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Genomic Instability in a Bcr-abl Leukemia Mouse Model

by

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"And, when you want something, all the universe conspires in helping you achieve it"

-A conversation between king Melchizedek and the young shepherd Santiago. From *The Alchemist* by Paulo Coelho.

To my parents.....

Hind and Fawzi Salloukh

Abstract

The Bcr-abl translocation arises from a reciprocal translocation between chromosomes 9 and 22 and results in the augmentation of the tyrosine kinase activity of c-Abl. Chronic myelogenous leukemia (CML) is one of several hematological malignancies associated with Bcr-abl expression. The pathogenesis of CML, associated with P210Bcr-abl, is bi-phasic consisting of an initial chronic phase followed by a severe terminal phase referred to as acute blast crisis. The chronic phase of the disease is characterized by granulocytic hyperplasia but in which normal hematologic maturation is still intact. Patients ultimately enter the terminal blast crisis where hematologic maturation is lost, resulting in the accumulation of immature blast cells and a severe immuno-compromised state. Progression to the blast terminal phase is associated with genomic instability demonstrated by the accumulation of genetic and cytogenetic abnormalities. Results from *in vitro* cell line systems expressing Bcr-abl have suggested that the loss of cell-cycle arrest and induction of apoptosis, as a result of genotoxic stress, might be responsible for this phenotype. In this study, I utilized a transgenic mouse model which expresses P190Bcr-abl to extend those observations to an *in vivo* model for leukemia. I observed normal cell-cycle arrest and induction of apoptosis following the induction of DNA damage. However, using the Big Blue *in vivo* mutagenesis mouse assay system, I evaluated genomic instability in P190Bcr-abl mice by measuring mutation frequencies *in vivo*. I observed an increase in mutation frequencies in spleens and kidneys from P190Bcr-abl mice. This Bcr-abl-induced mutator phenotype may explain the inherent genomic instability associated with the progression of CML and other diseases associated with the expression of activated tyrosine kinases.

Resumé

La translocation Bcr-abl provient d'une translocation réciproque entre les chromosomes 9 et 22, ce qui entraîne l'activation constitutive de l'activité tyrosine kinase de la protéine c-Abl. L'expression de Bcr-abl est associée à une grave maladie hématologique, qui est la leucémie myélogénique chronique (LMC). La pathogénèse de LMC, liée à la présence de la proteine P210Bcr-abl, est bi-phasique; elle comprend initialement une phase chronique, suivie par une grave phase terminale appelée crise blastique. La phase chronique de la maladie est caracterisée par une hyperplasie granulocytaire aigüe, où la maturation hématologique se trouve toutefois intacte. Les patients atteignent ultimement la crise blastique terminale, où dans ce cas, la maturation hématologique ne se fait plus. Les cellules blastiques immatures s'accumulent alors, compromettant ainsi gravement le système immunitaire des patients. L'accumulation de plusieurs anomalies génétiques et cytogénétiques, causée par une instabilité génomique, est associée avec la progression de la maladie vers la phase blastique terminale. Des expériences *in vitro*, sur des lignées cellulaires qui expriment Bcr-abl suggéraient initialement que, suite a un stress génotoxique, une perte de l'arrêt du cycle cellulaire et de l'induction de l'apoptose étaient la cause de ce phénotype. Dans l'étude que je présente, j'ai utilisé un modèle de souris transgéniques qui expriment la protéine Bcr-abl. Suite à l'induction de dommages à l'ADN, j'ai observé un arrêt normal du cycle cellulaire, ainsi qu'une induction normale de l'apoptose. Cependant, en utilisant le système *in vivo* de test de mutagénèse "Big Blue", j'ai étudié, dans les souris exprimant P190Bcr-abl, l'instabilité génomique, en mesurant *in vivo* la fréquence des mutations. J'ai observé, une augmentation de la fréquence des mutations, dans la rate, ainsi que dans les reins de ces souris. Ce phénotype mutationnel, induit par la proteine Bcr-abl, pourrait expliquer l'instabilité génomique inhérente associée avec la progression de la leucémie

mye'loge'nique chronique, ainsi qu'avec d'autres maladies associe'es à l'expression de tyrosines kinases active'es.

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Abreviation List

3BP-1:	3 binding protein-1
32Dcl3:	Mouse myeloid cell line
Abi-1:	Mouse abl-interactor
Abi-2:	Human abl-interactor
Abl:	Abelson
ALL:	Acute Lymphoblastic Leukemia
AT:	Ataxia telangiestacia
ATM:	product of the gene mutated in Ataxia telangiectasia
BAF3:	Bone marrow-derived murine IL-3 dependant pro-B cell line
BCA:	Bicinchonic acid
Bcl:	B cell lymphoma
BFU-E:	Blast froming units-erythroid
bp:	base pair
BrdU:	Bromodeoxyuridine
BSA:	Bovine serum albumine
c-Abl:	Cellular abelson
c-Cbl:	Cellular homologue of v-abl (Cas NS-1 retroviral induced pre-B lymphoma)
c-jun:	Protein member of AP1 transcription factor complex (Avian sarcoma virus 17)
c-Kit:	Tyrosine kinase receptor that binds Steel factor
CBC:	Cell blood count
Cdc2:	Cell division cycle 2 protein
Cdk:	Cyclin-dependant kinase
cDNA:	Complementary deoxynucleotidyl acid
CFU-GM:	Colony forming unit-granulocyte monocytes
CFU:	Colony forming unit

CML:	Chronic Myelogenous Leukemia
Co:	Cobalt
CrK:	Adaptor protein with SH2 and SH3 domains but no catalytic domains
CSF-1:	Colony stimulating factor
CT:	calcitonin
CTD:	Carboxy-terminal domain
Cyt-c:	Cytochrome c
DA-1:	IL-3 dependant murine myeloid progenitor cell line
DFF:	DNA fragmentation factor
DNA-PK:	DNA dependant protein kinase
DNA:	Deoxyribonucleic acid
E. coli:	*Escherichia coli*
ECL:	Enhanced chemiluminescence system
ECM:	Extracellular matrix
EDTA:	Ethylenedinitro tetraacetic acid
EGF:	Epithelial growth factor
EGTA:	Ethelenebis(oxyethylenenitrilo)tatraacetic acid
EPO:	Erythropoietin
F_1:	First generation mice
F-actin:	Filamentous actin
FACS:	Fluorescence-activated cell sorter
FAK:	Focal adhesion kinase
Fas:	(or CD95) A 45 kDa glycoprotein belonging to the tumor necrosis factor (TNF) receptor family
FICT:	Fluorescein isothiocyanate
FISH:	Fluorescent in situ hybridization
FN:	Fibronectin

fos:	Protein member of AP1 transcription factor complex (FBJ osteosarcoma)
Fps/Fes:	Cytoplasmic protein kinase that phosphorylate tyrosine residues (Fujinami avian sarcoma and feline sarcoma)
GADD45:	Growth arrest and DNA damage gene
G-CSF:	granulocyte colony stimulating factor
GAP:	GTPase-activating protein
GEF:	Guanine nucleotide exchange factor
GM-CSF:	Granulocyte macrophage colony stimulating factor
GTBP	G/T binding protein
GTP:	Guanosine tri-phosphate
GVDH:	Graft-versus-host disease
Gy:	Gray
HL60:	A promyelocytic cell line derived from patients with acute promytelocytic leukemia
HLA:	Human leukocyte antigen
HLH:	Helix-loop-helix domain
HNPCC:	Hereditary non polyposis colorectal cancer
HZ2-FSV:	Hardy-Zuckerman-2 feline sarcoma virus
IFN-α:	Interferon α
IGF2:	Insulin growth factor II gene
Il-2:	Interleukin 2
Il-3:	Interleukin 3
JAK:	Janus kinase
JNK:	Jun kinase
K562:	Erythromyeloid cell line derived from a CML patient and positive for p210Bcr-abl
L:	Leukemic
LacI:	*E coli* gene coding for a repressor that binds to the lactose operon

LacZ:	*E coli* gene coding for β-galactosidase activity and regulated by LacI
LAP:	Leukocyte alkaline phosphatase
LFA-3:	Lymphocyte fraction-associated antigen
LIZ:	Chromosomally-integrated λ bacteriophage shuttle vector
LOH:	Loss of heterozygosity
LOI:	Loss of imprinting
LZ:	Leucine-zipper domain
M-bcr:	Major break cluster region
m-bcr:	minor break cluster region
MAP:	Mitogen activated protein
MAPK:	Mitogen activated protein kinase
MCF-7:	Breast adenocarcinoma cell line with epithelial-like morphology
MEK:	MAP or extracellular-regulated kinase
MEM:	Eagle Modified Medium
MHC:	Major histocompatibility complex
MIP-1α:	Macrophage inflamatory protein 1α
MMR:	Mismatch repair
MO7e:	Human cytokine-dependant human myeloid cell line with a megakaryoblastic phenotype
mRNA:	Messenger ribonucleic acid
myc:	Transcription factor (Avian MC29 myelocytomastosis)
NF-κb:	Nuclear factor activates κ-immunoglobulin genes in B lymphocytes
NLS:	Nuclear localization signal
ODNS:	Oligodeoxynucleotides
$p53^{+/-}$:	p53 heterozygote null mice
$p53^{-/-}$:	p53 homogygote null mice
$p62^{dok}$:	Protein 62 downstream of tyrosine kinases

$P95^{vav}$:	A protein with signal transduction and transcription regulation characteristics
PARPS:	Adenine diphosphate [ADP]-ribose polymerase
PBS:	Phosphate buffered saline
PCR:	Polymerase chain reaction
PDGF:	Platelet derived growth factor
pfu:	Plaque forming units
PH:	Plekstrin homology
Ph:	Philadelphia Chromosome
PI3-K:	Phosphatidylinositol 3'-kinase
PI:	Propidium iodide
PL:	Pre-leukemic
PMSF:	Phenylmethylsulfonylfluoride
PolII-neo:	A polII fragment containing a neomyecin resistance gene
PVDF:	Polyvinylidene fluoride
Ras:	Guanine nucleotide binding protein with GTPase activity
Rb:	Retinoblastoma protein
RelA:	Avian reticuloendotheliosis
rhSF:	Recombinant human steel factor
RNA:	Ribonucleic acid
ROS:	Reactive oxygen species
RT-PCR:	Reverse transcriptase polymerase chain reaction
RTK:	Receptor tyrosine kinase
SCID:	Sever combined immuno-deficiency
SDS:	sodium dodecyl sulfate
SH1,2,3:	Src-homology domains
Sos:	Son-of-sevenless has ras-specific GEF activity
Src:	Cytoplasmic protein kinase that phosphorylate tyrosine residues (Rous avian

	sarcoma)
STAT:	Signal transducer and activator of transcription
TdT:	Terminal deoxynucleotide transferase
TE:	Tris-EDTA
U-937:	A cell line derived from a human histiocytic lymphoma exhibiting monocyte-like characteristics
v-Abl:	Viral abelson
WBC:	White blood cell
X-gal:	5-bromo-4-chloro-3-indol-ß-galactosidase
µ-bcr:	Third break cluster region in Bcr-abl

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I- Introduction

1.1 Etiology and Pathogenesis

Chronic myelogenous leukemia (CML) is a clonal myeloproliferative disorder arising from the neoplastic transformation of a multipotential hematopoetic stem cell. Clinically, the disease is characterized by a massive overproduction of normal appearing yet slightly defective granulocytes. CML passes through a biphasic course consisting initially of a long preliminary period termed chronic phase and characterized by myeloid hyperplasia. This is followed by acute transformation to a blast crisis marked by an increase of cellular immaturity in either the myeloid or lymphoid compartments [1]. The cytogenetic hallmark of CML is the Philadelphia (Ph) chromosome. This chromosome results from an unequal reciprocal translocation, t(9,22)(q34;q11), where most of the *c-abl* proto-oncogene (exons 2-11) situated on chromosome 9 is translocated to chromosome 22 (Fig. 1). The breakpoint on chromosome 22 is clustered around a 5.8 Kb "major break cluster region" (M-*bcr*) in what became known as the *bcr* gene. This region spans 5 exons referred to as exons b1 to b5 but better known as exons 12 to 16 of the *bcr* gene. This translocation results in a head-to-tail fusion of *c-abl* exon 2 to M-*bcr* exons 2 or 3 resulting in a hybrid *bcr-abl* gene encoding a 210 kDa (P210) fusion protein[1] (Fig.1). In Ph^- acute lymphoblastic leukemia (ALL) and in rare cases of CML, the *bcr* breakpoint is further upstream on chromosome 22 in a region referred to as m-*bcr* in the long intron between exons e1 and e2 resulting in the creation of a smaller 185 kDa (P185 or P190) protein[2] (Fig. 1). Bcr-abl fusion protein is present in

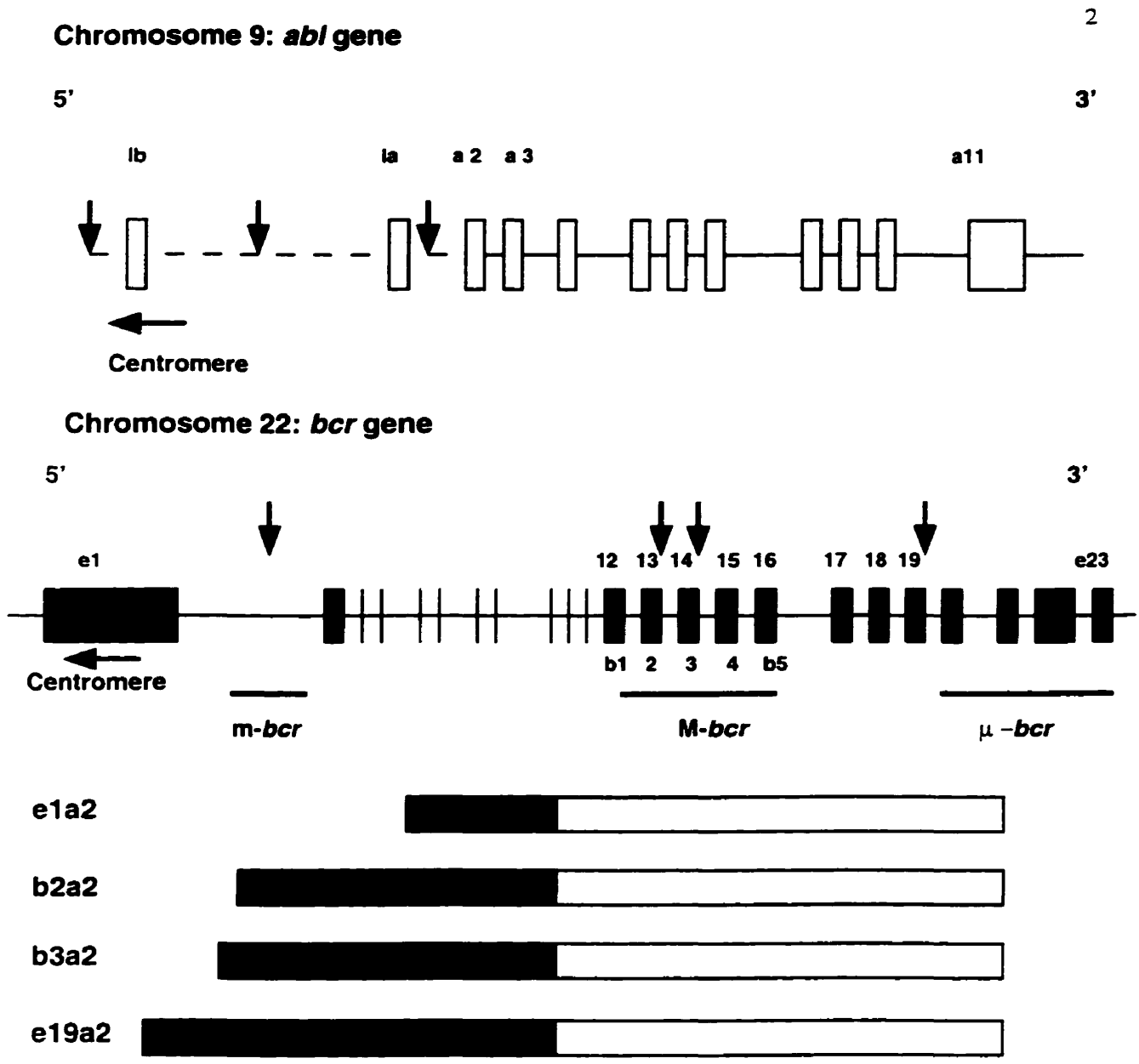


Figure 1 Schematic representation of possible Bcr-abl fusion products.

