2 that had undergone this rearrangement. Some of the subclones then proceeded to rearrange their other kappa allele. Clearly, tumor 4 contained cells which individually had differentiated along both the lymphoid and myeloid pathways.

Having observed kappa light chain rearrangements in macrophage subclones of tumor 4, we next examined the status of immunoglobulin expression by Northern blot analysis. Kappa light chain transcript could not be detected (data not shown). A mu heavy chain probe revealed a diverse range of RNAs in the macrophage subclones (Figure 5) that correspond in size to 1.9, 2.1, 2.3 and 2.9 kb transcripts reported to be initiated in the mu switch region of myeloid cell lines (Kemp et al., 1980). The R2 pre-B cell line possesses predominantly larger RNA species that include those that correspond in size to mature mu mRNAs of 2.4 and 2.7 kb. These species are diminished upon lineage switch. Comparison to a hybridization of the same blot with a probe for GAPDH (Figure 5) shows the R2 RNA to be underloaded and thus the diminution of mu transcription in the macrophages is even more dramatic than apparent from casual inspection of the data. Apparently, the macrophage subclones of tumor 4 lose the capacity to transcribe functional mu mRNA, even though the rearrangement of the kappa locus suggests progress in lymphoid differentiation.

Since CD45 isoforms have been reported to be lineage specific (Saga et al., 1987; Streuli et al., 1987; Ralph et al., 1987), the expression of this surface marker was examined among the subclones of tumor 4. An immunoperoxidase detection

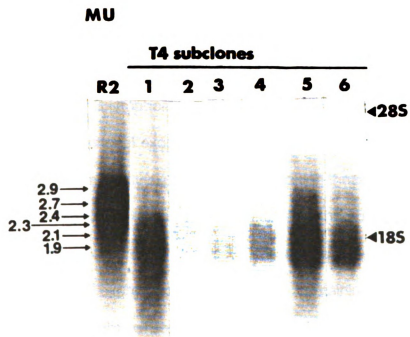

Figure 5. Expression of mu heavy chain RNA. Northern blot analysis was performed on poly A+ RNA from R2 and six subclones of tumor 4 (1-6). Each sample of RNA was the poly A+ fraction selected from 100 μg of total cytoplasmic RNA. The blot was probed for mu heavy chain. The positions of ethidium bromide-stained rRNAs are noted on the right. The positions of mu RNA species are marked on the left and denoted in kilobases. The lower panel shows the same blot probed for GAPDH as a control for loading.

procedure detected B220, the B lymphoid isoform of CD45, in tumor 4 cells. The expression of CD45 was further examined among the tumor 4 subclones in orderto determine the relative expression of the B220 isoform in comparison to the isoform that predominates in myeloid cells. Recently, Chang et. al. (1989) described the use of a reverse transcription-polymerase chain reaction (RT-PCR) technique to determine the pattern of alternate exon use in CD45 expression of hematopoietic cells. They found that B lymphoid cell lines uniquely expressed a form of CD45 mRNA possessing 3 optional exons, while two myeloid cell lines (a macrophage and a mast cell) predominantly expressed a form lacking these exons. We utilized RT-PCR to examine CD45 expression among R2 and the subclones of tumor 4 (Figure 6). All of the cell lines expressed multiple species of CD45 mRNA. Subclones 2,3,4 and 6 expressed a CD45 mRNA containing 3 optional exons, typical of B lymphoid cells, while subclones 1 and 5 predominantly expressed mRNA lacking these exons, typical of myeloid cells. R2 expressed the expected three exon B lymphoid isoform. Thus the pattern of CD45 expression is heterogeneous among macrophage subclones of the same tumor.

The subclones of tumor 4 can function effectively in antigen presentation. In order to test the ability of the tumor 4 subclones to present antigen, an assay system required an antigen-specific T helper cell line that could be stimulated to produce interleukin 2 (IL-2) upon presentation. For these experiments, the putative antigen-presenting cells were co-cultivated with a T cell hybridoma specific for chicken ovalbumin (cOVA) and restricted for I-Ad (the haplotype for BABL/c), DO.11.10/54.4. In the presence of cOVA, authentic macrophages such as those in a peritoneal exudate stimulate the hybridoma to produce IL-2 (Figure 7A). IL-2

production was assayed by the application of media supernatants from co-cultivations to an IL-2 dependent cell line, CTLL-2. All of the macrophage-like tumor 4 subclones displayed antigen presentation capacities comparable to peritoneal exudates (Figure 7A). Furthermore, all of the tumor 4 subclones showed antigen presentation capacities dramatically greater than either the parental R2 pre-B cell line or tumor 1, a B cell tumor derived from R2 (Figure 7A). IL-2 production was dependent on the presence of cOVA during co-cultivation of presenting cells with cells of the helper T cell hybridoma. Supernatants produced in the absence of cOVA were analyzed for all the cell lines and the values for IL−2 production were found to be near zero (data not shown). These control values were subtracted from those determined for supernatants produced in the presence of cOVA to generate the data presented in Figure 7A, B and C. IL−2 production was also dependent on the presence of T cell hybridoma cells. Supernatants produced by incubations of putative presenting cells with cOVA in the absence of T cell hybridoma cells had no detectable IL−2 (data not shown). The ability to present antigen was not stimulated by exposure to LPS for any of these cell lines (data not shown). A macrophage-like outgrowth from tumor 5 (also derived from R2) and the cells of a macrophage-like tumor derived from the pre-B cell line R1 (9) showed levels of antigen presentation similar to those observed for the tumor 4 subclones (Figure 7B). The ability to present antigen may therefore be a common phenomenon among v-Ha-*ras*-transformed B lymphoid cells that acquire macrophage-like characteristics.

Authentic antigen presentation should be MHC-restricted, so all of the putative antigen-presenting cells were also co-cultivated with a T cell hybridoma

specific for cOVA and restricted for I-Aq, 3Q023-24.4. As exemplified by subclone 4 of tumor 4 (T4.4) and the macrophage tumor derived from R1 (R1T), the antigen presentation observed is MHC-restricted (Figure 7C).

I-A Expression

Antigen presentation to T cells requires Ia expression and the observation of I-Ad-restricted presentation (Figure 7C) indicates that these "lineage switch" macrophages express I-Ad. In order to assess whether the acquisition of presentation capacity correlated with acquisition of Ia expression, in particular I-Ad, we performed flow cytometry with FITC-conjugated anti-mouse I-Ad on the macrophage cell lines and their pre-B cell precursors. While both R1 and R2 (pre-B cells) displayed no detectable I-Ad, the macrophage cell lines represented by R1T and T4.4 showed a low expression of I-Ad (Figure 8).

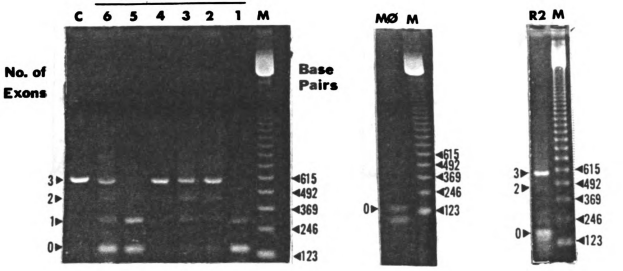

Figure 6. RT–PCR analysis of CD45. RT–PCR was performed on the polyA+ RNAs of R2, the six subclones of tumor 4 and P388D1 (a myeloid control). The products were electrophoresed through 2% agarose and stained with ethidium bromide. (C) 3-exon plasmid control; (1–6) T4 subclones; (M) 123 bp ladder; (Mϕ) P388D. PCR products smaller than the O exon product may represent an RNA species lacking an additional exon (Chang and Esselman, unpublished results).

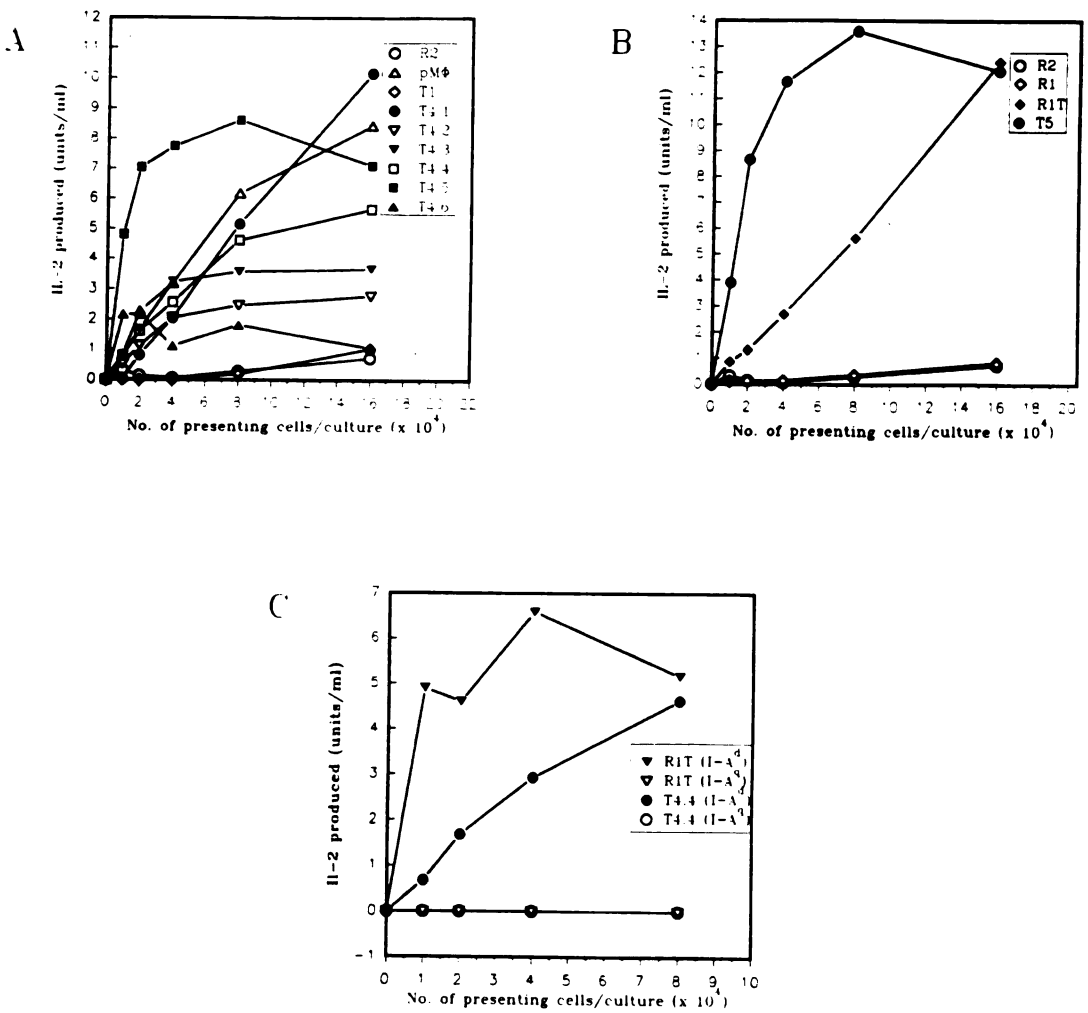

Figure 7. Antigen presentation. (A) Antigen presentation assays were performed on R2, the tumor 4 subclones (4.1–4.6), tumor 1 (a B cell tumor derived from R2), and peritoneal exudates (pMΦ). (B) Antigen presentation assays were performed on R2, a macrophage outgrowth of tumor 5 (T5; derived from R2 in a different animal), R1 (another pre-B cell line transformed by v-Ha-*ras*), and R1T (a macrophage tumor derived from R1). (C) Antigen presentation assays were performed on a tumor 4 subclone (T4.4) and R1T with DO-11.10/54.4 (I-Ad-restricted) or 3Q023-24.4 (I-Aq-restricted). Each point represents an average value obtained from a dilution series for each presentation supernatant, testing the response of CTLL-2 cells to IL-2 in those superantants. The results shown are representative of at least two experiments with each cell line.

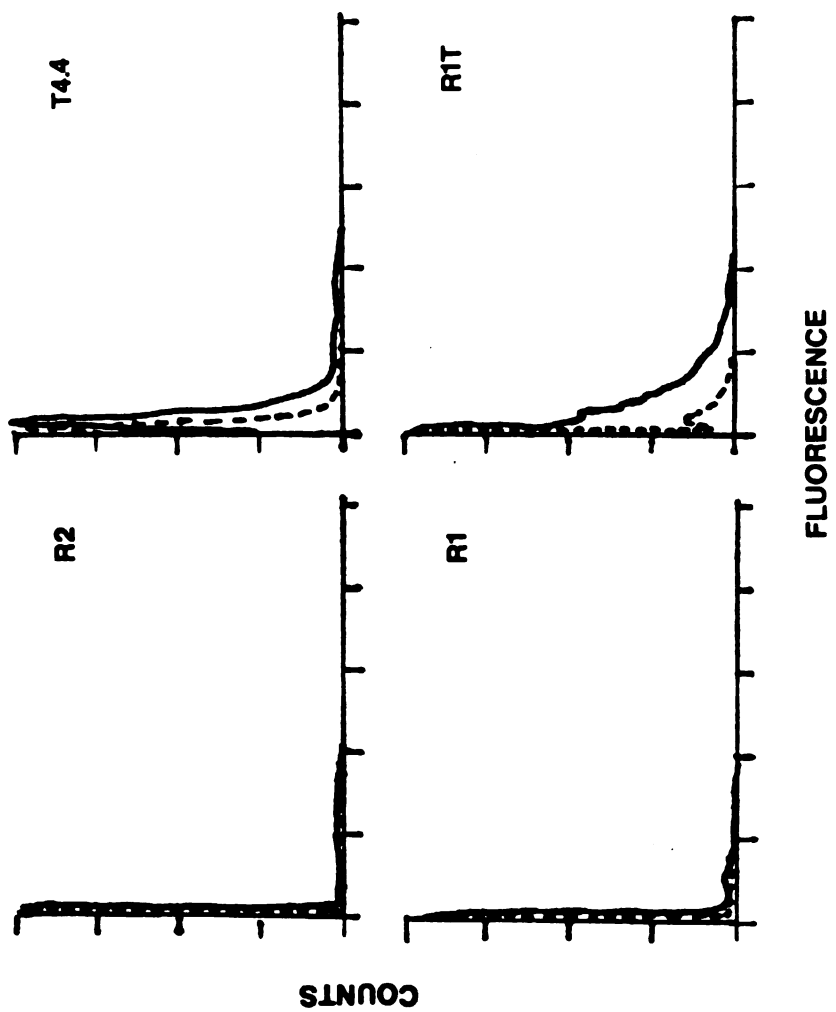

Figure 8. I-Ad expression. Flow cytometry was performed on R1, R2, T4.4 and R1T after reactions with FITC-conjugated anti-I-Ad(solid line)or, as a control, FITC-conjugated mouse IgG2b,K (dashed line). For R1 and R2 the plots of experimental and control are virtually coincidental.

DISCUSSION:

This study demonstrates the capacity of several macrophage-like tumor cell lines derived from v–Ha–*ras*-transformed pre–B cell lines to present antigen with MHC-restriction. This finding establishes that cells having undergone "lineage switching" can perform a function normally associated with a fully differentiated macrophage or B cell. While numerous examples exist of B lymphomas with the capacity to present antigen (Chesnut et al., 1982; Walker et al., 1982), neither the pre–B cell precursors of the macrophage-like cell lines nor B cell lymphoma cell lines derived from those precursors could present antigen. Thus the capacity to present antigen appears to correlate with the differentiation of these cells along the macrophage lineage. Indeed, two cell lines with the most dramatic level of antigen presentation had lost expression of the B cell isoform of CD45 and displayed a pattern of CD45 more typical of a myeloid cell (subclones 1 and 5, Figure 6; T4.1 and T4.5, Figure 7A). Perhaps, loss of the B cell isoform of CD45 is indicative of further maturation along the myeloid lineage. It may be worthwhile to investigate the role of CD45 in macrophage function. The fact that similar antigen presentation abilities were found in macrophage derivatives of two completely independent cell lines (R1 and R2) suggests the generality of this phenomenon.

Since it is well established that IL–1 along with antigen presentation is an important co-activator of T cells, it is surprising that the inducibility of cytokine release by LPS (Table 1) does not correlate with the effectiveness of antigen presentation by the T4 subclones (Figure 7A). Apparently the low levels of IL–1 that some of these macrophages are capable of elaborating is sufficient for T cell activation. The observation that two of the best lines for antigen presentation (T4.1

and T4.5) have a weak response to LPS suggests that LPS-induced cytokine release may not be an adequate measure in itself for evaluating macrophage function.

The "lineage switch" macrophages reported here express a low level of Ia (Figure 8). This is consistent with the previous report of Davidson et al. (1988). The precursor pre-B cells lack detectable Ia. Perhaps Ia expression is the critical property determining the capacity to present antigen among these cells. Certainly, Ia expression is necessary for antigen presentation, but its sufficiency for antigen presentation among the cell lines we have studied will require further experimentation.