Possible breakpoint positions in *c-abl* and *c-bcr* are represented by vertical arrows. The e1a2 fusion product results in a 190 kDa protein associated with acute lymphoblastic leukemia (ALL) while the b2a2 and b3a2 products code for a 210 kDa protein mainly associated with chronic myelogenous leukemia (CML). The e19a2 fusion transcript codes for a rare 230 kDa protein associated with a disease that largely resembles a chronic neutrophilic leukemia. The breakpoint in *c-abl* usually occurs in the second intron and results in the translocation of most of the gene (exons 2-11) to chromosome 22. *c-abl* exons are designated (a) for *abl*.

nearly all CML patients and in 25% of adults and 5% of children with ALL[1]. A third breakpoint region, μ-*bcr*, has been identified and lies downstream of M-*bcr*. Utilization of this breakpoint results in a large bcr-abl hybrid gene encoding a mRNA with an e19a2 junction coding for a 230 kDa protein (Fig. 1). P230Bcr-abl leukemias are rare and resemble a chronic neutrophilic leukemia rather than CML[3].

The exact mechanism by which the *bcr-abl* translocation occurs is not well understood although fusion products coding for P185 and P210Bcr-abl were produced *in vitro* by subjecting myeloid cell lines to high doses of irradiation[4]. Bcr-abl translocation is of historic significance as it represents an early realization of the link between an acquired chromosomal abnormality and a specific human malignancy. This linkage permits the analysis of the effects of genomic changes on cancer initiation and progression[5].

Clinical manifestations in CML appear years after clonal initiation. Studies on japanese survivors of the atomic bombing of Hiroshima indicate that the appearance of the first Ph^- cell precedes the symptomatic and clinically diagnosed stage by six years [6]. This may suggest the requirement for additional mutations during disease progression. Utilizing age incidence data, it has been estimated that three somatic mutations are required to establish chronic phase CML [7].

1.1.1 Symptoms and Clinical Features of Chronic Phase CML

Typical presenting symptoms in chronic phase are fatigue, weight loss, bleeding, and discomfort in the left upper abdomen attributed to splenic enlargement. Weight loss and anorexia are common but not severe. In a study involving 430 patients with CML seen at a

referral center over a 16 year period, the frequency of weight loss, sweats, and bone pain did not differ between patients in the chronic phase and those in advanced stages[8] but treatment relieved symptoms only in chronic phase patients. Haemorrhagic symptoms such as purpura and retinal haemorrhages were also reported in some patients. Splenomegaly and purpura were the predominant abnormalities on physical examination.

Hematologic findings indicate leukocyte count is often greater than $100x10^9/L$. All stages of the neutrophilic series from immature myeloblasts to mature segmented neutrophils are usually present accompanied by distinctive peak frequencies in the segmented neutrophil and myelocyte categories[9]. The absolute number of basophils are increased early in the course of the disease. At the time of diagnosis most patients have basophilia, eosinophilia, and monocytosis. The proportion of lymphocytes in the blood with T and B cell characteristics is normal, but an abnormally high T cell to B cell ratio is found in the spleen[10]. In untreated patients, a progressive increase in leukocyte count is observed although in most patients no ill effects are directly attributed to the number of leukocytes present in the blood[11]. Most patients have some degree of anemia at the time of diagnosis, and the severity of this condition is directly related to the level of leukocytosis[12].

Marrow examination reveals a hypercellular marrow with diminished fat space and an increase in the estimated number of marrow neutrophils and neutrophil precursors, as well as, megakaryocytes [13]. Marrow analysis also reveals an increase in myeloid to erythroid ratios with the preservation of orderly neutrophil maturation in most patients. The proportion of immature to mature neutrophils is higher in patient's marrow compared to peripheral blood indicating the retention, at least in part, of the barrier to release immature cells into

circulation[14]. Morphological analysis using electron microscopy reveals abnormality in the neutrophilic lineage. This is thought to occur due to a faster maturation rate of the cytoplasm compared to the nucleus in a process now known as discordant maturation. This is translated morphologically in the appearance of a greater than normal number of granules in promyelocytes with immature nuclei[15]. Discordant maturation has been suggested as a possible mechanism underlying leukocytosis in chronic phase CML. Such an imbalanced cytoplasmic-nuclear maturation is thought to give rise to cells with a capacity for more cell divisions and a longer life span. Other functional defects in circulating neutrophils include a decrease in granule enzymes, low leukocyte alkaline phosphatase (LAP) activity, as well as defective hydrogen peroxide production in mature neutrophils[16]. Defects also include subnormal adhesiveness to glass, plastic, or adherent stromal surfaces[17], delayed emigration to extravascular sites[18], and reduced phagocytic and bacterial cytocidal activities[19]. Although CML cells contain normal isoforms of actin, the level of actin in CML granulocytes is low. This explains defective microfilament-dependent phenomena such as chemotaxis[20]. CML neutrophils capable of emigrating to extravascular sites show a higher phagocytic and LAP activity when compared to CML cells trapped in circulation[21]. Prolonged intravascular circulation of CML neutrophils seems to correlate with lack of active emigration to extravascular sites suggesting a link between selectin-induced stickiness and transendothelial passage induced by integrins and considered the first step in extravascular migration[21].

Erythrocyte development and megakaryocyte maturation are morphologically normal but occasionally morphological abnormalities such as megaloblastic erythroid maturation are observed in chronic phase CML. Extramedullary hematopoiesis involving erythroid and

megakaryocytic lineages as well as the different classes of myeloid leukocytes is detected in spleen and liver of CML patients during chronic phase CML [22].

1.1.2 Cell Culture Studies

All committed hematopoietic stem cell compartments in CML are enlarged at diagnosis. Blast forming unit-erythroid (BFU-E) and colony forming unit-granulocyte-monocyte (CFU-GM) progenitors in circulating blood may increase by 180 and 9,000 folds respectivelly[23]. Expansion of terminally differentiated compartments is usually restricted to the granulocyte-monocyte lineage. Compared to normal counterparts, colony forming cells in the chronic phase of CML are actively cycling. This abnormal rate of replication in CML is apparent in studies utilizing pluripotential mixed colony forming cells. Normally over 92% of such cells from normal marrow survive short term poisoning by (tritiated) ^{3}H-thymidine indicating that most cells are in the resting phase of the cell cycle. In CML marrow, however, only 50-70% of such mixed colony forming cells survive such treatment indicating that a large proportion of pluripotential stem cells in CML are in cell cycle[24].

Defects in hematopoiesis are observed in CML. Neutrophils normally secrete lactoferrin which in turn inhibits the production of colony stimulating factors by monocytes and macrophages[25]. In CML, neutrophils produce abnormally little colony inhibitory lactoferrin[24]. Also, monocytes and macrophages in CML do not exhibit feedback regulation by prostaglandin E[26]. This is thought to be responsible for myeloid hyperproliferation and the cyclic variations in granulocyte levels and granulocytopoiesis often observed in CML patients. CML clones also exhibit resistance to negative regulators of hematopoiesis such as

macrophage inflammatory protein 1α (MIP-1α)[27].

1.1.3 Changes Associated with Blast Transformation

Progression to blast crisis in CML can occurs at anytime after or concurrent with diagnosis at a rate of 10% per year. Progression towards this malignant stage is inevitable in CML regardless of previous responsiveness to chemotherapy during the chronic phase and is the cause of death in 90% of CML-related deaths[28]. Blast crisis is further complicated by the development of multi-drug resistance in some patients where many blast cells show high levels of the multidrug transporter P-glycoprotein and thus become refractory to chemotherapeutic intervention[29]. The majority of myeloid forms of blast crisis are refractory to conventional therapy leading to the death of patients.

The clinical, morphological, and cellular characteristics of the blast phase are very heterogeneous. In terms of cell type, myeloblasts constitute the metamorphosed blast-cell population in over half of the cases[30]. In few cases myeloid blast crisis result from blastic transformation of promyelocytes[31] or eosinophil progenitors[32]. In almost a third of the cases the blast crisis is lymphoid. In such cases blast cells are usually of B cell lineage and show lymphoid marker profile and IgG rearrangements typical of acute lymphoblastic leukemia[33]. Lymphoid blast crisis with T cell phenotypes can also occur but at a much lower rate[34].

The lineage of the blast cell population is sometimes complex as myeloid blasts can acquire lymphoid surface markers including TdT as well as lymphoblast morphology. Also, myeloid-lymphoid mixed lineage blasts or granulocytic blast mixtures may evolve. In addition, megakaryocytic and erythroblastic transformation can occur in 20% and 10% of

blast crises respectively. Such a spectrum of blast transformation is not surprising given that the blast crisis arises from a lesion established in a multipotential stem cell which can support the clonal expansion of several cell lineages. Treatment strategies are usually unchanged if blasts are derived from stem cells committed to generating myeloblasts, erythroblasts, megakaryoblasts, or mixtures of those lineages. However, if the stem cell lesion triggers a lymphoid blast crisis in CML the disease becomes more responsive to ALL type treatment[35].

The onset of the blast crisis may be accompanied by symptoms that resemble initial symptoms at chronic phase presentation but rapidly become more severe. These may include fever, drenching night sweats, pain from osteolytic bone lesions, malaise, anorexia, weight loss, and deep depression. Lymphoadenopathy, hemorrhages, and recurrent infections, uncommon in the chronic phase may also mark transformation to blast crisis[36]. Presently, however, patients do not report any physical ill-being as regular laboratory analysis indicate entry into the blast crisis before the onset of such symptoms.

Hematologically and during chronic phase CML, the rank order according to cellular maturity is nearly normal in the myeloid compartment. As blast crisis sets, a growing population of arrested blast cells form rapidly and displaces the orderly ranks of maturing cell. Blasts and promyelocytes outnumber the intermediate forms; myelocytes and metamyelocytes. If patients remain untreated, a complete replacement of blood and marrow cells by myeloblasts and promyelocytes can occur. As the blast crisis progresses in the marrow, the original leukemic population may be replaced by immature cells that lack any evidence of differentiation. Such leukemic blasts freely traverse the marrow-blood barrier

resulting in a similar morphological display when blood and marrow smears are compared[28].

1.2 Prognosis, Course, and Causes of Death

In "good risk" Ph-positive non-blastic patients, the clinical course of the disease and responsiveness to treatment are uniform. Spleen size and the percentage of circulating blasts are the best indicators of early transformation to the blast crisis. Other unfavorable predictive indicators are increased age and platelet level above 700,000/μl. Basophilia, eosinophilia, marrow blasts above 5%, and karyotypic anomalies in addition to the Ph chromosome are considered as unfavorable prognostic variables[37]. The death rate in CML patients in the first year after diagnosis is 5% and rises to 14% in the second year. From 2-8 years after diagnosis the rate of deaths averages at 25% a year reflecting the high rate of entry into the terminal blast crisis. Most CML patients (85-90%) die of complications encountered during the blast crisis. The most common causes of death are thrombocytopenic bleeding, infection involving septicemia and pneumonia, and marrow aplasia. At a lower rate, patients may die of therapy related aplasia, infection, myocardial arrest, pulmonary fibrosis, or a combination of such complications[38].

1.3 Detection

Many molecular methods for the detection of Bcr-abl are available. *bcr-abl* can be detected *in situ* on chromosomes by Fluorescent In Situ Hybridization (FISH) which does not require the arrest of cells during mitosis[39]. At a genomic DNA level and using Southern blot techniques, the translocation can be demonstrated by looking at rearrangements in the

bcr gene. Bcr-abl transcripts can be detected by Northern blots or using RT-PCR techniques which is a more sensitive assay that requires minimal amounts of RNA as a template[40].

Bcr-abl can also be detected by antibodies raised against the amino-terminus of Bcr or the the carboxy-terminus of Abl by immunoprecipitations[41] or Western blots[42].

Detection of the *bcr-abl* translocation is important diagnostically where qualitative RT-PCR is utilized to confirm the presence of the fusion transcript and the type of junctions utilized in its formation. Also following treatment, especially in the case of bone marrow transplantation, detection of the fusion product is necessary to provide an estimation of the number of bcr-abl transcripts present in order to evaluate the presence of minimal residual leukemia. For such an estimation quantitative RT-PCR is utilized where the patients cDNA is co-amplified with a series of known amounts of an artificial cDNA. The competition between the two cDNA templates for the primers and other PCR reagents is a measure of the relative proportion of each cDNA in the reaction. The reaction where equal amounts of PCR products are produced is termed the equivalence point and indicates the amount of bcr-abl transcripts in the patient's sample. In most cases CML cells carry the b2a2 or b3a2 (Fig. 1) junction although in few cases (5-10%) both b2a2 and b3a2 can be formed by alternative splicing[43].

Atypical bcr-abl transcripts are sometimes encountered in CML patients. Transcripts where *abl* exon a3, instead of a2, is fused to *bcr* exons b3 or b2 resulting in a smaller PCR product amplifying shorter transcripts[44]. Other atypical transcripts observed contain e1a2 junction and in rare cases an e6a2 junction [40;45]. The presence of atypical transcripts in CML

has important implications. First, as all the fusion transcripts are in frame, a functional fusion protein is expressed, which maintain an oncogenic characteristic as all those patients have CML. Second, the presence of atypical transcripts can result in false negative diagnosis if inappropriate primers are used. Atypical transcripts in CML can result in an altered disease profile as observed in rare cases of CML involving the minor breakpoint cluster region (m-*bcr*) of the *bcr* gene producing a P190Bcr-abl product. In such patients monocytosis is observed accompanied by a low neutrophil to monocyte ratio[45].