The six macrophage-like subclones of tumor 4, while possessing a common rearranged kappa allele, displayed a variety of kappa light chain gene rearrangements at their other kappa allele. Compared to their parental cell line, these cells have progressed along the B as well as the monocyte/macrophage lineage. The varying rearrangements of one kappa allele suggests rearrangement subsequent to macrophage conversion and that at least certain elements of lymphoid and macrophage differentiation programs are not mutually exclusive. The fact that the R2 cell line can also generate a lymphoma (T1, Figure 7A) that expresses both mu and kappa chains (data not shown) demonstrates the potential of this cell line to differentiate quite far along either the lymphoid or macrophage pathways. The relationship between lymphoid and macrophage differentiation revealed in these cells differs somewhat from that seen in cases of "lineage switch" previously reported. Klinken et al. (1988) found "lineage switch" macrophages at both the pre-B and B cell stages of immunoglobulin rearrangement. However, they

did not find macrophages that had progressed in their immunoglobulin rearrangement compared to their lymphoid cell precursors, as we have. Davidson et al. (1988), examining v–Ha–*ras*-transformants similar to those reported here, could induce those cells to differentiate into either lymphoid or macrophage cells upon exposure to LPS. The lymphoid derivatives they reported did not progress beyond the pre-B cell stage, while we have identified an immunoglobulin producing tumor derived from a pre-B cell line that also gave rise to a macrophage tumor. Perhaps the more complex environment provided during tumor challenge allowed the cells described here to more fully develop along the lymphoid lineage when that pathway was selected. At any rate, the v–Ha–*ras*-transformed pre-B cells described here seem truly bipotential.

The ability of these cells which undergo an apparent "lineage switch" to perform a fully differentiated function presents the possibility that they may represent an unusual but normal subset of hematopoietic cells rather than an oddity induced by transformation. The existence of both lymphoid and macrophage characteristics in a cell fully capable of antigen presentation suggests greater plasticity in hematopoietic lineage commitment than conventionally thought to be the case.

MATERIALS AND METHODS:

Cell Lines. R1 and R2 are v–Ha–*ras*-transformed murine pre–B cell lines described in Schwartz et al. (1986a). Tumors derived from R1 and R2 were generated as described in Schwartz et al. (1986a,b) in syngeneic BALB/c mice and in BALB/c athymic nude mice. Briefly, cells were washed twice in RPMI 1640 and

were then resuspended in the same at $8x10^6$ cells per ml. Five week old mice were injected intraperitoneally with 0.25 ml of the cellular suspension. Tumor 4, in particular, was isolated from an inguinal lymph node at 74 days post injection. Tumor cell lines were readily produced from explanted tumors by dispersal and transfer to feeder cultures of adherent bone marrow cells (Whitlock et al., 1983). All of these cell lines were cultured over feeder cells in RPMI1640 supplemented with 5% fetal calf serum and $5x10^{-5}$ M 2-mercaptoethanol.

The subclones of tumor 4 were generated from single colonies grown in soft agar medium as described by Whitlock et al. (1983). The T cell hybridoma, DO-11.10/54.4, was a generous gift of Drs. Philippa Marrack and John Kappler (University of Colorado, Denver) (White et al., 1983). This hybridoma is specific for chicken ovalbumin in the context of I-Ad and crossreacts weakly with chicken ovalbumin in the context of I-Ab. 3Q023-24.4, another T cell hybridoma, was also a gift of Drs. Marrack and Kappler. This hybridoma is specific for chicken ovalbumin in the context of either I-Aq or I-E. CTLL-2 is a T cell line responsive to IL-2 and was obtained from the ATCC. All of these cell lines were cultured in RPMI1640 supplemented with 10% fetal calf serum and $5x10^{-5}$ M 2-mercaptoethanol in the absence of any feeder cells.

Peritoneal exudates containing macrophages were produced from BALB/c mice treated 1 week previously with a 0.5 ml intraperitoneal injection of pristane.

Nucleic Acid Analysis. Cytoplasmic RNA was isolated from actively growing cells by a sodium dodecyl sulfate-urea procedure as described by Schwartz et al. (1981). Poly A+ RNA was selected by oligo-dT cellulose chromatography (Rave et al., 1979). RNA was denatured, electrophoresed in a formaldehyde-1% agarose

gel (15), and transferred to Nytran (Schleicher and Schuell) (Thomas, 1980).

High molecular weight DNA was isolated from nuclei collected in the preceding RNA isolation procedure as described in Schwartz et al. (1986a). DNA was digested with restriction enzymes as noted in the figure legends, electrophoresed through 0.8% agarose and transferred to Nytran (Southern, 1975).

Hybridization probes were prepared by nick translation (Rigby et al., 1979) through the incorporation of [α-32P] dATP (3000 Ci/mmol; ICN). The v–Ha–*ras* probe was the replicative form of phage M13mp10 containing a 0.46 kb *Eco*RI fragment corresponding to v–Ha–*ras* encoding sequences (Ellis et al., 1980). The env probe was a 0.8 kb *Bam*HI fragment from the env region of Friend murine leukemia virus and is specific for the env sequences of murine ecotropic retroviruses (Silver and Kozak, 1986). The c–*myc* probe was the 4.7 kb genomic *Hind*III fragment of murine c–*myc* (Stanton et al., 1984). The murine c–*myb* probe was a cloned 2.4 kb cDNA (a generous gift of Dr. Timothy Bender, University of Virginia, Charlottesville). The *fms* probe was a cloned 2.7 kb *Cla*I-*Bam*HI fragment of the McDonough strain of feline sarcoma virus (Donner et al., 1982). The murine ß2-microglobulin probe was a cloned 0.5 kb cDNA (Parnes et al., 1981). The rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was a cloned 1.3 kb cDNA (Fort et al., 1985). The murine kappa light chain probe was the replicative form of phage M13mp10 containing a genomic 0.48 kb *Hpa*I–*Bgl*II fragment extending from a point about 50 base pairs within the 5' terminus of the kappa light chain constant region gene to the poly A addition site (Seidman and Leder, 1978). The murine mu heavy chain probe was a cloned cDNA (μ12) which extends from CH2 to the 3'-untranslated region of the secreted form of mu mRNA (Rogers et al.,

1980). All hybridizations were performed under aqueous conditions in 5 x SSC at 65° C and washed to a stringency of 0.1 x SSC at 65° C.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). RT-PCR was performed according to the procedure of Chang et al. (1989; 1991) using poly A+ RNA as substrate. The primers were a sense primer specific to exon 2 (GCCCTTCTGGACACAGAAGT; base positions 167–186) and an anti-sense primer specific to exon 9 (AATTCACAGTAATGTTCCCAAACAT; base positions 764–740) of the cDNA of murine CD45 (Thomas et al., 1987). cDNA was prepared by incubating 1 μg of poly A+ RNA for 60 min at 37° C with 200 units of MoMuLV reverse transcriptase in a 20 μl reaction volume containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 5 mM DTT, 100 μg/ml BSA, 40 units RNasin, 500 μM dNTP and 200 ng of anti-sense primer. A 5 μl aliquot was used directly for PCR amplification in a 50 μl reaction volume containing 50 mM KCl, 10 mM Tris-HCl (pH 9.3), 3 mM MgCl2, 0.1% w/v gelatin, 500 μM dNTP, 400 ng of sense and anti-sense primers and 2.5 units of Taq polymerase. PCR was performed in a DNA Thermal Cycler (Perkin-Elmer-Cetus, Inc.) for 24 cycles. Each cycle consisted of 40 s at 94° C for denaturation, 15 s at 55° C for annealing and 30 s at 72° C for elongation. The first cycle was preceded by a 5 min incubation at 94° C and the last cycle followed by a 4 min incubation at 72° C.

Cytological Analyses. Cells were cytocentrifuged onto a microscope slide and allowed to air dry overnight. The cells were then incubated with either rat anti-B220 (monoclonal 14.8) or rat anti-MAC–1 (Boehringer Mannheim). Goat anti-rat immunoglobulin-horseradish peroxidase (Boehringer Mannheim) was used in a secondary incubation for detection. The presence of α-naphthyl acetate

esterase was determined by cytochemical staining (Yam et al., 1971) with a Sigma research kit. Nonspecific phagocytosis of latex beads was assayed by the method of Raschke et al. (Raschke et al., 1978).

Antigen Presentation. Assays for antigen presentation were performed in a manner similar to that described by Marrack et al. (1989). Briefly, the cell lines to be assayed for antigen presentation were titrated into 200 μl microcultures containing 10^5 cells of either the T cell hybridoma DO-11.10/54.4 or 3Q023-24.4, both of which produces IL-2 in response to the presentation of chicken ovalbumin (cOVA) in the context of I-Ad or I-Aq, respectively. These assays were carried out in RPMI 1640 supplemented with 10% fetal calf serum, $5x10^{-5}$ M 2-mercaptoethanol and, where required, cOVA at 1 mg/ml. After 24 hours, incubation supernatants from these cultures were assayed for IL-2 using CTLL-2, an IL-2-dependent cytotoxic T cell line. Two-fold serial dilutions of supernatants were added to $5x10^3$ CTLL-2 cells in 100 μl microcultures and incubated for 48 hours at 37° C. MTT (Sigma), a substrate for production of a colored product indicative of cell survival (Mosmann, 1983), was added at 0.5 mg/ml and the cultures incubated for an additional 4 hours at 37° C. Acid-isopropanol (40 mM HCl) was then added to dissolve the MTT formazan reaction product. The optical density of each well was quantitated by an ELISA reader at a wavelength of 540 nm. The specific activity of IL-2 in the supernatants was determined by comparison to a standard curve produced through the use of purified recombinant IL-2 (Cetus Inc.).