An important point in *bcr-abl* cytogenetics worth considering is the frequency by which this anomaly occurs. Experimental reconstruction of the fusion protein has been reported in non CML cell lines by high doses of X-irradiation[4]. Bcr-abl fusion proteins resulting from such experiments were not all functional as both in-frame and out of frame transcripts were produced. Using sensitive methods of detection, it has been reported that *bcr-abl* expression can be detected at a level as low as 1 to 10 transcripts per 10^8 cells in normal individuals[46]. This suggests that *bcr-abl* translocation and perhaps other translocations are continuously formed in the marrow and blood of normal individuals, only when the correct fusion protein is formed in the appropriate primitive hematopoietic progenitor cell that cell gains a proliferative advantage and may give rise to a malignant clone.

In addition to the Bcr-abl fusion protein, the t(9;22) translocation gives rise to a reciprocal *abl-bcr* hybrid gene that has been previously shown to be expressed in about two thirds of CML patients. Transcripts are the product of fusion between one of the first *abl* alternative exons usually exon 1b and *bcr* exons b3 or b4[47]. Although the junctions of the

abl-bcr transcripts are in frame allowing for the translation of a functional Abl-bcr protein, the presence of such a protein has been hard to demonstrate in cells from CML patients. An interesting feature of the Abl-bcr fusion protein is that it will contain the carboxy-terminal *bcr* sequences that code for a racGAP domain[48] (Fig. 2), fused to *abl* amino-terminal sequences which may contain a myristoylation sequences that can relocate the fusion protein to the cell membrane. Abl-bcr has the potential of affecting the regulation of the small GTP binding protein, Rac, which is involved cytoskeletal organization, cell adhesion[49], and the oxidative respiratory processes of neutrophils[50]. However, it is unlikely that the *abl-bcr* gene is involved in CML given that it is not expressed in 40% of patients.

1.4 Treatment

In the chronic phase of CML, therapy is directed towards reducing the proliferating myeloid mass and relieve complications created by hyperleukocytosis, thrombocytosis, and splenomegaly. Such strategy is successful in most patients who are at the chronic phase during presentation. Granulocyte counts can be reduced to normal levels utilizing cytotoxic drugs or biologic modifiers. However, none of those treatments eradicate the clonal founder cells. As CML is not chemo-curable, such treatments improve the patients' quality of life but do not represent a curative therapy[51].

1.4.1 Busulfan (1,4-dimethanesulfonyloxybutane)

Although rarely used now, for several decades this alkylating agent has been the treatment of choice for the chronic phase of CML. Busulfan can control CML-related symptoms for 3 months or more in most patients. Treatment results in a drop in the white

blood cell count accompanied by reduction in spleen size and a general feeling of well-being. Leukocyte doubling time is usually an indication of whether treatment should be discontinued[52]. Unfortunately, with each relapse of the disease the leukocyte doubling time becomes shorter and leukemic repopulation rates accelerates reaching a peak during blast crisis transformation where the leukemic clone may double in total mass every 2 days[53]. Busulfan has several drastic side effect which heralds careful monitoring during administration. Busulfan depresses platelet counts, can cause pulmonary fibrosis, and in patients receiving prolonged treatment can cause cellular atypia leading to the deterioration of the marrow micro-environment. Following blast crisis transformation busulfan treatment is discontinued for its ineffectiveness towards leukemic blasts but also its selective targeting of the remaining normal hematopoiesis[52].

1.4.2 Hydroxyurea [54]

The ribonucleotide reductase inhibitor is a cell cycle-specific antagonist of DNA synthesis during S phase. Hydroxyurea seems to be more effective in sustaining remission during the chronic phase when compared to Busulfan. Hydroxyurea is also superior to busulfan in that it can be used to treat the blast crisis, is more toxic to CML cells than normal cells, and suppresses early blast expansion. In chronic phase, hydroxyurea results in the reduction of leukemic cells as well as a drop in white blood cells. Hydroxyurea, however, is incapable of inducing cytogenetic remission and therefore similar to most chemotherapeutic agents it cannot prevent or control myeloid blast crisis in CML .

1.4.3 Interferon-α [55]

Recombinant interferon (IFN)-α can greatly modulate the course of the disease in some patients. IFNα possesses pleiotropic activities including anti-viral and anti-proliferative abilities. INF-α stimulates the expression of 2'-5'-oligoadenylate synthetase which in turn can activate Rnases which can specifically target and degrade mRNAs of growth promoting genes. IFN-α induces degradation of growth factor mRNAs and interferes with the proliferation of actively cycling stem cells and progenitors such as those encountered in patients with hematologic malignancies. The molecular basis of INF-α in treating CML, however, seems to be related to its effect on cell adhesion. Bcr-abl is suspected to alter the β-1 integrin function, rendering CML clones defective in adhesion to the stroma. INF-α has been shown to correct this defect *in vitro*[56]. In newly diagnosed chronic phase CML patients, IFN-α treatment results in complete or partial hematologic remission in 31% to 81% of cases, a major cytogenetic response (>65% Ph^-) in 10% to 38% of cases, and complete cytogenetic response (100% Ph^-) in 7% to 26% of cases. CML patients experiencing a major or complete remission have a longer survival term than patients with no cytogenetic remission. However, IFN-α therapy does not appear to be a cure for CML. Sensitive molecular genetical examination of the marrow of complete cytogenetic responders (100% Ph^-) reveals the presence of the *bcr-abl* translocation. INF-α treatment results in adverse side effects which requires the discontinuation of therapy in 15%-25% of patients and its reduction in 30%-50% of patients .

1.4.4 Allogenic Transplantation Therapy for CML[57]

Although few patients qualify, allogenic bone marrow transplantation remains the only known curative treatment modality for CML . Patients in the chronic phase of the disease who receive human leukocyte antigen (HLA)-matched sibling donor transplant have up to 45-70% probability of long-term disease-free survival. Patients transplanted in more advanced stages of the disease have a 15-40% probability of disease free survival. Transplants from matched unrelated donors have also been shown to be successful in some CML patients especially with the advent of methods aimed at improving donor-recipient major histocompatibility complex (MHC) matching. Also alternative sources of allogenic stem cells for transplantation such as peripheral blood progenitor cells and umbilical cord blood cells are being developed. Nevertheless, many CML patients are not eligible for allogenic transplantation as suitable donors are not available or because of the advanced age of the patients. Allogenic transplantations, especially from unrelated donors, may be sometimes accompanied by life-threatening conditions such as infections and acute and chronic graft-versus-host disease (GVHD). Clinical relapses, some occurring as late as 5-10 years after transplantation, may also occur.

1.4.5 Autologous Transplantation Therapy for CML [58]

As most CML patients are not suitable for allogenic transplantation and only few of those treated with IFN-α can expect a longer survival term, autologous transplantation is emerging as an alternative therapy for CML. The existence of benign hematopoietic progenitors, naturally or induced by treatment, alongside leukemic counterparts in the

marrow of CML patients formed the basis of autologous transplantation in CML. Such therapy is still largely experimental and involves the recovery of benign progenitors from marrow or peripheral blood of the patient which is then used to reconstitute hematopoiesis.

A study involving 21 chronic phase CML patients transplanted with autologous peripheral blood mononuclear cells demonstrated that the five years survival of autografted patients was significantly higher compared to 636 aged matched controls treated with conventional chemotherapy. Of the 21 patients, 9 exhibited some degree of Ph^- hematopoiesis including two patients who achieved complete Ph^- hematopoiesis late after transplantation. In another study from autografting in 49 CML patients in the chronic phase, engraftment was faster when peripheral blood mononuclear cells (PBMNC) were transplanted compared to marrow. Out of 34 evaluable patients, 15 had a major cytogenetic response (>65% Ph^-) and 10 had a complete cytogenetic response which lasted for 6 month to 3 years after transplantation. Numerous studies suggest that autologous transplantation is associated with an increase in survival when compared to conventional therapy especially in patients in the chronic phase of the disease. Data from a study following 200 autotransplanted CML patients at different stages of the disease and involving eight different transplant centers in Europe and North America was recently reported. The probability of survival at four years for 141 patients with chronic phase CML was 66%, suggesting that autotransplantation may result in an increase in survival when compared to conventional chemotherapy treatment. Also, patients in the chronic phase of the disease when receiving autotransplantation had an improved survival rate compared to patients in the accelerated phase or in the blast crisis. The same study also concluded that shorter time intervals from

diagnosis to transplantation and younger age of recipient are favorable variables for a successful autologous transplantation.

Most patients who successfully receive autologous transplantation therapy experience cytogenetic or hematologic relapse indicating that such a procedure is not curative and improved survival is probably due to reduction rather than elimination of the malignant progenitors after autografting. Such a scenario favors the reduction of the malignant clone from transplant material by *ex vivo* purging to increase the efficacy of the transplant. Many methods for *ex vivo* treatment of autologous transplant material have been developed. In one study, marrow from CML patients was treated with cyclophosphamide derivatives (4-hydroperoxycyclophosphamide) that selectively exert a killing effect on Ph^+ progenitors and increase Ph^- stroma-adherent progenitors. Successful engraftment of marrow treated *ex vivo* using this method was observed in 10 patients, 5 of which were in the chronic phase of the disease and the other five in the advanced stage. Marrow recovered after transplantation showed complete cytogenetic remission (100% Ph^-) in 6 out of 9 evaluable patients. However, cytogenetic remission was short lived and only one patient remained Ph^-.

Other groups have utilized chemotherapy to induce Ph^- hematopoiesis before collecting marrow or peripheral blood for autografting. In one study from the Swedish CML group, newly diagnosed CML patients were treated with IFN-α and Hydroxyurea for at least 6 month. Fifteen patients were autografted when in full cytogenetic remission while 3 were in partial remission. Nine out of 16 evaluable patients were Ph^- following transplantation suggesting that intensive chemotherapeutic treatment reduces the Ph^+ clone in CML and that autografting can result in Ph^- hematopoiesis.

Unfortunately, cytogenetic remission after autologous transplantation is not durable and Ph^- cells can be detected within the first year following transplantation. The relapse might be the caused by the persistence of leukemic cells in the autologous material used for transplantation, or the persistence of such cells in systemic tissue following intensive chemotherapy treatment in preparation for transplantation. More ambitious attempts are underway for graft purging including using antisense oligodeoxynucleotides directed at the breakpoint junction of bcr-abl mRNA, ribozymes that bind to and cleave sites on bcr-abl mRNA, and Bcr-abl specific tyrosine kinase inhibitors.

1.5 Cytokinetic Abnormalities

Electron microscopic studies have demonstrated evidence of asynchrony in maturation between the cytoplasm and the nucleus of CML cells whereby the cytoplasm mature more rapidly than the nucleus[59]. As a result of such a discordant development it seems that CML cells go through one or more divisions when passing through intermediate and later maturation compartments compared to normal progenitor cells. Also, maturing CML granulocytes seem to live longer than normal counterparts resulting in the expansion of the leukemic population mostly in the later stages of maturation[60]. This has detrimental effects on normal hematopoiesis in that once the normal cell density in the marrow is exceeded, normal stem cells are suppressed[60]. Numerous cytokinetic studies have shown that in the chronic phase of CML, the leukemic precursors have lower mitotic indices, a lower proportion of cells in the S phase of the cell cycle, longer generation times, slower emergence times of the maturing cells, and longer transit times in the different maturation

compartments compared to normal counterparts[61]. Even though there is an increase in the number of committed myeloid precursors or colony forming unit cells (CFUs) in CML patients compared to CFUc in normal individuals, the proportion of such cells in the S phase of the cell cycle is half in CML patients compared to normal individuals. The slower proliferative rate of CML intermediate level precursors (CFUc, blasts, and promyelocytes) is usually attributed to the high cell density of CML marrow which reaches three fold or greater than in normal individuals. Although CML cells always show a tendency towards progressive expansion, untreated patients exhibit stable white blood cell (WBC) counts as well as a normal spleen size for several month. This demonstrates that CML cells are still responsive to feed back mechanisms whilst at a higher cell level than in marrows of normal individuals [62].

Spontaneous and treatment-induced cyclic oscillations of blood granulocytes as well as longer duration of the cell cycle are observed in CML patients favoring the model by which granulocytic expansion in CML occurs by progenitor cells undergoing additional divisions while passing through later maturation compartments[63]. Mature granulocytes in chronic phase CML have been shown to have a slower rate of disappearance from the blood compared to normal mature granulocytes. This may be attributed to an intrinsic abnormal characteristic of CML cells, allowing them to have an extended life span as well as an increase in granulocyte production in CML[64]. Extramedullary hematopoiesis in the spleen, which normally does not occur after birth, is prominent in CML. This causes the spleen of CML patients to increase from a normal 150 g to 1-5 kg consisting largely of leukemic cells. In some patients with an extensive splenic involvement, the majority of immature circulating

granulocytes may originate from the spleen[65]. The long preclinical course of CML also supports a model by which leukemic cells appear to have only a relatively marginal proliferative advantage over normal cells. It has been calculated that only 41 to 42 doublings are required for the Ph^- clone to reach several trillions or more cells which are present when the disease is diagnosed[66]. At the time of diagnosis normal cells are still present in the marrow of CML patients but are dominated by the leukemic population from which the majority of dividing cells in the marrow is derived[67]. Following treatment, the normal stem cell population expands again and produces normal mature cells. However the leukemic population increases in size again and suppresses normal hematopoiesis[60].

A mathematical model comparing normal and leukemic granulocytopoiesis has been described[60; 68]. This model took advantage of short-term culturing of core bone marrow biopsies obtained from normal individuals and patients in the chronic phase of CML to compare the growth characteristics of normal and leukemic progenitors. These studies have concluded that in granulopoiesis a large proportion of CML progenitors show reduced proliferative potential. This is caused by the fact that fewer recycle as primitive progenitors compared to normal counterparts probably due to the premature cytoplasmic maturation they exhibit. On the other hand, slow nuclear maturation leads to the expansion of granulocytic population by enabling the Ph^- granulocyte progenitors to undergo a few additional divisions when passing through intermediate and later maturation compartments as well as perhaps by increasing the life span of the maturing Ph^- granulocytes. The proliferative rate of CML granulocyte progenitors is comparable or even slower than normal counterparts resulting in a slow expansion of the CML population over several years to reach a population that will

lead to symptomatic disease.

1.6 The Molecular Basis of Bcr-abl Oncogenicity

The Bcr-abl tyrosine kinase contains a number of functional domains which suggests its ability to affect different growth regulatory pathways in order to exert its oncogenic potential. The *in vivo* role of such domains and their contribution in transformation largely depend on the assay preformed. For example, Tyrosine 177 in the Bcr sequences of the fusion protein (Fig. 2) is indispensable for transformation of Rat-1/*myc* fibroblasts [69] but is not required for the transformation of bone marrow cells [70]. The Src-homology (SH) domain 2 of Abl in Bcr-abl is required for the transformation of both Rat-1/*myc* cells and bone marrow cells, but is not required for the abrogation of IL-3 dependence[71]. Both forms of Bcr-abl (P190 and P210) exhibit an increase in tyrosine kinase activity by the Abl moiety of the fusion protein. This tyrosine kinase activity is essential in all *in vitro* transformation assays such as morphological transformation of rodent fibroblasts [72], the abrogation of interleukin-3 dependence[73], and the induction of long-term survival of bone marrow cells in culture [74].

1.6.1 Normal and Oncogenic Characteristics of c-Abl

An important factor in Bcr-abl oncogenesis is the disruption of the normal function of c-Abl. The *c-abl* gene was originally defined as the cellular homolog of the *v-abl* oncogene residing in the transforming retrovirus the Abelson murine leukemia virus. The

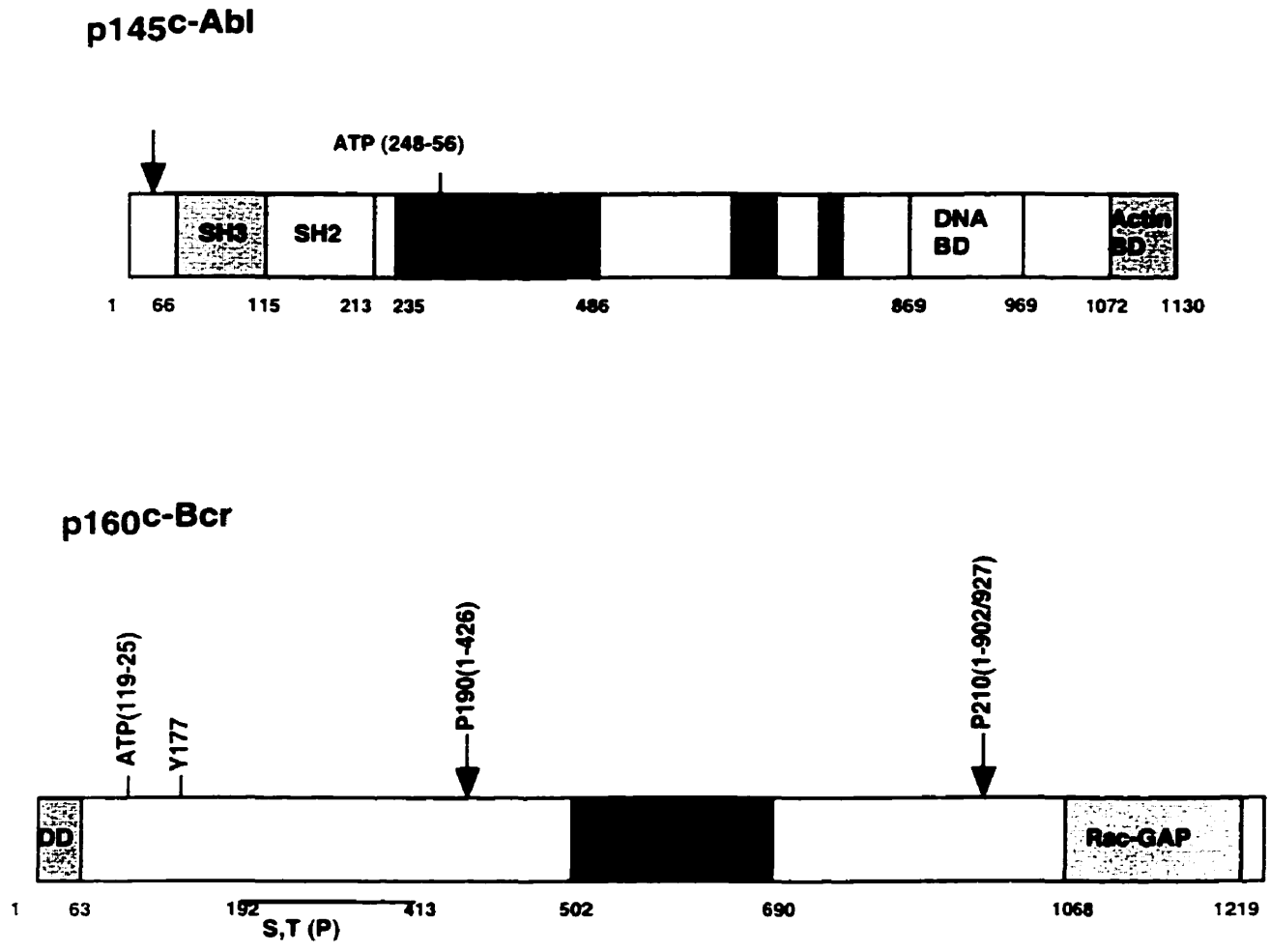


Figure 2 Structural characteristics of Abl and Bcr.

Critical structural features of Abl are shown and include the Src-homology domains SH1,2, and 3, a nuclear localization signal (NLS), a nuclear export signal (NES), a DNA binding domain (BD), and an actin binding domain. In Bcr the amino most terminal region functions as a dimerization domain (DD) followed by an ATP binding site, a critical tyrosine (Y) at amino acid 177 that binds SH2 domains when phosphorylated, a stretch of phospho serine and threonine residues that exhibit SH2 binding, a Rho guanine nucleotide exchange factor (Rho-GEF) homology domain, and a Rac-GTPase activating protein (Rac-GAP) domain. Numerical indications correspond to amino acid residues. The break points for the Bcr-abl translocation are indicated by vertical arrows.

cellular homologs of *v-abl* have been cloned in human, mouse, fruit fly, and nematode. The *abl* family of genes all encode intracellular tyrosine kinases and share several common features with the Src family members including amino terminus myristoylation site and the arrangement of the SH domains 3, 2, and 1 which codes for the tyrosine kinase catalytic function (Fig.2). The Abl family is distinctive, however, in that it has a large carboxy-terminal segment beyond the kinase domain that shows weak conservation between the different family members. This unique carboxyl- terminal domain encoded by a single exon contains several structural and functional sequences including a nuclear localization signal (NLS), proline-rich sequences that can bind SH-3 domain-containing proteins, a DNA binding domain, and an actin-binding domain (Fig. 2). Although c-Abl cannot select DNA sequences to which it binds, it can be recruited to specific protein-DNA complexes through protein-protein interaction. By direct interaction with RB, c-Abl is recruited to the transcription factor E2F-DNA complexes[75]. Recently, c-Abl was found to contain a functional nuclear export signal enabling it to shuttle between the cytoplasm and the nucleus[76]. This shuttling of c-Abl between the nucleus and the cytoplasm is an important regulatory property of the tyrosine kinase as will be discussed later. Serine and threonine sequences within the carboxyl-terminal domain are phosphorylated by cdc2 kinase as well as by protein kinase C[77].

Members of the Abl family are thought to be indispensable for the viability of post-embryonic organisms. *c-abl* knock-out mice have high frequency of neonatal lethality with the survivors showing defects in B and T cell development, developmental abnormalities in the spleen, cranium, and eye, and a high susceptibility to infections[78]. This suggests that *c-abl*

plays an important role in the development of lymphocytes. Another type of transgenic mice for *c-abl* has been reported. Those mice are homozygous for a truncation mutant which disrupts the 3' exon coding for the large carboxyl-terminal segment but retain the tyrosine kinase function and result in a similar phenotype as *c-abl* knock-out mice indicating the importance of the long carboxyl- terminus in the function of *c-abl*[79].

The tyrosine kinase activity of c-Abl is believed to be tightly regulated *in vivo*. Over-expression of c-Abl does not lead to transformation but rather to cell cycle arrest. However when altered forms of c-Abl are over-expressed cell transformation accompanied by elevated tyrosine kinase activity ensues. Oncogenic forms of *c-abl* exhibit a constitutively activated tyrosine kinase function and result from structural alterations in the amino or carboxyl-terminal sequences. Whereas normal c-Abl is found both in the nucleus and in the cytoplasm, where it is associated to the plasma membrane or bound to actin filaments, oncogenic forms of c-Abl are found exclusively in the cytoplasm[77]. The kinase activity of nuclear c-Abl is regulated during cell cycle progression[80] (Fig. 3). In quiescent and G_1 cells, nuclear c-Abl is kept in an inactive state by the tumor suppressor retinoblastoma (RB) protein that binds to the tyrosine kinase domain. The interaction between c-Abl and RB results in loss of c-Abl kinase activity[80]. Phosphorylation of RB at G_1/S by cyclin dependent kinases releases c-Abl from the complex leading to the activation of its kinase activity. The active nuclear c-Abl kinase can then modulate transcription by phosphorylating the carboxyl-terminal repeated domain (CTD) of RNA polymerase II[81].

The cytoplasmic pool of c-Abl is not regulated by cell-cycle progression. Cytoplasmic c-Abl is active both in G_0/G_1 and S phase cells and this activity seems to depend

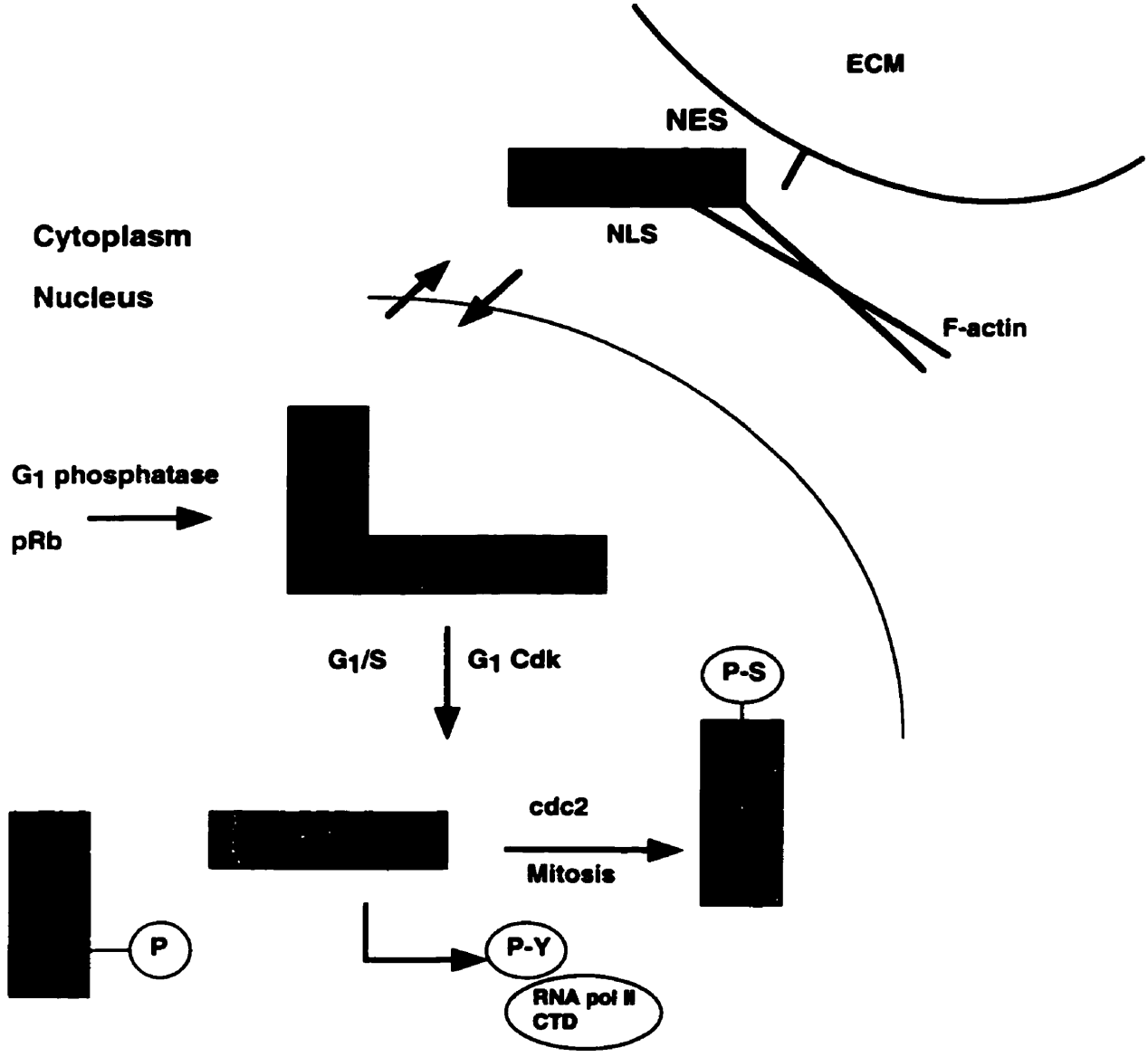


Figure 3 The regulation of c-Abl function.

The activity of c-Abl is regulated in a cell cycle dependent manner. Phosphorylation of pRb at G_1/S frees Abl which can phosphorylate substrates involved in gene expression. The phosphorylation of c-Abl by cdc2 at mitosis leads to its dissociation from DNA. An alternative mechanism of c-Abl regulation seems to involve cell adhesion. c-Abl may translocate from the nucleus following cell adhesion probably through the recently described nuclear export signal (NES) where an increase in its kinase activity is observed. The activated c-Abl can then relocalize to the nucleus through the nuclear localization signals (NLS) it posseses.

on cell adhesion to the extra cellular matrix (ECM)[82] (Fig. 3). When fibroblasts are detached from the ECM, loss of c-Abl tyrosine kinase activity is observed and is only recapitulated by adhesion to fibronectin matrix. In such detached cells, a 3-5 fold decrease in the kinase activity of cytoplasmic c-Abl is observed. Reactivation of the kinase activity of c-Abl as a result of plating on fibronectin (FN) suggests that cytoplasmic c-Abl is regulated by cell adhesion[82]. Nuclear c-Abl showed an increase in kinase activity only after a lag phase following attachement to FN. The recovery of nuclear c-Abl kinase activity coincided with the re-entry of c-Abl to the nucleus after a transient loss from the nucleus following cell adhesion suggesting that inactive nuclear c-Abl is transported to the cytoplasm where it is reactivated by integrin-dependent signals and returns to the nucleus[82]. Translocated nuclear c-Abl has been found to localize to focal adhesions raising the possibility of a role for c-Abl in the formation of focal contacts and/or the transduction of adhesion signals and integrating those signals with cell cycle regulation. Binding of c-Abl to actin is thought to be essential for such a role as c-Abl activity is inhibited in cells treated with cytochalasin which disrupts F-actin [76].

The mechanisms by which oncogenic transformation of *c-abl* occurs are different in mouse, cat, and human. In mice *v-abl* activation requires the deletion of the SH3 domain and the fusion of viral *gag* sequences at the amino terminus[83]. In feline, *v-abl* activation in Hardy-Zuckerman-2 Feline sarcoma virus (HZ2-FSV) requires in addition to the fusion of viral sequences, the deletion of the large carboxyl-terminal segment but the retention of the SH3 domain[84]. Activation of c-Abl in humans results from the addition of Bcr sequences leading to the formation of a Bcr-abl fusion proteins[85; 86]. In humans, c-Abl is also activated

by the fusion of part of Tel, a member of the *ets* family of transcription factors, upstream of c-Abl exon 2 forming a Tel-Abl fusion protein found in some patients with acute leukemia[87].

At least two possible mechanisms have been proposed to explain the tight regulation leading to the normally low level of c-Abl kinase expression. The c-Abl kinase domain is thought to be normally repressed by mechanisms that involve the SH3 domain as the deletion of such a domain leads to an increase in tyrosine kinase activity[88]. Also, the deletion of the entire carboxyl-terminal segment in HZ2-FSV v-Abl leads to the activation of the kinase[84]. Hence, it is proposed that a cis-inhibitory domain in the carboxyl-terminus segment may interact with SH3, locking c-Abl in an inactive conformation. In support of this model, new activating mutants in Abl randomly generated in a rodent fibroblast transformation assay, contained in frame deletions in proline-rich region located in the middle of the last exon of Abl which codes for the long carboxyl-terminal segment[89]. It is possible that this region can bind in cis to the SH3 domain locking Abl in a closed conformation which will prevent the kinase domain from interacting with cellular substrates[89]. However, direct binding between the Abl carboxyl-terminal and the Abl SH3 domain has not yet been clearly demonstrated.