Flow Cytometry. Cells were stained in PBS, 2% FCS with either FITC-conjugated monoclonal antibody AMS-32.1 (anti-mouse I-Ad) (Phar Mingen) or

FITC-conjugated mouse IgG2b, K (Phar Mingen) as an isotype-matched control. Cells were then fixed in PBS, 2% FCS, 0.5% formaldehyde and stored at 4° C until analysis. Flow cytometry was performed using an Ortho Diagnostics Cytofluorograph 50-H.

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APPENDIX B

Introduction:

This appendix presents "data not shown" pertinent to the manuscript that comprises Chapter 2. These include data that demonstrated (i) that the tumors derived from R2 possess a more transformed phenotype than the R2 cell line; (ii) that alternative mechanisms of c-*myc* gene activation may occur in tumors derived from R2; (iii) the capability of the pre-B cells to undergo further differentiation during the process of tumorigenesis. A list of probes of oncogenes, tumor suppressor genes, growth factor genes, and sites of frequent viral integration used to screen for additional genetic lesions in the tumor cells is also included (Table 2).

Materials and Methods:

Cell culture. Cells were cultured under conditions described in Chen et al. (1991)(Chapter 2). Recombinant IL–7, a generous gift of Dr. Steven Gillis (Immunex Corp., Seattle), was used in the culture of cells in the absence of feeder layers.

Tumor challenges. Cells were washed twice in RPMI 1640 and were then resuspended in the same at $8x10^6$ cells per ml. BALB/c mice, 4 weeks old, were injected intraperitoneally with either 0.25 ml or 0.5 ml of the cellular suspension. Animals were observed for a maximum of 10 weeks postinjection. Animals were sacrificed and autopsied when they become moribund or at 10 weeks. Latency in these experiments refers to the time until the animals became moribund.

Nucleic acid analyses. All hybridization analyses were performed as described in Chen et al. (1991)(Chapter 2). The kappa probe is a 3.4 kb *Hind*III restriction fragment derived from a plasmid (pcK), which contains a genomic fragment corresponding to kappa constant region sequences (Seidman and Ceder, 1978).

Nuclear run-on experiments. Nuclear run-on experiments were performed essentially as described in Stewart et al. (1987) with the following modifications. Labeling was accomplished through the incorporation of (α-32P)UTP (600 Ci/mmol; Amersham). Isolated RNA was partially hydrolyzed in NaOH in order to allow differential analysis of initiation and elongation of c–*myc* transcription. RNA was resuspended in 250 μl of 20 mM HEPES (pH 7.5), 5 mM EDTA. 62.5 μl of 1M sodium hydroxide was added and the mixture was incubated on ice for 10 minutes. The reaction was quenched with 125 μl of 1M HEPES (free acid; pH 5.5). Hybridizations were carried out in 50% formamide, 5 x SSC, 5x Denhardt's solution, 0.1% SDS, 50 mM sodium phosphate (pH 6.8), 250 μg/ml single-stranded salmon sperm DNA, 5% dextran sulfate for at least 40 hours at 42° C. Hybridizations were washed to a stringency of 0.1 x SSC at 55° C. Single-stranded M13 probes were a generous gift of Dr. Alain Nepveu (Ludwig Institute for Cancer Research, Montreal). The exon 1 c-*myc* probe contained the 0.5 kb *Bam*HI-*Bgl*II fragment of the murine c–*myc* gene. The exon 2 and 3 probe contain a 3.4 kb *Bam*HI-*Hind*III fragment of the murine c–*myc* gene. The antisense control contained the 145 bp *Hae*III-*Hind*III fragment at the 5' end of the murine c–*myc* gene. The rGAPDH probe contained the 1.3 kb cDNA of rGAPDH.

mRNA turnover assay. R2 cells and tumor cell lines were cultured as described in Chen et al. (1991)(Chapter 2), and maintained in an actively growing state by

addition of fresh media 24 hours prior to harvest. Cells were harvested, and counted with trypan blue staining to assess their viability. 1.5 x 10^8 cells were resuspended in 60 ml standard medium supplemented with Actinomycin D at a concentration of 50 micrograms per ml, and then split into three portions. The first 20 ml were immediately subjected to cytoplasmic RNA preparation by methods described in Chen et al. (1991)(Chapter 2), while the other two portions were incubated for either 1/2 hour or 1 1/2 hours after the addition of Actinomycin D. RNA of R2 and the tumor cell lines isolated at each time point was analyzed by a Northern blotting procedure as described in Chen et al. (1991)(Chapter 2). Northern blots were sequentially hybridized to probes for c-*myc* and rGAPDH. The stability of c-*myc* RNA in R2 and the tumor cell lines was assessed by their relative decrease in abundance over time by the method described in the table legend.

Results:

The tumors possess a more transformed phenotype than their parental cell line.

As described in the Chapter 2, the infrequent occurrence and latency of tumors derived from the R2 cell line raised the question of whether tumor progression required the acquisition of genetic lesions in addition to v-Ha-*ras* expression. Other explanations for the weak tumorigenicity of the R2 cell line are plausible. First, it is possible that R2 cells are normally able to grow into tumors if they do not acquire other genetic lesions, which may somehow stimulate the host immune system to eliminate or inhibit their growth. This scenario is unlikely, since Schwartz et al. (1986b) found R2 to be weakly tumorigenic in athymic nude

mice. Second, the infrequent occurrence may relate to the variation of epigenetic factors among hosts, and the long latency may only reflect the *in vivo* slow growth properties of R2 cells. In order to ascertain whether the tumor cells had gained growth properties consistent with tumor progression, the growth properties of early passage tumor cell lines were compared to the R2 parental line. Two growth properties were tested: the ability to grow *in vitro* independent of adherent feeder cells and the ability to generate tumors in syngeneic mice.

R2 had previously been found to be dependent on an adherent cellular feeder layer for growth (Schwartz et al., 1986a). All of the tumor cell lines grew well on feeder layers. In order to test whether the tumors had gained some level of growth factor independence, the tumor cell lines and R2 were cultured in the absence of feeder layers. While R2 was incapable of growth in the absence of a feeder layer, all of the tumor cell lines exhibited growth (Figure 1). This is most consistently the case at higher cell density.

Since IL-7 has been reported to be required for the growth of pre-B cells (Namen et al., 1988), it was of interest to test the IL-7 responsiveness of R2 and the tumor cell lines derived from it. R2 (Figure 1) and other v-Ha-*ras*-transformed pre-B cell lines (data not shown) are both dependent on and dramatically responsive to IL-7 for growth in the absence of a feeder layer. Several of the tumor cell lines, although independent of cellular feeder layers and IL-7 for growth, retained some responsiveness to IL-7 (Figure 1).

The abilities of the tumor cell lines to form tumors in syngeneic BALB/c mice were compared to that of the parental R2 cell line. Tumor cell lines 1, 2, 3, 4 and

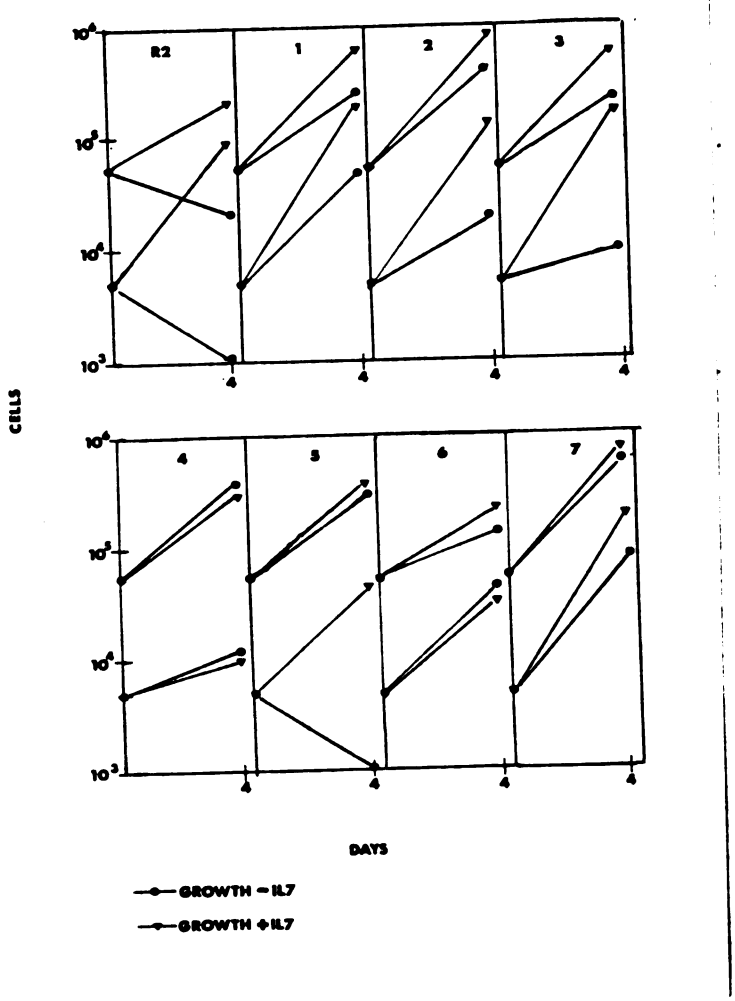


Figure 1. Growth of the R2 and seven tumor cell lines (1-7) in the presence and absence of IL-7. Cells were removed from feeder layers and resuspended in fresh medium with and without 10 units/ml IL-7. Either $5x10^3$ or $5x10^4$ cells were plated in suspension in 1 ml. On day 4, viable cells were counted in the presence of trypan blue. The graph plots the average total cells of duplicate cultures.

Table 1. Tumor challenges in BALB/c mice.

Cell Line	Frequency (animals)		Average Latency Till Moribund (days)	
	$2x10^6$ cells[a]	$4x10^6$ cells[a]	$2x10^6$ cells[a]	$4x10^6$ cells[a]
R2	1/4	1/4	81	70
1	1/5	5/5	44	25
2	2/5	5/5	41	24
3	1/4	5/5	44	27
4	4/4	N.D.	22	N.D.
5	N.D.	3/5	N.D.	50
6	N.D.	1/5	N.D.	22
7	2/2	3/4	16	45

N.D. = Not determined
[a] Two inocula were used.

7 were dramatically more tumorigenic than R2 (Table 1). Tumor cell lines 5 and 6 had lower frequencies of tumor induction than the other tumor cell lines. This is consistent with their lack of growth in the absence of a feeder layer in experiments of longer duration than those presented in Figure 1 (data not shown). All of the mice challenged with tumor cell lines did have a shorter latency till moribund than did those challenged with R2 (Table 1).

The data on feeder layer independence and tumorigenicity demonstrate that the tumor cell lines have acquired transformed growth potential which exceeds that of their parental cell line.

To examine the molecular events that underlie the transformed phenotypes of tumor cells, we have used a set of probes of oncogenes, tumor suppressor genes, growth factor genes, and sites of frequent viral integration (Table 2) to test for their involvement. Gene rearrangement or altered expression of these genes or sites

Table 2. Probes that were used in searching for the putative secondary events that may be involved in the tumor progression of R2 tumors.

Gene	Range(Kb)	Rg.[a]	Enzyme[b]	Probe(fragment)	Vector	Reference[c]
v-abl	>22.0	-	BHI	2.0 kb XbI-RI	pUCabl2.3	Witte O.
v-src	>16.0	-	BHI,RI	0.8 kb PvuII	pBR322	Slamon D.
hck	>20.0	-	BHI,RI	1.7 kb RI	pUC18	Perlmutter R.M.
lck	>22.0	-	RV	1.7 kb RI	pUC13	Perlmutter R.M.
v-fms	>10.0	-:+	RI;RNA	2.7 kb ClaI-BHI	pBR322	Sherr C.
N-myc	>24.0	-;+	BHI,RI;RNA	0.5 kb RI	pSP65	Alt F.
c-myb	>20.0[d]	-;+	BHI,RI;RNA	2.4 kb RI	pBR322	Bender T.P.
c-raf	>20.0[d]	-;-	RI;RNA	2.9 kb RI	pUC13	ATCC
GMCSF	22.0	-	BHI,RI	0.53 kb AvaII	pCDSV40	Frank
IL-3	>9.0	-	BHI,RI	0.6 kb PstI-NcoI	pCDSV40	Frank
CSF-1	11.0	-	BHI,RI	0.5 kb ScI	pBluescrib	Cole M.
IL-7		-	RNA	0.5 kb PstI	cos plasmid	Witte O.
kit	>20.0	-;-	KpnI;RNA	3.7 kb XhoI	pGEM72f(+)	AMGEN Co.
Rb	>20.0	-	RI,BHI	0.7 kb RI	p11-4	Imperiale M.
p53	>19.0	-	RI	2.7 kb BHI	prb17d	Imperiale M.
MHCclassI Ag	>20.0	-	RI	2.4 kb RI	pBR327	Fluck M.
B220	>20.0	-	RI,BHI	3.0 kb RI	pUC19	Esselman W.
max	>20.0	-;-	RI,RNA	0.55 kb RI	pvZ1	Eisenman R.
mlvi-1	21.8	-	BHI	1.2 kb RI-PvuII	pBR322	Tsichlis et al.,1983a
mlvi-2	18.4	-	BHI,ScI	2.0 kb H3	pBR322	Tsichlis et al.,1984
mlvi-3	>24.0	-	BHI	3.5 kb H3	pUC	Copeland N.G.
mlvi-4	>18	-	RI,BHI,RV, KpnI	1.0 kb H3-RI	pLE18	Copeland N.G.
pim-1	22.0	-	RI,RV	0.93 kb BHI	pBR322	Cuypers et al.,1984
pim-2	29.0	-	KpnI	1.4 kb PstI-RI	pSP64	Breuer M.
pvt-1	>20.6	-	KpnI,BHI	0.7 kb RI	pUC9	Graham et al.,1985
evi-1	7.0	-	RI	1.0 kb H3-PstI	pUC1	Mucenski et al.,1988a
ahi-1	7.5	-	BHI,RV	0.8 kb PstI-H3	pUC18	Jolicueur P.

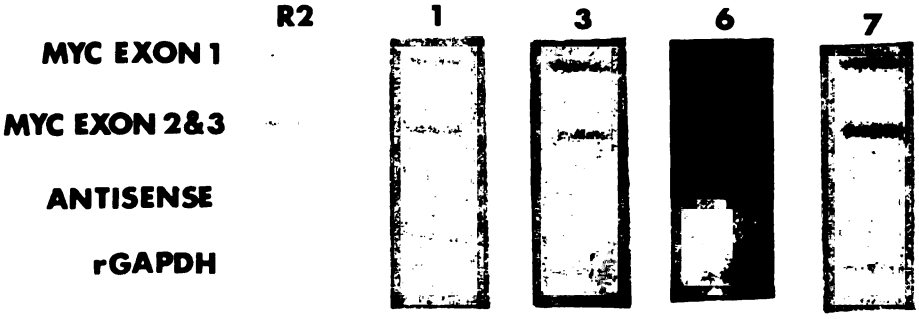
Abbreviation:
[a]: DNA rearrangement detected on southern blots or abnormal expression, on northern blots.
[b]: DNA blots with various restrictionenzyme digestion or RNA blots.
[c]: Original references or persons from which the vectors were obtained are listed.
[d]: Only Tumor 4 was examined for DNA rearrangement or abnormal expression.
RI: EcoRI, RV:EcoRV, BHI:BamHI, H3:HindIII, ScI:SacI, XbI:XbaI

was examined by Southern and Northern blotting analyses. Tumor 4 showed altered expression of several genes, and was described in detail in Bretz et al. (1992)(Appendix A). The c-*myc* gene was aberrantly expressed in four of the seven tumor cell lines tested (two of these also showing retroviral integration at c-*myc*) as described in Chen et al. (1991)(Chapter 2). No gene rearrangement was found in about a 20 kb region surrounding most of the other oncogenes or frequent viral integration sites tested in the tumor cells. No altered expression of other tested genes was detected in the tumor cells, with the exception of Tumor 4 (see Chapter 4).

Studies of mechanisms of elevated c-*myc* expression

We have observed a 3-fold increased level of c-*myc* mRNA in tumors 1 and 3, presumably as a result of the proviral insertion, and 4-fold elevated levels of c-*myc* mRNA in tumors 6 and 7 in the absence of any obvious genetic alteration (Figure 6 in Chapter 2). The c-*myc* mRNAs were all approximately 2.4kb in length, suggesting normal promoter usage and an unaltered RNA structure (Figure 6 in Chapter 2). RNAse protection studies confirmed normal promoter usage (data not shown).