A second trans- acting inhibitory model suggests that a trans inhibitor might interact with SH3 resulting in the blocking of the kinase activity[90]. c-Abl SH3 binding proteins have been described and include 3 (binding protein) BP-1 [91] and 3 BP-2[92]. Both proteins contain a proline rich binding motif which can bind to SH3 domains. 3BP-1 shares homology with a GTPase-activating protein (GAP) domain found in rho-GAP, Bcr, and n-chimerin. However, the binding of 3BP-1 has not been shown to inhibit the tyrosine kinase activity of c-Abl and neither 3BP-1 nor 3BP-2 have been shown to interact with full length c-Abl *in*

vivo. Two other proteins, mouse Abl-interactor (Abi) -1[93] and human Abi-2[94] were shown to interact with Abl carboxyl terminus of Abelson leukemia virus and human c-Abl respectively through their SH3 domains. Both are substrates for Abl tyrosine kinase domain and both can suppress the transforming activity of Abl. Abi-2 which was cloned from a human lymphocyte library can also interact with the SH3 domain of c-Abl via a proline rich region which may result in the locking of c-Abl in an inactive form. Abi-2 mutants that lack the SH-3 binding site activate the transforming activity of c-Abl in the absence of any structural alterations to c-Abl[94]. The suggested inhibitory role of Abi-2 on c-Abl kinase include stabilizing the inactive form of c-Abl or blocking the access of the Abl kinase to cellular substrates. The over-expression of truncated form of Abi-2 that does not bind SH3 of Abl might convert c-Abl into a transforming protein by displacing full length Abi-2 leading to an open c-Abl conformation with free access to constitutively phosphorylate cellular substrates[94].

The SH3 domain was also found to be necessary for the inhibition of the kinase activity of c-Abl by the human proliferation-associated gene (PAG) [95]. PAG is a member of a family of proteins with antioxidant activity implicated in the cellular response to oxidative stress and control of cell proliferation and differentiation[95]. Interestingly, PAG weakly inhibits the *in vitro* kinase activity of Bcr-abl which also contains the SH3 domain. The addition of Bcr sequences in the translocation may confer a conformation change rendering the SH3 domain inaccessible to PAG inhibitory effects[95].

The role of c-Abl as a negative regulator of growth [96]extends to the regulation of cell growth in response to DNA damage (Fig. 4). Wild type c-Abl, and a kinase defective c-Abl

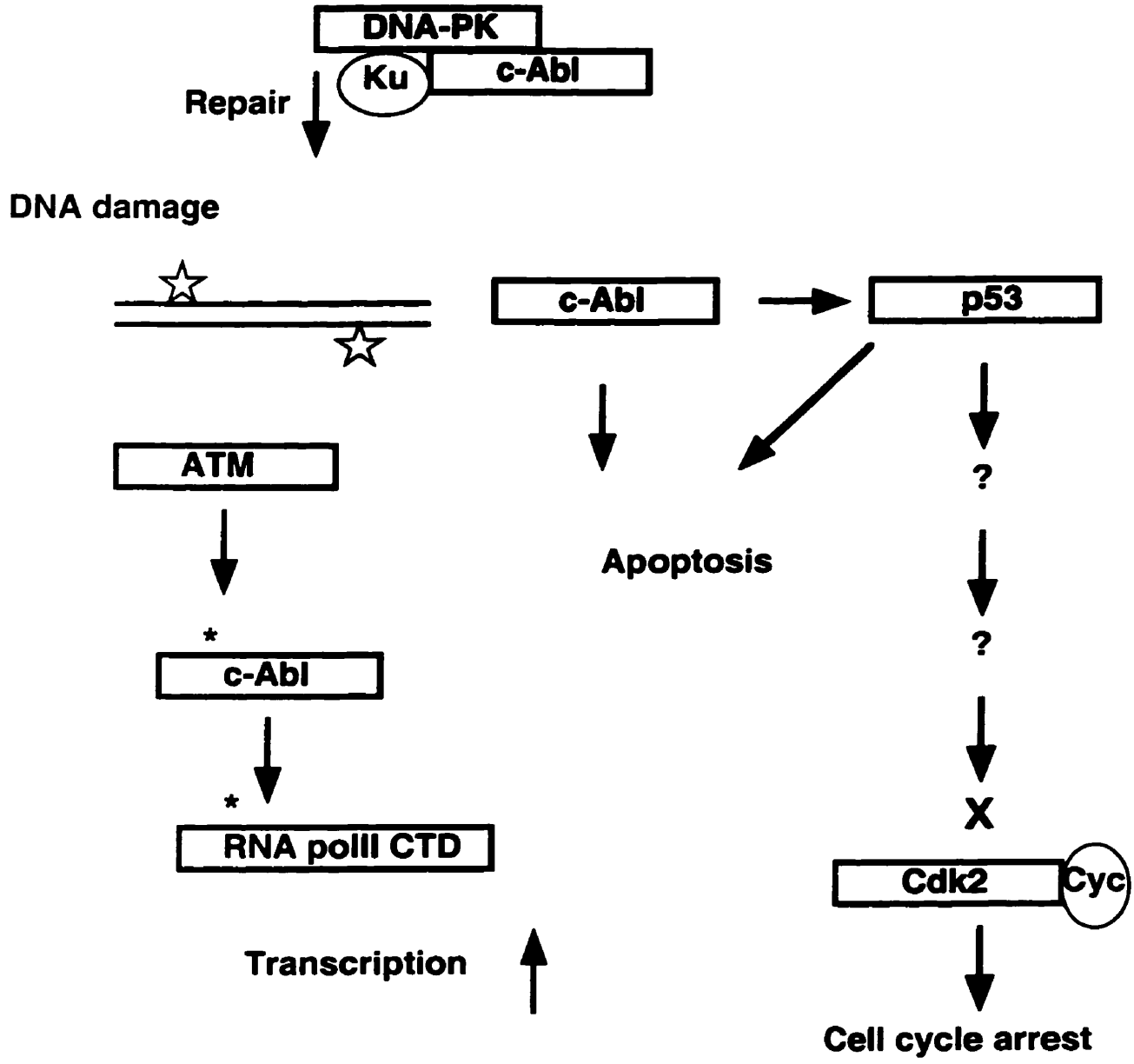


Figure 4. The role of c-Abl in preserving genomic integrity of cells.

c-Abl is thought to induce cell cycle arrest as a result of DNA damage. This process is p53 dependent but does not require the presence of $p21^{WAF-1/CIP1}$. c-Abl can also induce apoptosis partly by a p53 independent mechanism. c-Abl also interacts with DNA-dependent protein kinase (DNA-PK) which is implicated in DNA repair. Following the induction of DNA damage, c-Abl is phosphorylated and activated by ATM and in turn can down regulate transcription by phosphorylating the carboxyl terminal domain of RNA polII.

but not a c-Abl mutant defective in p53 binding was able to activate the expression of $p21^{WAF-1/CIP1}$ in promoter transplant experiments[97]. $p21^{WAF-1/CIP1}$ is an inhibitor of cyclin-dependent kinase 2 (Cdk2) and can arrest the cell cycle at G_1/S phase in a p53-dependent fashion following the induction of DNA damage by irradiation [98]. Cdk2 was down-regulated only in cells expressing wild type c-Abl but not with the other two mutants correlating with the ability of only wild type c-Abl to inhibit growth in fibroblast cells[97]. This suggests that the kinase activity of c-Abl and the p53 binding domain are both involved in growth arrest, and that such a phenotype is not exclusively $p21^{WAF-1/CIP1}$-dependent as the kinase inactive mutant of c-Abl was able to activate $p21^{WAF-1/CIP1}$ but did not arrest growth (Fig. 4).

Following the irradiation of fibroblast cells expressing different mutants of c-Abl, the kinase activity of c-Abl was not found to be required for transactivation of $p21^{WAF-1/CIP1}$ and GADD45 (growth arrest and DNA damage) by p53 but was required for Cdk2 down-regulation[97]. Also, wild-type c-Abl but not the kinase defective mutant was able to arrest cells at G_1 following irradiation[97]. Growth arrest and the inhibition of Cdk2 activity by c-Abl were p53 dependent as no change in Cdk2 levels were observed in $p53^{-/-}$ fibroblasts transfected with c-Abl[97]. c-Abl mediated inhibition of Cdk2 activity was confirmed not to be $p21^{WAF-1/CIP1}$ -dependent, as transfection of c-Abl into $p21^{-/-}$ cells inhibited Cdk2. In addition to cell cycle arrest, wild type c-Abl but not a kinase defective c-Abl mutant was also found to result in apoptosis when overexpressed in the breast adenocarcinoma cell line MCF-7 [99]. Cells expressing a dominant negative c-Abl demonstrated resistance to ionizing radiation-induced apoptosis compared to normal cells[99]. The link between the c-Abl kinase and apoptosis was confirmed in that $Abl^{-/-}$ cells were resistant to apoptosis following ionizing

radiation. However, p53 was still induced in those cells indicating that c-Abl is dispensable for the induction of p53 following the induction of DNA damage. Expression of the dominant negative mutant of c-Abl in $p53^{-/-}$ cells lead to resistance to apoptosis following irradiation suggesting that c-Abl regulated apoptosis might be, at least in part, p53-independent [99](Fig. 4).

A direct role for c-Abl in DNA repair was suggested after an interaction between c-Abl and DNA-dependent protein kinase (DNA-PK) was observed following the induction of DNA damage[100] (Fig. 4). DNA-PK is involved in double-strand break repair and was detected to bind c-Abl probably through the SH3 domain following induction of double strand breaks by ionizing radiation[100]. Ku antigen is a DNA end-binding protein and transcription factor which activates DNA-PK . There was no interaction observed between Ku and c-Abl in the unirradiated human histiocytic lymphoma cell line U-937. However, following irradiation complexes containing c-Abl and Ku were detected[100]. Induction of DNA damage by alkylating agents also resulted in promoting the interaction between DNA-PK and c-Abl. Incubation of purified heat activated c-Abl and purified DNA-PK and Ku in the presence of sonicated DNA, resulted in the phosphorylation of c-Abl on serine. The phosphorylation of c-Abl by DNA-PK lead to the increase of the kinase activity of c-Abl *in vitro*[100]. In the same study c-Abl was shown to tyrosine phosphorylate DNA-PK resulting in the inhibition of DNA-PK binding to DNA and hence its down regulation. This suggests a feed-back mechanism where the accumulation of c-Abl as a result of DNA damage leads to the phosphorylation of DNA-PK resulting in the blocking of its ability to bind DNA.

c-Abl was also shown to interact with ATM, the product of the gene mutated in the

autosomal recessive disorder *ataxia telangiestacia* (AT). AT is a disorder with pleiotropic phenotypes including neuronal degeneration, immune dysfunction, premature ageing and a higher risk for cancer development[101]. ATM is a member of phosphatidylinositol-3-kinase-like enzymes[102] that are involved in cell-cycle control[103], meiotic recombination, telomere length monitoring and DNA-damage response[104]. AT cells have been demonstrated to be hypersensitive to radiation and defective in the G_1/S checkpoint after radiation damage, a phenotype similar to cells lacking c-Abl[105]. ATM was shown to constitutively bind c-Abl in control cell lines but not in AT cell lines[15]. This interaction was confirmed to be direct by the yeast two-hybrid system and was found to occur through the SH3 region of c-Abl[15]. Following irradiation, an increase in c-Abl kinase activity was observed in control cells but not in AT cell lines suggesting that ATM might be involved in the activation of c-Abl by DNA damage and this interaction may in part mediate DNA damage induced G_1 arrest by c-Abl. In another study the tyrosine kinase activity of c-Abl was observed to increase when co-expressed with a functional ATM kinase domain[106]. The carboxyl-terminal kinase domain of ATM is homologous to the kinase domains of DNA-PK, phosphatidylinositol-3-OH kinase, and the yeast check-point regulators MEC1, TEL1, and Rad3[102; 107]. Most AT mutations affect the kinase domain, and the expression of a functional ATM kinase domain reverses the radiosensitivity of cells from AT patients[108]. ATM kinase domain was found to phosphorylate c-Abl at serine 465 similar to DNA-PK. As mentioned before, the CTD of RNA polymerase II is a known substrate of the c-Abl kinase. As a result of ionizing radiation, the tyrosine phosphorylation level of RNA polymerase II increases in normal lymphoblasts but not in AT lymphoblasts. This suggests that the ATM/c-Abl pathway might

be responsible of transducing signals to RNA polymerase II and thus regulating the transcription machinery following DNA damage[106] (Fig. 4).

1.6.2 Structure-Function Analysis of Bcr and Abl Motifs Involved in Transformation

The activation of the Abl kinase activity in Bcr-abl is largely due to the N-terminal coiled-coil domain of Bcr[86; 109](Fig. 2). These *bcr* sequences are common to the three alternative forms of Bcr-abl, P190 or 185 Bcr-abl, P210 Bcr-abl, and the rare P230Bcr-abl. The leucine zipper of the yeast regulatory protein GCN4 was recently shown to be able to replace the Bcr coiled-coil sequences in Bcr-abl transformation assays[110]. This suggests that activation of Abl tyrosine kinase in human leukemia is caused by the clustering of kinase domains in a way similar to the ligand-mediated activation of receptor tyrosine kinases[110]. Also, Tel sequences in Tel-Abl, found in some patients with acute leukemia, code for a putative helix-loop-helix that can potentially function in a similar manner to bcr in that it facilitates the oligomerization of the Abl protein[87]. When transfected into IL-3 dependent hematopoietic cell lines, Tel-abl resulted in the emergence of IL-3 independent clones[111]. Tel-abl expression in such cells also resulted in the phosphorylation of substrates as well as the activation of signal transduction pathways similar to that encountered after the expression of P190 and P210Bcr-abl[111]. Tel-abl, similar to Bcr-abl, is localized at the cytoskeleton in transformed cells [112]. This suggests that Bcr and Tel sequences contribute to transformation by primarily clustering the Abl kinase and ensuring its proper intracellular localization.

The smaller form of Bcr-abl associated with ALL contains an internal deletion of 501

Bcr-derived residues (Fig. 2) which seem to account for the difference between the oncogenic potential of both forms of Bcr-abl[88]. While both activated tyrosine kinases can transform primary bone marrow cells, P190Bcr-abl is more potent in oncogenic transformation of hematopoietic cells than P210Bcr-abl[113]. Using recombinant retroviruses to express the two forms of Bcr-abl in Rat-1 fibroblasts, P190Bcr-abl was found to be more effective in eliciting transformation in those cells than P210Bcr-abl [114]. The kinase activity of both forms of Bcr-abl were compared by measuring the autophosphorylation activity of equimolar amounts of both proteins prepared by *in vitro* translation in reticulocyte lysate. The amount of ^{32}P incorporated into P190Bcr-abl was 5-10 times more than that incorporated into P210Bcr-abl although tryptic phosphopeptide maps show fewer autophosphorylation sites in P190Bcr-abl compared to P210Bcr-abl. This suggests that ^{32}P incorporation into equimolar samples of both proteins correspond to the number of molecules undergoing autophosphorylation and hence is a measure of the kinase activity of the proteins. In the same study the specific activity of P190Bcr-abl towards an exogenous substrate was 5 times higher than that of P210Bcr-abl. These studies suggested that the loss of the 501 additional internal Bcr-derived residues in Bcr-abl lead to a more profound Abl tyrosine kinase activity in P190Bcr-abl [114].

Recently the specific role of those variable Bcr residues was directly addressed[110]. The *bcr* gene has in its center a Dbl-homology domain which encodes a guanine nucleotide exchange factor [115]. This Dbl domain can activate the small ras related GTP binding protein Rho [116]. The activated Rho in turn promote the formation of F-actin stress fibers [117]. This Dbl-homology domain is only present in the P210 form of the fusion protein but not in

P190Bcr-abl [118] (Fig. 2) and was found to contribute to the stabilization of actin stress fibers in transformed cells[110]. The C-terminal region of the *bcr* gene contains an GTPase-activating function for another small GTP-binding protein, Rac, involved in cytoskeletal architecture[119]. The activation of Rac has been shown to stimulate membrane ruffling [117], pinocytosis [117], and play a role in ligand-mediated activation of oxidative burst in neutrophils[120]. The Bcr Rac-GAP function, which is not present in either Bcr-abl fusion proteins, can therefore play a role in the down regulation of membrane ruffling and pinocytosis[110] . Expression of Bcr-abl lacking the Dbl sequences may be of clinical importance. In P210Bcr-abl-positive ALL , a disease more aggressive than CML, 100% of the P210Bcr-abl cells express P190Bcr-abl at a mean P190/P210 ratio of 2×10^{-3} compared to 2×10^{-4} in some chronic phase CML patients. The increase in P190Bcr-abl expression in P210Bcr-abl-positive ALL may explain the more aggressive form of the disease perhaps due to the lack of the Dbl homology domain leading to the disruption of the cytoskeleton[110]. These results should be viewed with caution, however, given the similar clinical history of $P190^-$ and $P210^-$ ALL.

The N-terminal 63 amino acids of Bcr which form the coiled-coil domain of Bcr-abl were also shown to be necessary for the binding of Bcr-abl to the actin cytoskeleton[86] (Fig. 5). The binding of Bcr-abl to the actin cytoskeleton is essential for transformation and is thought to bring Bcr-abl to the vicinity of important substrates required for transformation[71]. Mutations in the actin-binding domain encoded by the last 58 amino acids of Abl can also abolish transformation by Bcr-abl[71].

In addition to the Dbl-homology domain, Bcr sequences in Bcr-abl which are capable

of binding the SH2 domain of Abl are also important for transformation [85]. This region lies within the Bcr first exon and is common to both P210 and P190Bcr-abl (Fig. 2). Two separate domains in this region extending from amino acids 192-242 and 298-413 are capable of binding Abl SH2 domain[85]. Both of those domains are highly rich in serine/threonine residues. The binding of those two regions to Abl SH2 requires the presence of phosphoserine and phosphothreonine but not phosphotyrosine residues [85].

Bcr-abl is capable of forming hetero-oligomers with P160Bcr via the Bcr N-terminal coiled-coil sequence [121] (Fig. 5). A mutant Bcr-abl protein which is not capable of recruiting P160Bcr shows a reduced capability of transforming fibroblasts suggesting that the recruitment of Bcr through the coiled-coil domain may augment the transformation capability of Bcr-abl[110] .It is plausible that serine and threonine residues in the SH2 binding region of Bcr may be phosphorylated by P160Bcr binding to Bcr-abl through the coiled coil domain. The mechanism by which the binding of those two domains to the SH2 domain of Abl potentiate transformation is not well understood. The binding of the Bcr residues intermolecularly and/or intramolecularly may interfere with the binding of an inhibitor to Abl regulatory domains. Alternatively, the binding of Bcr residues may induce a conformational change in the Bcr-abl leading to the augmentation of its tyrosine kinase activity[109]. Another region in the Bcr sequences of Bcr-abl critical for transformation include a tyrosine at amino acid 177 (Fig. 2). When autophosphorylated, this tyrosine can bind the adaptor protein Grb-2 which is usually in a complex with the Ras activating guanine nucleotide exchange factor Son-of-sevenless (Sos)[69]. In fibroblast cells the binding of Bcr-abl to Grb-2 is necessary for transformation and is believed to activate the mitogenic Ras-Raf-MAPKinase pathway[69]

(Fig. 5).

SH1 and SH2 domains of Abl in the Bcr-abl fusion protein are essential for cell transformation and are required for substrate phosphorylation and binding to phosphotyrosine proteins respectively[122]. The SH3 domain of Abl in Bcr-abl was recently shown to affect cell-cell and cell-extracellular matrix interactions allowing for efficient lodging of leukemic cells in tissue. This correlated with a reduced ability of Bcr-abl mutants carrying a deletion in the SH3 domain to induce the expression of α2 integrin, suggesting that wild type Bcr-abl activates the expression of α2 integrin via proline rich proteins that normally interact with SH3 domains. In the same study the mouse myeloid cell line 32Dcl3, expressing Bcr-abl mutants lacking SH3, demonstrated a reduced ability to home into bone marrow and spleen when injected into mice. Mice injected with 32Dcl3 cells expressing the SH3 mutant Bcr-abl exhibited a marked survival prolongation compared to mice injected with the same cells expressing wild type Bcr-abl. In addition, the SH3 domain did not influence intracellular signaling or regulate the proliferation and the survival of Bcr-abl transfected cells but was necessary for full leukemic potential *in vivo*. It appears that the impaired leukemogenic potential of Bcr-abl SH3 deletion mutants are due to changes in the motility and adhesion of cells expressing such mutants brought about by the disruption of signals generated by SH3-dependent protein-protein interactions[123].

1.7 The Influence of Bcr-abl on Signal Transduction Pathways

The presence of multiple functional domains important for Bcr-abl transformation in addition to the large number of Bcr-abl substrates indicates the capacity of this activated

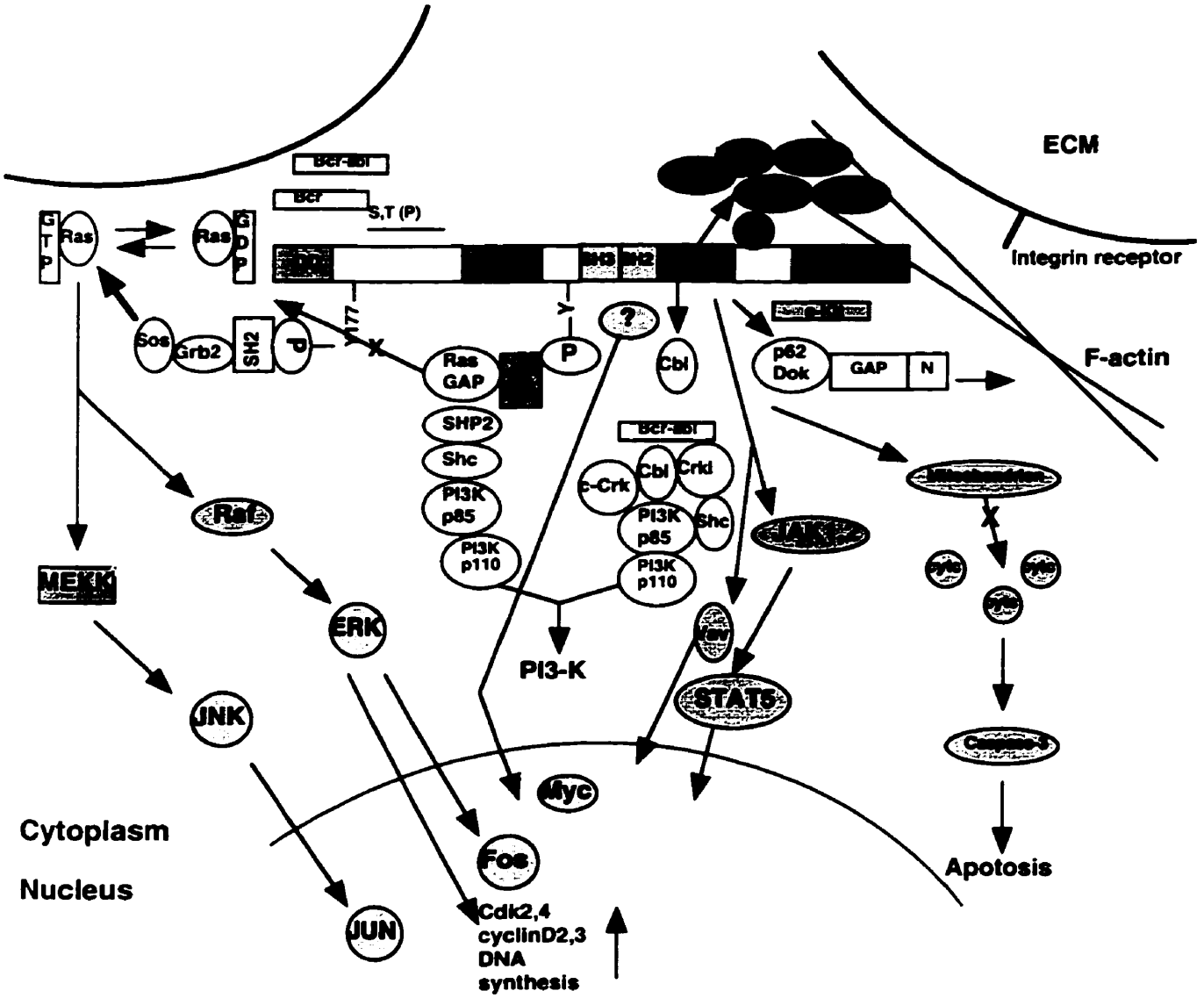


Figure 5 Bcr-abl exerts its oncogenic effect by influencing several biological processes.

Bcr-abl mediated oncogenicity is largely dependent on its ability to affect different signal transduction pathways involved in cell proliferation and growth, apoptosis, and cell adhesion.

tyrosine kinase to exert its influence on different signal transduction pathways (Fig. 5). Oncogenic forms of c-Abl such as v-Abl, P210Bcr-abl, and P190Bcr-abl cause malignant disease *in vivo*[77; 124; 125], transform cells *in vitro*[126], and convert growth dependent hematopoietic cells to growth independence[73; 127]. These diverse effect of Bcr-abl are probably due to its interaction with different cellular components involved in signaling pathways controlling growth, apoptosis, and cell adhesion.

1.7.1 Bcr-abl and the Ras Pathway

Bcr-abl expression in murine growth factor-dependent cells results in an increase in GTP-bound Ras and its activation to a high level[128]. The role of Ras in Bcr-abl mediated transformation was clearly demonstrated in experiments where simultaneous retroviral expression of Bcr-abl and either the catalytic domain of RasGAP, which converts active GTP-bound Ras to inactive GDP-bound Ras, or a dominant negative Ras block transformation by Bcr-abl measured by soft agar colony formation assays [128]. The mechanisms by which Bcr-abl activates the Ras pathway are believed to be mainly dependent on SH-2 -containing adaptor proteins such as Grb2 and Shc, and the tyrosine phosphatase Shp-2 (also known as Syp, and SHPTP-2 or 3). Bcr-abl can tyrosine phosphorylate Shc and Shp2 which in turn recruit the adaptor protein Grb2[126; 129] . The Grb2 adaptor protein is associated with Sos, a Ras guanine nucleotide exchange factor (GEF), which converts Ras from the inactive GDP- bound state to active GTP-bound state[129]. Tyrosine 177 within the Bcr residues of the Bcr-abl fusion proteins was shown to bind SH2 domain containing proteins such as Grb2 when phosphorylated[69]. Bcr-abl mutants in which tyrosine 177 is

mutated to phenyl alanine lose the ability to transform fibroblasts but can still transform primary bone marrow cells[70]. In the same study, the over-expression of Shc was able to complement transformation by Grb2 binding site-deleted P190Bcr-abl [70]. P210 Bcr-abl mutants lacking the Grb2 binding site can, therefore, still associate with Grb2 through interacting with Shc which in turn can bind Grb2[130]. Bcr-abl can also activate the Ras pathway by interacting and recruiting Shp-2 [122]. Shp-2 has two adjacent SH2 domains which are important in linking the phosphatase with activated receptors such as the platelet-derived growth factor (PDGF) receptor and the epidermal growth factor (EGF) receptor.

P160 Bcr can be found in complex with Bcr-abl probably through the Bcr-encoded amino terminal coiled-coil region [131]. P160Bcr can be transphosphorylated by Bcr-abl on tyrosines predominantly in the first exon of Bcr corresponding to the same residues that are autophosphorylated in Bcr-abl [131]. Also, P160Bcr tyrosine phosphorylated by c-Abl[132] or the Fps/Fes protein tyrosine kinase [133]can bind Grb2-Sos complex. This presents an additional mechanism by which Bcr-abl can activate the Ras pathway by forming a complex with P160Bcr, which when tyrosine phosphorylated, binds the Grb2-Sos complex.

It is therefore clear that Bcr-abl can activate the Ras pathway through employing different strategies that will ultimately lead to the deregulation of normal growth pathways.

The downstream consequences of Ras activation in Bcr-abl transformed cells are not well understood. Constitutive activation of the immediate early genes c-*jun*, c-*fos*[141] and c-*myc*[142] was observed after the introduction of Bcr-abl into murine cell lines and correlated with transformation or growth factor independence. Bcr-abl activates the JNK [Jun N(amino)- terminal kinase] pathway resulting in the activation of Jun[143]. The activation of

Jun by Bcr-abl requires Ras, MEK (MAP or extracellular-regulated kinase) kinase, and JNK. Bcr-abl transformation has been shown to be impaired by the expression of a dominant negative c-Jun. c-*myc* has also been shown to be involved in the transformation potential of Bcr-abl[142]. Whereas c-Abl is involved in the normal transcriptional regulation of c-*myc*, c-Myc over-expression can rescue the transformation capability of a Bcr-abl SH2 mutant rendered non-transforming[142; 144]. Also, a dominant negative Myc mutant can block transformation by activated Abl suggesting that c-Myc is an important downstream target of Bcr-abl induced transformation[142]. Transcriptional activation assays have revealed that a kinase active wild type P210Bcr-abl can stimulate NF-κB (nuclear factor activates κ immunoglobulin genes in B lymphocytes) activity in an IL-3-dependent murine myeloid progenitor (DA1) cell line[145]. Electrophoretic mobility shift experiments revealed the presence of p65 protein (RelA) DNA binding activity in P210Bcr-abl transformed DA1 cells but not in parental DA1 cells. After the inhibition of RelA by antisense oligonucleotides, P210Bcr-abl transfected cells did not survive following the removal of IL-3 compared to cells only transfected with P210Bcr-abl. In addition, inhibition of cellular growth was observed following the treatment of P210Bcr-abl transformed DA1 cells with p65 antisense oligonucleotides. These observations suggest that NF-κB may be a downstream effector for P210Bcr-abl induced transformation[145].