Nuclear run-on experiments were performed in order to examine (i) whether the elevated c-*myc* mRNA levels reflected increased transcription and (ii) the role of transcriptional attenuation (Bentley and Groudine, 1986) in modulating c-*myc* expression in these tumors. Transcriptional initiation of c-*myc* was evaluated by hybridization of labeled RNA to a probe for exon 1 of c-*myc*, while elongation of transcription was evaluated by hybridization to a probe for exons 2 and 3 of c-*myc*.



TRANSCRIPTION LEVELS					
MYC EXON 1	1.0	3.1	3.1	1.6	1.0
MYC EXON 2&3	1.0	2.0	1.5	2.0	1.2

Figure 2. Nuclear run-on transcription. Labeled nuclear run-on products from R2 and tumors 1, 3, 6 and 7 were hybridized to probes for c-*myc* exon 1, c-*myc* exons 2 and 3, antisense upstream of c-*myc* and rGAPDH. Relative levels of transcription were determined by densitometry and normalized to rGAPDH.

Densitometric values were normalized to the amount of hybridization detected to a probe for rGAPDH. Background hybridization was evaluated with a probe for anti-sense transcripts upstream of the c-*myc* locus. In comparison to R2, tumors 1 and 3 showed about 3-fold increased transcriptional initiation and about 1.5 to 2-fold increased elongation (Figure 2). Since these values average the activity of the retrovirally activated allele(s) with the normal c-*myc* allele, they are likely to underestimate the degree to which the presence of the MoMuLV LTR affects c-*myc* transcription. The elevated transcription detected in this experiment is consistent with the 2 to 3-fold elevated steady state levels of c-*myc* mRNA observed by Northern analysis (Figure 6 in Chapter 2). Since initiation of transcription increases more than elongation, increased initiation rather than decreased attenuation is the probable mechanism by which retroviral integration increases c-*myc* mRNA expression. Tumor 6 showed about 1.5-fold increased initiation and about 2-fold increased elongation (Figure 2). This is consistent with the 3-fold increased steady state levels of c–*myc* mRNA observed for tumor 6 in Figure 6(of Chapter 2). On the other hand, tumor 7 did not show significant increases in either parameter. The data offer no evidence for a transcriptional mechanism leading to the elevated c-*myc* mRNA levels observed for tumor 7 (Figure 6 in Chapter 2).

Determinations of the half-life of c-*myc* mRNA in the presence of actinomycin D did not reveal significant differences between R2 and tumors 1, 3, 6, and 7 (Table 3). R2 and tumor cell lines were expanded and maintained in an actively growing state, and then treated with 5 microgram/ml Actinomycin D. Portions of cells were then harvested at three time points: 0 hour, 1/2 hour, and 1 1/2 hour after adding Actinomycin D. RNAs of each cell line were isolated at the

three time points and subjected to Northern hybridization analyses with a c-*myc*-specific probe and a rGAPDH gene probe. Densitometric measurements were taken for both c-*myc* and rGAPDH specific signals on the resulting autoradiogram. Since the level of rGAPDH remained relatively unchanged among R2 and tumor cell lines during the 1 1/2 hour time period, we normalized the level of c-*myc* mRNA in each time point to that of rGAPDH at the same time point under the assumption that the degradation rate of rGAPDH mRNA is the same in R2 and tumor cell lines. The stability of c-*myc* mRNA was determined by the assessment of the relative half-life assigned to R2 and each tumor cell line by the method described in the table legend. The relative T 1/2 of c-*myc* mRNA is 32 minutes, whereas those of tumor cell lines ranged from 28 to 40 minutes. Therefore, we concluded that alterations in the stability of c-*myc* mRNA did not play a role in the increased c-*myc* mRNA levels observed in these tumor cells.

Table 3. Relative stability of c-*myc* mRNA in R2 and tumors

cells	relative T 1/2 (min)*
R2	32(29,34)
T1	40(35,46)
T3	36(31,42)
T6	28(26,29)
T7	38(35,41)

*These values were determined by the following calculation procedure. The signal of c-*myc* was normalized to rGAPDH for a loading control, and then the resulting values were plotted on a semilog graph (log of hybridization intensity versus time (minutes)). The relative T 1/2 was determined by the time required for the loss of half of the original hybridization intensity.

Observation of light chain rearrangement in tumor cell lines

One other interesting observation obtained in these studies was the detection of immunoglobulin light chain rearrangements in most of the tumors (Figure 3). As mentioned in Chapter 2, all tumor cell lines possess the same immunoglobulin (Ig) heavy chain rearrangements as that of the parental R2 cell line (Figure 4). A southern blot hybridization analysis with a probe specific to the J region of Ig heavy chain revealed hybridizations to a 6.0 kb germ line fragment (Figure 4, lane 1) on *Eco*RI digested liver DNA and to one or two rearranged fragments (7.0 kb and 3.5 kb, Figure 4, lane 2-9) on DNA of the R2 and tumor cells. DNA from the original tumor sample (a portion of which may consist normal cells) of tumor 6 was used in the analysis, therefore, a germ line fragment was also observed (lane 8). When the same DNAs were digested with *Bam*HI, and probed with a DNA fragment from the constant region of the kappa chain, all the tumor cell lines showed dramatically different banding patterns (Fig. 3, lanes 2, 4, 5, 7, and 8) from the germ line fragment observed in the R2 cell line and liver (Fig. 3, lanes 1 and 9, respectively). Tumor cell lines 2 and 5 (Fig. 3, lanes 3 and 6, respectively) both retained the germ line fragment, but a portion of these cells may already have undergone kappa chain rearrangements as judged by the presence of some minor hybridized bands. No lambda light chain gene rearrangement was found in the R2 and tumor cell lines (data not shown). Immunoprecipitation experiments with both anti-mu and anti-kappa chain anti-sera revealed that the rearrangements successfully produced authentic immunoglobulin heavy and light chain proteins(data not shown).

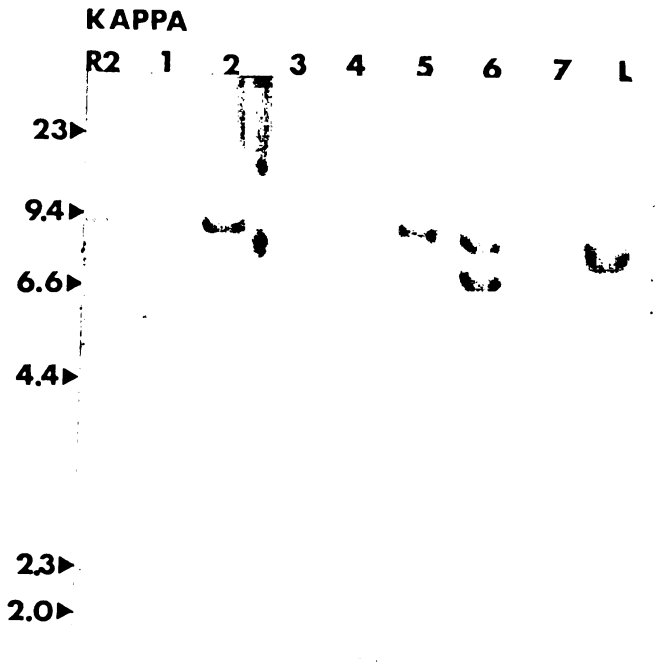


Figure 3. Kappa chain gene rearrangement. *Bam*HI digested R2 (lane 1), liver (lane 9), and tumor cell DNAs (lane 2-8) were subjected to gel electrophoresis through 0.8 % agarose, blotted to nytran paper, and hybridized with a nick-translated probe of a kappa-chain-specific DNA fragment. Size markers are the positions of an ethidium bromide-stained *Hind*III digest of bacteriophage lambda and are denoted in kilobases.

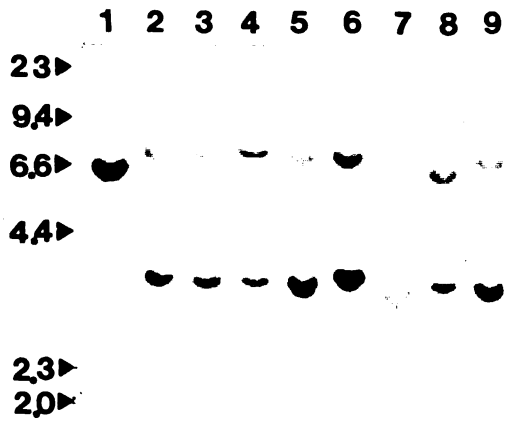


Figure 4. Mu chain gene rearrangement. *Eco*RI digested liver (lane 1), R2 (lane 2), and tumor DNAs (lane 3-9) were electrophoresed through a 0.8 % agarose gel. The blot was probed for the J region of mu heavy chain gene. Size markers are the positions of an ethidium bromide-stained *Hind*III digest of bacteriophage lambda and are denoted in kilobases.

Discussion:

The data presented here confirm that the R2 tumors had acquired a more transformed phenotype as evidenced by their high tumorigenicity and their growth factor independence. The fact that these tumors were not uniformly tumorigenic (i.e. did not cause tumors in all the tumor-challenged mice) suggested the possibility of tumor regression through a variety of mechanisms. These may include stimulation of host immune system perhaps by generating a recognizable tumor specific antigen, or growth inhibition by some intrinsic factors that evolved in tumor cells during the course of tumor challenge. The intermediate transformed phenotypes of these tumors may provide tools for studying tumor progression.

The regulatory mechanisms responsible for increased levels of c-*myc* mRNA probably occur at both transcriptional and posttranscriptional levels as described by Klein and Klein (1985). In the case of tumors 1 and 3, MoMuLV had integrated immediately 5' to c-*myc* in a reverse transcriptional orientation, suggesting enhancer activation. Nuclear run-on experiments showed that these tumors with MoMuLV integration near c-*myc* displayed increased levels of transcription consistent with the increased steady state level of c-*myc* mRNA. The nature of the events leading to elevated levels of c-*myc* mRNA in the other two tumors is unclear. Tumor 6 displayed increased transcription, but no gross structural alterations were observed on either the DNA or RNA levels. The attenuation of transcription in the first exon of c-*myc* was also unaffected. Tumor 7 showed no increase in transcription and, as in the case of tumor 6, there were no structural abnormalities observed in either the c-*myc* gene or its transcription products. Southern blot analyses of genomic restriction fragments extending approximately

15 kb upstream and 30 kb downstream of c-*myc* detected no rearrangements in either tumor 6 or 7 (data not shown) suggesting that retroviral integration is unlikely to play a direct role in the activation of c-*myc* in these tumors. On the other hand, MoMuLV integrations at *Mlvi*-1 and *Mlvi*-4 can affect c-*myc* over a long distance (Lazo et al., 1990). However, we have not detected rearrangements at these sites (data not shown). The increased c-*myc* mRNA level in tumor 7 may be due to posttranscriptional mechanisms such as increased maturation rate of poly A^+ RNA and increased transport rate through the nuclear membrane. Studies of mRNA half-life show that these mechanisms are not involved.

The mechanism by which c–*myc* activation promotes tumorigenesis is unclear. Expression of v-*myc* has been reported to abrogate the dependence of lymphoid and myeloid cell lines on IL-2 and IL-3 for growth (Payne et al., 1982). Since all the tumors with and without c–*myc* activation show reduced dependence on IL-7, it is difficult to conclude that a similar causal relationship exists between c-*myc* activation and alleviation of IL–7 dependence.

The fact that pre-B cells were able to continue to undergo light chain rearrangements and to successfully produce kappa chains suggests that introduction of the v-Ha-*ras* oncogene does not block B cell differentiation. It also implies that B cell lymphomas may result from a gradual acquisition of genetic lesions by a precursor B cell that can progress along the differentiation pathway. Similar results had been shown in Eμ-*myc* transgenic mice, in which tumors of both pre-B and B cells were obtained (Adams et al., 1985; Harris et al., 1988). On the other hand, the majority of tumors induced by v-*abl* seem to be frozen at a pre-B stage (Sklar et al., 1974; Sklar et al.,1975; Weimann, 1976). Interestingly,

long term cultures (as long as the period of tumor challenge) of the R2 cell line did not show evidence of light chain rearrangement *in vitro* (data not shown). It seems that some *in vivo* factors may be important for light chain rearrangement in addition to a successful heavy chain rearrangement. *In vitro* triggering of light chain rearrangements of R2 cells may help to identify these factors, and provide more insights on B cell differentiation.

Although no gross gene aberrations in genes other than c-*myc* were detected in the R2-derived tumor cells, we can not exclude their roles in the additive transformed phenotypes of these cells. Mutations of other types such as point mutation, small deletion, or viral integration in regions outside of the tested regions may have occurred. Further investigations will be required to elucidate the secondary events in the tumor progression of v-Ha-*ras* transformed pre-B cells.

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Summary and Discussion

The goals of my thesis study were to search for oncogenes that were capable of cooperating with the v-Ha-*ras* oncogene in transforming murine B cells, and to examine the differentiation status of tumor cells. Two model systems were used. The first model system tested for genes that could cooperate v-Ha-*ras* in transforming murine B cells *in vivo*, whereas the second model system examined this issue *in vitro*.

In the first model system, we found that tumors had acquired more malignant growth properties by comparing the growth characteristics of R2, a v-Hr-*ras*-expressing pre-B cell line, and tumor cells derived from it. The tumor cell lines exhibited greater tumorigenicity and shorter latency in tumor development than the parental R2 cell line. In addition, tumor cell lines had acquired growth factor independence. Two major molecular events have been shown to be associated with these transformed phenotypes: an increased number of virus-associated restriction fragments on southern blot analysis, and alteration of c-*myc* expression.

First, studies on the viral integration patterns of R2 cells and tumors suggested that 6/7 tumors were an outgrowth of a single R2 subclone, which contained unique viral related fragments. These unique virus-associated fragments could have resulted either from recombination of the original integration sites of the parental cell line, or from new viral integrations, or both. Oncogene activations had been found previously through each mechanism. We found two tumors that had activated c-*myc* by viral integration.

Many, if not all, proviruses near c-*myc* have sustained deletions of the viral genome, probably as a result of homologous recombination between the two

two LTRs. Sometimes little more than a solitary LTR is left intact (Robinson and Gagnon, 1986). A mechanistic explanation of the requirement for these deletions was given by Cullen et al. (1984), who showed that transcription starting in the 3' LTR of an intact provirus is quenched by transcription driven by the 5' LTR. By artificial termination of transcription within the provirus, or by the deletions as found in tumors, the activity of the 3' LTR becomes sufficient to act as a strong promoter (Fujisawa et al., 1985) and thus to activate oncogenes. The c-*myc* rearrangement in two of the R2 tumor cell lines resembles the above observation in that the genome of MoMuLV integrated in the c-*myc* gene in these two tumors has undergone a further rearrangement (data not shown). This viral genome rearrangement was assessed by a similar approach to that described in Figure 4 (Chen et al., 1991), with the exception that restriction mapping utilized enzymes that cut inside the viral genome rather than in the LTRs. However, we do not know the significance of this viral genome rearrangement, since the activation of c-*myc* gene in this case is presumably through an enhancer activation rather than a promoter activation.

The reintegration of MoMuLV and avian leukosis virus (ALV) into the cellular genome of infected cells has been well documented in the tumors they generate, and are thought to be causative for tumor formation through the activation of nearby cellular oncogenes (Jaenisch, 1976; Payne et al., 1982). Studies on transgenic mice carrying a single copy of MoMuLV DNA sequences has shown an increase to two MoMuLV specific DNA copies per haploid mouse genome in preleukemic tissues, and a further increase to 3-4 copies in leukemic tissues (Jaenisch, 1979). This amplification of MoMuLV is seen in target organs but not

in nontarget organs, and thus appears to be related to leukemic transformation.

At least one tumor specific virus-related fragment (*bonc*-1) in our studies belonged to the aforementioned class (reintegration), since DNA of the parental R2 cell line showed a germ line pattern at this locus. The nature of other tumor specific virus-related fragments is unknown. We were unable to detect any transcriptional activity over a 20 kb region in the vicinity of the *bonc*-1 locus. However, sequence homology of *bonc*-1 was found to genomes of many mammalian species and chicken. The high conservation of *bonc*-1 suggests that this region has an important function. On the other hand, activation of protooncogenes through distal provirus integrations (270 kb) has been observed (Lazo et al., 1990), and it is possible that a putative oncogene is located beyond the 20 kb region studied. Whether this putative gene would indeed be able to cooperate with v-Ha-*ras* gene in course of tumor progression, and whether it would have B cell specificity in the induction of neoplasia remains to be tested.

Although I was not able to definitively identify a novel oncogene during the period of my thesis study, the fact that MoMuLV was highly mobilized in infected cells provides a method to identify new protooncogenes involved in hematopoietic diseases. In retrospect, modifications of the insertional mutagen (MoMuLV) might have been added to facilitate the identification of viral integration sites. These modifications might include the addition of a bacterial *supF* gene or some other bacterial indicator gene to the MoMuLV sequences. Tagging with a *supF* gene would allow the construction of an integration library in a amber mutant λ phage that could contain only clones with sequences of *supF* integrated proviruses and their flanking cellular loci as described by Shih et al. (1988).

The second molecular event found among the tumor cell lines derived from the R2 cell line was the increased steady state level of c-*myc* mRNA in four of the seven tumors. Studies of c-*myc* activation have revealed that the c-*myc* gene can be deregulated by several mechanisms: increased transcription, increased elongation, and perhaps increased transport through nuclear membranes (for review see Klein and Klein, 1985). Our findings are consistent with varied mechanisms for deregulation of c-*myc*. Two of the four tumors studied here had suffered a MoMuLV integration at about 150 bp 5' of the first exon of the c-*myc* gene in a reverse transcriptional orientation, presumably driving the expression of the c-*myc* gene through enhancer activation and increased transcription. The exact mechanism of c-*myc* activation of the other two tumors is unknown, although one tumor appears to be transcriptionally activated.