1.7.2 Bcr-abl and the Phophatidylinositol 3'-kinase Pathway

Both P190Bcr-abl and P210Bcr-abl can activate the phosphatidylinositol 3'-kinase (PI3K) pathway through several alternative mechanisms, similar to Ras activation[146; 146].

Direct evidence of the importance of this pathway in Bcr-abl transformation came from experiments where the expression of p85-regulating subunit of PI3K or the p110 catalytic subunit were inhibited by antisense oligodeoxynucleotides and wortmanin respectively[147]. The loss of either subunits resulted in the inhibition of the proliferation of Bcr-abl transformed hematopoietic cell lines and colony formation by primary CML cells. Although the activation of PI3K in a hematopoietic cell line expressing a temperature sensitive Bcr-abl requires the tyrosine kinase activity of Bcr-abl, no direct interaction between the p85 SH2 domain and its potential target binding motif (YXXM) on Bcr-abl was detected or required for activation[148]. Bcr-abl can activate the PI3K pathway probably through intermediary proteins such as Grb2[149], Shc[150], and SHP-2[151]. Tyrosine phosphorylated Shc can bind to the SH2 domain of the p85 subunit and the amino-terminal proline rich region of Shc can also interact with the SH3 domain of p85[150]. Also, SHP-2 can form a complex with p85, probably through the interaction with Grb2, in Bcr-abl transformed hematopoietic cells.

c-Cbl is a 120 kDa proto-oncogene which is normally cytoplasmic and partially associated with the cytoskeleton[152]. A carboxyl-terminal truncated form of c-Cbl is the oncogene in the CAS NS-1 retrovirus which induces pre-B cell lymphomas and some myelogenous leukemias in mice[153]. c-Cbl is tyrosine phosphorylated in v-Abl and Bcr-abl transformed cells[154]. Recently c-Cbl was found to form a signaling cluster involving Bcr-abl and leading to the activation of PI-3K[155]. In Bcr-abl transfected cells, c-Cbl was found in a complex with Bcr-abl and adaptor proteins including c-Crk, and CrkL[155]. CrkL and c-Crk have similar structures including a SH2 domain and two SH3 domains[156]. The tyrosine phosphorylated c-Cbl can bind to the SH2 domain of the p85 subunit of PI-3K leading to the

increase of PI-3K lipid kinase activity[155]. The structural domains involved in those interactions have been partially defined[155]. The SH2 domains of CrkL, c-Crk, $p85^{PI-3K}$ and Abl, either in v-Abl or Bcr-abl, can directly bind to phosphotyrosine residues on c-Cbl. Also, the SH3 domain of $p85^{PI-3K}$ binds to a proline rich region on c-Cbl. These experiments suggest that a primary role of c-Cbl in cells transformed by Bcr-abl is to link Bcr-abl to the PI-3K pathway[155]. This interaction seems to require auxiliary factors such as c-CRK and CrkL. CrkL is a major substrate for Bcr-abl in primary CML cells and also co-precipitates with Bcr-abl[157]. On the other hand, c-Crk is minimally phosphorylated on tyrosine in CML cells. However, c-Crk has been shown to bind c-Abl through its SH3 domain, and also c-Abl has been suggested to be the cellular tyrosine kinase which is activated in response to v-Crk[158]. In CML transformed cells, CrkL and c-Crk may function to link Bcr-abl to c-Cbl which is in complex with PI-3K. The consequence of such an interaction ultimately results in an altered regulation of PI-3K. This can be of important consequences given that the PI-3K pathway regulates biological functions such as apoptosis[159].

1.7.3 Bcr-abl and Cytokine Signaling Pathways

Cytokines regulate the growth of hematopoietic cells by binding to their respective receptors and induce intracellular signal transduction pathways. Leukemia cells can abrogate this growth requirement for cytokine by constitutively activating signaling pathways controlled by cytokine receptors[160]. Cytokine receptors are generally divided into two categories. Those that contain a tyrosine kinase domain (receptor tyrosine kinase or RTK) and those which do not[161]. Cytokines such as colony stimulating factor (CSF-1) and Kit

ligand, that bind to RTK, usually induce the dimerization of their respective receptors leading to the activation of the tyrosine kinase activity of the receptor and the propagation of mitogenic signals. This results in the activation of Ras, mitogen activated protein kinase (MAPK), and nuclear transcription factors[161]. Cytokines such as the granulocyte/monocyte colony stimulating factor (GM-CSF) and erythropoietin (EPO) that do not bind to RTKs stimulate growth by Janus family of cytoplasmic tyrosine kinases (JAK) [162]. Following ligand stimulation of non-RTKs, JAK kinases bind to the receptor and are rapidly activated. JAK kinases can phosphorylate and activate STAT proteins, a family of transcription factors with signaling properties, which in turn translocate to the nucleus and activate the transcription of target genes. The level of specificity is thought to occur in the activation of different STATs as a result of stimulation with different cytokines[162]. STATs are also activated in cells expressing activated tyrosine kinases such as v-Src[163] and v-Abl[164]. A major characteristic of CML cells expressing Bcr-abl is the expansion of myeloid precursor cells. Cytokines such as GM-CSF, IL-3, and granulocyte colony stimulating factor (G-CSF) involved in the regulation of myeloid cells activate JAK/STAT pathway[165]. In cell lines derived from CML patients and in hematopoietic cells acutely transformed by Bcr-abl, STAT5 was found to be constitutively activated[160]. The activation of STAT5 correlated with the ability of Bcr-abl to confer cytokine independent growth. In K562 cells, an erythromyeloid cell line derived from a CML patient that expresses Bcr-abl, both JAK1 and 2 were found to be constitutively activated on tyrosine [160]. Transient transfection assays of Bcr-abl in non-hematopoietic cells directly demonstrated the activation of at least JAK-1[160]. The Grb-2 binding site, the SH2 domain, and the major tyrosine autophosphorylation site on

Bcr-abl were found not to be important for STAT activation. Those same sites were also found not to be required for growth of Bcr-abl expressing cells in the absence of cytokines.

Along the same lines, Bcr-abl introduction into the factor-dependent human myeloid cell line MO7e resulted in factor independent proliferation and constitutive tyrosine phosphorylation of $p95^{Vav}$ which correlated directly with the level of Bcr-abl tyrosine kinase activity[166]. $P95^{Vav}$ is selectively expressed in hematopoietic cells[167] and has structures similar to transcription factors such as helix-loop-helix (HLH) domain and a leucine-zipper (LZ)-like domain and other structures similar to those in signaling molecules such as two SH3 domains, one SH2 domain, and an internal Dbl-homologous domain[168]. When introduced into fibroblasts, $P95^{Vav}$ is phosphorylated in response to epidermal growth factor and platelet derived growth factor. In MO7e cells, Vav was also tyrosine phosphorylated in response to GM-CSF, IL-3, and Steel factor stimulation and was found associated with JAK2 in the same cells after treatment with GM-CSF[166]. These results suggest that Vav is phosphorylated in response to hematopoietic growth factors as well as after the expression of Bcr-abl. An important biologic effect of Bcr-abl expression is the reduction or elimination of IL-3 requirement for the proliferation or viability of hematopoietic cells [73]. Whereas Vav can be involved in normal signaling by growth factors, its constitutive phosphorylation in myeloid cells expressing Bcr-abl suggest that its activation may be required for abrogation of growth-factor requirement in CML cells.

Also, Bcr-abl has been shown to co-immunoprecipitate with and constitutively phosphorylates the common β_c subunit of the IL-3 and GM-CSF receptors resulting in the constitutive phosphorylation of JAK2 [169]. IL-3 binding to its receptor can activate two

distinct signaling pathways. The first is initiated by the tyrosine phosphorylation of Shc following IL-3 binding to its receptor. Shc can then form a complex with β_c subunit of the receptor and recruit Grb2-Sos complex resulting in the activation of the Ras pathway[170]. Another potential signal transduction pathway involves JAK2 which is constitutively associated with the β_c subunit of IL-3[171]. In response to IL-3 binding, JAK2 is activated and signals to the nucleus through the activation of STATs[170]. Bcr-abl can, therefore, activate both pathways independent of the presence of growth factors[169].

IL-3 and GM-CSF in normal hematopoiesis induce the proliferation of early myeloid progenitors and also stimulate the differentiation of later committed myeloid lineage cells. The ability of Bcr-abl to activate signal transduction pathways from the receptors of such growth factors independent of ligand binding suggests that Bcr-abl expression can mimic the mitogenic effect of such factors. Early progenitor cells expressing Bcr-abl can therefore divide many more times than normal precursors in the bone marrow, or during differentiation Bcr-abl cells may divide at later stages of maturation than normal cells. In both cases, Bcr-abl expression will lead to the increase in the number of myeloid cells consistent with the clinical presentation of CML patients[169; 170].

1.8 Inhibition of Apoptosis by Bcr-abl

Apoptosis plays a key role in the regulation and maintenance of homeostasis in multicellular organisms. The large increase in mature granulocytes in the chronic phase of CML unaccompanied by an increase in the mitotic rate suggests that Bcr-abl might inhibit apoptosis[172]. The Bcr-abl expressing cell line K562, derived from a CML patient in the blast

crisis, shows resistence to apoptosis induced by Fas activation[173]. Those cells were also shown to express high levels of the antiapoptotic Bcl-x_L but not Bcl-2[174]. In the pre-B BaF3 cell line, introduction of P190 and P210Bcr-abl resulted in the an increase in Bcl-2 and inhibition of apoptosis induced by growth factor withdrawal[175]. Inhibition of Bcl-2 expression by a vector expressing anti-sense Bcl-2 results in the restoration of apoptosis and the inhibition of tumorigenic potential of those cells in nude mice[175]. Also, BaF3 cells expressing P210Bcr-abl were shown to be resistant to apoptotic induced by irradiation. This phenotype was reversed by anti-sense oligodeoxynucleotides (ODNS) targeted against Bcr-abl junction-specific sequences. In such cells, typical apoptotic characteristics such as DNA fragmentation following irradiation was observed [176]. In contrast Bcr-abl expression does not seem to protect against apoptosis induced by cytotoxic T cells, natural killer cells, and lymphokine-activated killer cells[177].

Cellular response to DNA damage usually entails the induction of cell cycle arrest at G_1/S and G_2/M transitions. The G_1 arrest in response to irradiation is dependent on the p53-mediated induction of the $p21^{WAF1/CIP1}$ inhibitor of cyclin-Cdk complexes[178]. In the condition of continued replication in the presence of damaged DNA p53 can induce apoptosis [179]. BaF3 cells expressing P210Bcr-abl exhibited a normal G_1/S cell cycle response and induction of $p21^{WAF1/CIP1}$ induction following irradiation[176]. However, those cells accumulated at the G_2/M boundary with no sign of apoptosis following irradiation. Similarly, IL-3 dependent myeloblastic 32D Cl3(G) cell line transformed to IL-3 independence by retroviral gene transfer and expression of P210Bcr-abl resulted in increased fraction of cells in the G_2/M phase of the cell cycle[73]. Bcr-abl mediated inhibition of apoptosis can allow the

inappropriate retention of cells with damaged DNA leading to the propagation of unrepaired cells which can result in the progressive accumulation of additional secondary genotypic alterations. CML cells have a predisposition to secondary genetic changes that lead to the clonal evolution of the blast crisis. Bcr-abl-induced inhibition of apoptosis may define an important mechanism leading to such a phenotype[176].

Structural dissection of Bcr-abl has revealed that the tyrosine kinase activity of Bcr-abl is required for its antiapoptotis effect[180]. Three different Bcr-abl mutants carrying either a single amino acid substitution from tyrosine to phenylalanine in the Grb2 binding site (Y177F), or a mutation in Src homology 2 domain (SH2) (R552L) which abolishes binding to phosphotyrosine containing sequences, or a mutation in the autophosphorylation site in the tyrosine kinase domain (Y793F), do not show a diminished antiapoptotic capability[180]. Also, the transforming capability of those Bcr-abl mutants was unaffected in hematopoietic cells but resulted in a drastic reduction of fibroblast transformation[179] .The expression of those mutants in 32D myeloid cells resulted in inhibition of apoptosis and growth factor-independent proliferation following IL-3 withdrawal. In contrast cells expressing the kinase defective (K671R) and the Y177F/R552L/Y793F triple mutant underwent apoptosis. 32D cells expressing a deletion mutant (Δ176-427) in the Bcr region of Bcr-abl that lacks the Grb2 binding site, Bcr serine kinase activity, and a serine/threonine rich region, resulted in partial inhibition of apoptosis, but did not proliferate following IL-3 withdrawal.

Interestingly, the expression of the triple Bcr-abl mutant and the (Δ 176-426) mutant had a different effect in BaF3 (lymphoid) cells. BaF3 cells expressing these mutants were resistant to apoptosis following IL-3 withdrawal. These results indicate that the Bcr-abl

structural requirements for the inhibition of apoptosis following IL-3 withdrawal are different in different cellular contexts[180]. In the absence of IL-3, gamma irradiation induced apoptosis was inhibited in BaF3 cells expressing all the Bcr-abl mutants except the tyrosine kinase defective mutant (K671R). In contrast, 32D cells expressing the triple mutant were not resistant to gamma-irradiation induced apoptosis while the Δ176-427 deletion mutant conferred resistance but not as effective as the wild type Bcr-abl consistent with results obtained following growth factor withdrawal. BaF3 cells expressing all the Bcr-abl mutants except the kinase defective mutant were able to form tumors when injected into nude mice. 32D cells expressing the Δ176-427 mutant did not cause tumors in half of the injected mice while cells expressing the triple mutant showed a severe reduction in tumorigenic potential. These experiments demonstrated that viability and transformation signals elicited by Bcr-abl are different in that the Δ176-427 mutant expressed in 32D cells inhibit apoptosis but does not cause tumorigenic growth in some injected mice.

In the same study Ras activation correlated with the antiapoptotic activity of the different Bcr-abl mutants. An Increase in Ras-GTP was detected in all Bcr-abl mutants that demonstrated an antiapoptotic effect including Y177F and Δ176-427 mutants that loss the ability to bind Grb-2. The mutants which have lost the Grb-2 binding site can associate with Grb2 through Shc which was found to be tyrosine phosphorylated and associated to Grb2 in all mutants except the kinase-defective mutant. The triple mutant caused the formation of the Shc-Grb2 complex only in BaF3 cells but not in 32D cells correlating with the ability of this mutant to activate Ras and inhibit apoptosis in BaF3 cells only. The Δ176-427 Bcr-abl mutant was able to activate Ras and inhibit apoptosis but has severely impaired

transformation ability suggesting that apoptosis is necessary but not sufficient for transformation that may require additional signaling components[180].

The relevance of the biological properties of Bcr-abl in tissue culture system is hard to evaluate. Although CML progenitor cells are actively cycling probably at a rate higher than normal progenitor cells, they are entirely growth factor-dependent for proliferation *in vitro*. Introduction of Bcr-abl into growth factor dependent cell lines in tissue culture systems results in the production of cell lines which are growth factor independent[127]. Nevertheless, and as discussed later, resistence to apoptosis is not detected in Bcr-abl$^-$ CML progenitor cells[224].