Our finding of MoMuLV integration into the c-*myc* gene in B cell lymphomas is novel. MoMuLV integration in the c-*myc* locus has been commonly found in murine thymomas (Selten et al., 1984). Feline leukemia virus integration near c-*myc* has been observed to occur only in T cell lymphoma (Neil et al., 1984). Mucenski et al. (1987) reported that viral integration in the c-*myc* locus occurs only in murine T cell lymphomas from studies on a panel of B cell and T cell lymphomas from AKRD mice. Our finding suggests that the tissue tropism of MoMuLV (and perhaps other MLVs) in naturally occurring tumors (i.e., thymomas) may not be attributable to tissue specific integration sites. The observation that viral integrations into *pim*-1, originally identified as a common T cell integration site, were also detected in pre-B, and B cell lymphomas supports this idea (Mucenski et al., 1987). Although some virus integration sites such as *mlvi*-1, *mlvi*-2, *fis*-1,

and *pvt*-1 have been shown only found in T cell lymphomas (Mucenski et al.1987), the significance of these loci in tumorigenesis is not clear since no gene products have been found.

C-*myc* gene alterations have been associated with many B cell neoplasias and have been proven to be able to initiate or promote B cell malignancy in transgenic mice (Adams and Cory, 1991). Cooperation of the c-*myc* gene with the v-Ha-*ras* gene in transformation has been shown in both fibroblasts (Land et al., 1983a) and B lymphoid cells (Schwartz et al., 1986a). The occurrence of c-*myc* alteration in tumor challenges with a v-Ha-*ras*-expressing pre-B cell suggests the validity of this model system in identifying genes that are associated with the *in vivo* tumor progression of B cell neoplasia.

Besides retroviral integration and c-*myc* activation, we did not detect other gene rearrangements in tumors when probes of other known oncogenes, tumor suppressor genes, growth factor genes, and flanking genes of frequent viral integration sites (as listed in Appendix B) were used in southern hybridization analyses. Another approach applicable to the search for non-virally related secondary events involved in tumor progression of these tumors is the transfection of tumor DNA into indicator cells. If this approach were taken, care would have to be taken in choosing a appropriate indicator cell, since the tumor cells all posses a v-Ha-*ras* gene which is known to be dominant in focus formation assays in NIH3T3 cells.

Two other interesting features were observed among the v-Ha-*ras* tumors: a continuation of B cell differentiation and a lineage switch to macrophage-like cells. As discussed in appendix B, most tumors derived from the pre-B cell line,

R2, had undergone kappa chain rearrangements and produced kappa chain proteins. However, we did not observe detectable light chain rearrangements in R2 cells that had been cultured *in vitro* for a similar or longer period of time as that required for tumorigenesis. This discrepancy was particularly interesting to us, since it is generally thought that light chain rearrangements will be triggered once a successful heavy chain rearrangement has been generated in the same cell. These results suggested that additional factors are required for light chain rearrangements besides the production of an authentic heavy chain protein. This factor was absent in the Whitlock-Witte culture system but present in mice. A similar lack of *in vitro* light chain rearrangement was also found in another v-Ha-*ras* transformed cell line, R1, which was generated by the same procedure as that of R2. Tumors derived from R1 cells also exhibited light chain rearrangement (data not shown), suggesting the generality of this phenomenon.

On the other hand, it is also possible that some inhibitory factors may be present in the Whitlock-Witte culture system to prevent differentiation of these pre-B cell lines. A similar phenomenon has been demonstrated by Alt et al. (1981) on cell lines derived after *in vitro* infection of bone marrow or fetal liver cells with Abelson murine leukemia virus (A-MuLV). Most, if not all, of the cell lines were pre-B cells and they rarely went through light chain rearrangement, even after a long period of cultivation *in vitro*. In contrast, tumors induced by A-MuLV often display light chain rearrangements (Sklar et al., 1975; Weimann, 1976). However, the fact that certain subclones of pre-B cell lines established in the Whitlock and Witte are capable of undergone light chain rearrangements *in vitro* (Denis and Witte, 1986) argues against this possibility. Perhaps, this *in vitro* inhibition of light chain

rearrangements in both v-Ha-*ras* and v-*abl* expressing pre-B cells are associated with these oncogene expression. An *in vivo* factor may bypass this inhibitory effect of oncogene expression in tumors carrying the same oncogenes. At any rate, these v-Ha-*ras* transformed cell lines provide a good model system to search for factors that may interfere or participate in the differentiation process of B cells.

Another interesting feature of these studies was the discovery of a lineage switch of certain B cell tumor lines to macrophage-like cells. Although other groups have reported a similar phenomenon (Klinken et al., 1988; Davidson et al., 1988; Borzillo et al., 1990), the studies presented here uniquely establish the functionality of these lineage switch macrophages: LPS-induced cytokine release and the ability to present antigen. These macrophage cell lines may be the first macrophage lines ever reported to present antigens *in vitro* (John Cohen, U. Colorado, personal communication). Therefore, they are potentially useful in research areas such as of B cell activation, clonal expansion of T cells, and other cell-cell interactions that require macrophages with an antigen presenting capacity. They and their antecedent cells (the R1 and R2 cell lines) may prove useful in studying the lineage determination of lymphoid and myeloid cells.

A second model system which involved the introduction of oncogenes into v-Ha-*ras* transformed cells or the co-infection of bone marrow cells with retroviruses carrying a v-Ha-*ras* oncogene and another oncogene of interest provided a convenient way to test the oncogenic potentials of various oncogenes. With this model, we have shown that expression of IL-7 was not sufficient to cause a fully tumorigenic phenotype in a v-Ha-*ras* expressing cell line. These findings

were consistent with what had been reported by Young et al. (1991) and Overell et al. (1991). Both groups reported that IL-7 can participate in the tumorigenesis of murine B cell neoplasia, with the requirement of other secondary events. The insufficiency of IL-7 expression to confer tumorigenicity may imply the existence of a tightly regulated mechanism of IL-7 induced growth *in vivo*.

Another important finding from this model system was the discrepancy between the highly tumorigenic phenotype of cells transformed by a co-infection of bone marrow with v-Ha-*ras* and *myc*, or v-Ha-*ras* and IL-7 and the non-tumorigenic phenotype of cells sequentially infected with the same two oncogenes. The clonality of the outgrowing cell lines in the co-infection of bone-marrow and their high frequency and short latency for generation of tumors contrast sharply with the properties of v-Ha-*ras* cell lines superinfected with v-*myc* or IL-7 viruses. The clonal outgrowths of co-infections of bone marrow may represent the dominance of a rare and more highly transformed cell over many other cells carrying one or both oncogenes in this heterogeneous population. While IL-7 and v-Ha-*ras* expression may contribute to the transformed phenotype, the cooperation of other rare oncogenic events is critical to the tumorigenic phenotype. The more homogeneous population in the sequential infection of a single cell line does not as easily allow selection for other rare events. Therefore, interpretation of experiments of this sort should be made carefully. To ensure that the synergistic effect of two oncogenes in any particular system is indeed derived from the two oncogenes and not other "co-selected events", the two oncogenes should be sequentially introduced into a cell line rather than a heterogeneous population. Cells with an intermediate transformed phenotype derived from the

these experiments can be used as targets for testing the involvement of other oncogenic events.

In summary, results from both of our model systems for B cell neoplasia suggested that multiple events (at least three) were required for the induction of a fully malignant B cell phenotype. Both the c-*myc* gene and IL-7 gene were capable of cooperating with the v-Ha-*ras* oncogene in transforming murine B cells. However, to achieve a fully tumorigenic phenotype, a tertiary event(s) was required in both instances. The two models systems provide the opportunity to identify this tertiary event (s) as well as other independent steps involved in the tumor progression of B cell neoplasia. They also provide good tools to study B cell differentiation and lineage determination of myeloid and lymphoid cells.

In the future, if particular gene alterations are found to be restricted to the development of murine B cell neoplasia, and a correlation is found between murine and human B cell neoplasia, detection of early-stage neoplastic cells may be possible. Detection protocols may operate through the identification of mutant (quantitatively or qualitatively) gene products secreted into the blood or other body fluids, or through the detection of antibodies to the mutant gene products. In addition, the presence of genetic alterations in tumors may provide a molecular tool for improved prognostic evaluation of patients, as is now possible with colorectal cancer (Vogelstein et al., 1989; Kern et al., 1989). Finally, the identification of mutant gene products in tumors may provide targets for new chemotherapeutic agents.