The mechanism by which Bcr-abl expression inhibits apoptosis in cell lines was recently investigated [181]. A variety of apoptotic stimuli cause the pre-apoptotic mitochondrial release of cytochrome c (cyt c) into the cytosol. This mediates the cleavage and activation of caspase-3 which is associated with mitochondrial permeability transition resulting in the loss of membrane potential and increase in reactive oxygen species (ROS)[182]. Activation of caspase-3 results in the degradation of a number of substrates including poly (adenine diphosphate[ADP]-ribose) polymerase (PARP) and lamins, producing the morphologic features of apoptosis[183]. Caspase-3 activity can also cleave and activate a DNA fragmentation factor (DFF) which results in the DNA fragmentation typical of apoptosis[184]. In the human acute myelogenous leukemia HL-60 cells stably transfected with P190Bcr-abl and the chronic myelogenous leukemia blast crisis K562 cells which express P210Bcr-abl, induction of apoptosis by treatment with anti-leukemic agents such as Ara-C, etoposide, and sphingoid bases did not result in the cytosolic accumulation of cyt c, activation of caspase-3, or the

other mitochondrial perturbations associated with apoptosis [181]. An induction of Bcl-x_L but a reduction in the level of Bcl-2 accompanied the expression of Bcr-abl in HL60 cells and reflected the normal profile found in K562 cells[181]. Both Bcl-2 and Bcl-x_L are structurally similar to pore-forming domain of the bacterial toxins and might be channel proteins that regulate the transport of ions and small proteins such as cyt c across the outer mitochondrial membrane[185]. The over-expression of both Bcl-2 and Bcl-x_L blocks the mitochondrial release of cyt c preventing by that the activation of caspase-3 and apoptosis[182]. Although the exact mechanism is still ambiguous, Bcr-abl expression seems to block apoptosis due to diverse apoptotic stimuli by preventing the cytosolic accumulation of cyt c and other pre-apoptotic mitochondrial perturbations and thereby inhibit the activation of caspase-3 and apoptosis [181].

1.9 Bcr-abl - Induced Cell Adhesion Defects

In normal hematopoiesis, direct adhesion of progenitor cells to bone marrow stroma through fibronectin receptors (α4, α5, and β1 integrins), appears to confer negative regulatory control on proliferation[186]. Hematopoietic progenitor cells in patients with CML have been shown to have impaired adherence to bone marrow stromal cells allowing unregulated proliferation of primitive CML progenitor cells even when the stromal marrow cells are normal and unaffected by the disease[187]. Ph^- primitive progenitor cells fail to adhere to normal stromal layers and fibronectin although such progenitor cells do not exhibit any changes in the expression of fibronectin receptors[187]. This suggests that functional defects in such receptors, including its downstream signaling pathways, may exist in CML progenitors and thus contribute to the pathogenesis of the disease[187]. Failure to adhere to

stroma can be improved in CML patients by interferon-α treatment which probably acts by correcting impaired β1 integrin receptor function[188]. Also treatment of stromal cells from CML patients and donors with interferon-α prior to transplantation restores the adhesive properties of CML cells. This is associated with the production of macrophage inflammatory protein-1α by the stroma[189]. When adhesion to fibronectin was stimulated by α4β1 activating monoclonal antibodies a decrease in proliferation of CML progenitors and K562 cells was observed[190]. Also, CML stroma was shown to reduce the growth of normal long term culture-initiating cells by a mechanism involving malignant stromal macrophages[191]. The effect of abnormal interaction between normal stroma and CML progenitor cells and between leukemic stroma and normal progenitor cells may lead to the selective expansion of Bcr-abl progenitor cells.

On a molecular level, Bcr-abl interacts with signaling pathways, regulatory factors, and cellular structures involved in regulating cell adhesion. Focal adhesion kinase (FAK) plays a role in integrin-mediated-signaling[192]. FAK has been shown to be tyrosine phosphorylated and activated in cells expressing P190 and P210Bcr-abl but not by the stimulation of growth factor-dependent cells with hematopoietic growth factors[193;194]. Bcr-abl is exclusively present in the cytoplasm where it is mostly associated with actin stress fibers probably through the actin binding domain present in the carboxyl-domain of Abl[71]. This interaction with actin stress fibers is required for transformation by Bcr-abl[71]. In transformed cells, Bcr-abl induces a redistribution of filamentous (F) actin into punctate, juxtanuclear aggregates[71]. This altered integrity of the microfilament network may play a role in the Bcr-abl-induced phosphorylation and activation of FAK and the phosphorylation of other

components of focal adhesion such as paxillin, vinculin, and tensin[194]. Bcr-abl can, therefore, disrupt the normal structure and signal transduction capacity of various components of the cytoskeleton[194]. The discrete punctate structures where Bcr-abl and actin were observed to be concentrated in myeloid cell lines are similar to focal adhesions of epithelial cells[194]. Focal adhesions are believed to be the sites where integrin function is regulated partly through the focal adhesion molecules that receive and transmit signals through phosphorylation and dephosphorylation on tyrosine residues. Focal adhesion molecules are targets for activated tyrosine kinases such as v-Src[195], v-Crk [196], and v-Abl[197]. The tyrosine phosphorylation of focal adhesion molecules by activated tyrosine kinases is believed to be important for transformation by resulting in reduced adhesion to extracellular matrix proteins, decreased contact inhibition, and acquisition of the ability to metastasize [196]. Similar to v-Src, and in cells expressing Bcr-abl, phosphorylation of focal adhesion proteins such as paxillin, $p125^{FAK}$, tensin, talin , and vinculin was observed[194]. In P210Bcr-abl cells, paxillin was constitutively associated with vinculin, FAK, tensin, and talin while in control cells only vinculin was coprecipitated with paxillin following treatment with IL-3.

Crkl, which binds to Bcr-abl through its SH3 domain and links Bcr-abl to the PI-3K pathway, was found to be associated with paxillin in 32D cells expressing Bcr-abl through the interaction of the Crkl SH2 domain and tyrosines 31 and 118 of paxillin[198] . Crkl, therefore, might be the molecule that links Bcr-abl with focal adhesion proteins leading to altered cell adhesion properties.

In another study, more profound abnormalities of the cytoskeletal function were observed as a result of the expression of both P190 and P210 Bcr-abl in BaF3 cells[199]. An

increase staining of filamentous actin and an enhanced rate of formation and retraction of actin-containing protrusions such as pseudopodia and filopodia was observed. In addition, cells expressing Bcr-abl had an increased level of spontaneous motility and shorter periods of quiescence compared to normal non-transformed cells. The observed abnormalities required the tyrosine kinase activity of Bcr-abl and were detected in both BaF3 Bcr-abl transformed cells and primary CML progenitor cells isolated from patients with active leukemia[199]. The increased motility observed as a result of Bcr-abl expression can explain some of the clinical aspects of the disease. CML is characterized by the early release of myeloid cells from the marrow and the accumulation of myeloid cells at all stages of differentiation. It is possible that the increased motility observed due to the expression of Bcr-abl is a result of the activation of cytoskeletal-dependent signal transduction pathways by Bcr-abl. Most cells require a variety of signals from cell surface receptors to support growth and viability. In addition to mitogenic signals from growth factor receptors, cells might also depend on signals from adhesion and co-stimulatory receptors for proliferation and viability[199]. CML cells may, therefore, be receiving false signals that indicate that certain adhesion receptors such as integrin are stimulated even in the absence of ligand binding[199]. These cells still need hematopoietic growth factors for growth and viability but, because of increased motility, can migrate into capillaries at an immature stage and divide in tissue where myeloid cells are not found. Treatment of BaF3 cells expressing Bcr-abl as well as CD34+ cells from CML patients with interferon-α resulted in reduced spontaneous motility and a reduced number of protrusions and pseudopodia per cell[199]. Also, interferon-α was found to correct the defective adherence of CML progenitors to stromal cells by increasing

the expression of the adhesion molecule lymphocyte function-associated antigen (LFA)-3, which is usually down regulated in CML cells[188], and by correcting β1 integrin function[186]. Recently, a constitutively tyrosine phosphorylated 62 kDa protein prominently present in hematopoietic progenitor cells of CML patients has been identified[134]. $p62^{dok}$ ($p62$ protein downstream of tyrosine kinases) was isolated from a hematopoietic cell line expressing P210Bcr-abl. $P62^{dok}$ has structural features typical of a signaling molecule such as a plekstrin homology (PH) domain, which is found in a number of signaling molecules and also in molecules involved in cytoskeletal organization. PH domains are believed to mediate protein-protein interactions and also interactions with cellular membranes possibly by binding different inositol phosphate components of the lipid bilayer[134]. Most of the tyrosine residues in $p62^{dok}$ are located in the carboxyl-terminal half and when phosphorylated can bind SH2 containing proteins. $p62^{dok}$ also contains ten proline rich motifs which can interact with SH3 domains[134]. $p62^{dok}$ binds *in vitro* with the amino-terminus of GAP. The amino-terminal domain of GAP has a different biological role than the catalytic carboxyl-terminus half which is a Ras GTPase activating protein that leads to Ras down-regulation[135]. The amino-terminal domain of GAP, when over-expressed, can disrupt the actin cytoskeleton and focal contacts, decrease fibronectin binding, as well as cell adhesion[136]. The GAP-associated $p62^{dok}$ is also rapidly tyrosine phosphorylated upon the activation of the c-Kit [137], EGF and PDGF [138] receptors suggesting that it plays a role in distinct signal transduction pathways initiated by receptor or membrane associated tyrosine kinase. The constitutive tyrosine activation of $p62^{dok}$ in hematopoitic progenitor cells containing P210Bcr-abl and by c-Kit is interesting given that both appear to manifest similar biological effects in a relatively mature

stem cell population at the level of lineage commitment rather than at the level of a self-renewing stem cell[139]. c-Kit has been shown to be constitutively active and form a complex with P210Bcr-abl in P210Bcr-abl transfected myeloid cell lines[140]. Colony assays with bone marrow from Bcr-abl positive CML patients showed that hematopoietic precursors of most patients did not respond to recombinant human Steel Factor (rhSF), a growth factor that binds and activate c-Kit. This suggests that c-Kit is already activated, presumably by P210Bcr-abl[140]. It is therefore possible that the aberrant and tyrosine phosphorylation of $p62^{dok}$ is a key step in the progression of CML. Bcr-abl presumably can amplify signals leading to $p62^{dok}$ by binding and activating c-Kit. The amino-terminal domain of GAP might also be part of this complex cascade as it can potentially control processes, such as cell adhesion and actin organization, both important in Bcr-abl transformation. As mentioned above Bcr-abl might therefore induce abnormal adhesion, not only by affecting the expression and function of integrin receptors, but also by the redistribution of the actin cytoskeleton and the phosphorylation of proteins associated with the cytoskeleton.

1.10 Bcr-abl and the Induction of Cell Proliferation

As mentioned previously, CML cells do not demonstrate an increase in either mitotic index or in proliferative capacity. However, evidence of an induction of cellular proliferation accompanied by the deregulation of cell cycle by activated Abl is emerging. In soft agar colony formation assays, the transformation potential of both v-Abl and P190Bcr-abl were drastically increased in the presence of cyclin D1 over-expression[200]. Progression through late G_1 in the cell cycle coincides with the period where cells are no longer dependent on

mitogens and commit to DNA synthesis [178]. This transition is controlled by cyclins D and E and their cyclin-dependent kinases (Cdks) which, when activated, can phosphorylate substrates and facilitate entry into S phase [178]. D cyclins bind to Cdk4 and 6 to form holoenzymes whose activity is detected in mid-G_1 and increases as cells reach G_1/S boundary. Cyclin D-dependent kinases are active as long as mitogenic stimulation is present, hence, acting as mitogenic sensors. Cyclin E is expressed late in G_1 and combines with Cdk2 and maximally induce its activity at G_1/S. Cyclin D1 probably acts by targeting Cdk4 and 6 to Rb. Phosphorylation of Rb by Cdk4 and 6 leads to its inactivation and loss of its ability to bind transcription factors, such as E2F, whose activity is required for S-phase transition[178]. Cyclin D1 but not cyclin E over-expression was able to increase the transformation efficiency of v-Abl[200]. This function of cyclin D1 required the amino-terminal motif essential for the binding of Rb. Synergy between cyclin D1 and activated Abl therefore appears to depend in part on an Rb-mediated pathway. A transformation defective SH2 deletion mutant of P190Bcr-abl was rescued by cyclin D1 but not cyclin E, suggesting that cyclin D1 compensates for loss of signaling from Abl SH2 domain[200]. Also, primary mouse bone marrow cells were transformed by a transformation-defective P190Bcr-abl mutant with a point mutation in the SH2 domain in the presence of cyclin D1[197]. Those transformed cells resulted in normal *in vivo* transformation efficiency when introduced into SCID mice. Cyclin D1 can, therefore, cooperate with Abl oncogenes to induce *in vitro* and *in vivo* transformation by replacing an SH2-dependent signal which seems to involve Rb [200].

Other studies also support the effect of Bcr-abl expression on the cell-cycle machinary. Fifty per cent of patients with lymphoid blast crisis in CML exhibit homozygous

deletions in the tumor suppressor gene $p16^{INK4a}$ compared to only 26% of patients with *de novo* acute lymphoblastic leukemia [201]. p16 deletions were detected in acute leukemia cells but not in cells from chronic-phase leukocytes, indicating that the deletions are somatically acquired and not inherited thus implicating those deletions in the pathogenesis of CML. p16 inhibits Cdk4 by competing with cyclin D binding and is considered a principal regulatory element in cell-cycle progression[178].

In the untransformed IL-3 dependent myeloid progenitor cell line 32D, Bcr-abl expression by retroviral infection results in growth factor independent proliferation, accompanied by the activation of mitogenic signals represented by the activation of the Erk family of MAP kinases[202]. 32D cells, expressing a kinase-inactive Bcr-abl and grown in low serum and IL-3 conditions, accumulate with unreplicated DNA. Conversely, the same cells expressing a kinase active Bcr-abl and grown under the same conditions, continued to cycle[202]. This reduced growth factor requirement for cell cycle progression was associated with the sustained activation of cyclin-dependent kinases controlling the G_1-to-S transition. Cyclins D2 and D3-associated Cdk4 and Cdk6 decreased rapidly in 32D cells grown under reduced growth factor conditions but were maintained, at a level similar to that obtained with growth factor stimulation, in 32D cells expressing kinase active forms of Bcr-abl. This suggest that Bcr-abl signals prevent cells from exiting the cell cycle by inhibiting the down regulation of mitogenic signals following growth factor deprivation[202]. In the same study, a 32D cell line clone expressing inducible P210 and P190Bcr-abl fused to glucocorticoid-responsive promoter was used to determine whether the induction of mitogenic signals is a primary response after the expression of Bcr-abl. Induction of Bcr-abl expression by

dexamethasone in this system resulted in increased level of GTP-bound Ras, hyperphosphorylation of Raf, activation of Erk, and an enhanced DNA synthesis. Bcr-abl induced cell cycle entry was associated with the increase in Cdk2, and cyclin D2 and D3 associated kinases, suggesting that such an induction is associated with the activation of the cell cycle machinary. Bcr-abl-mediated transformation may therefore, in part, be mediated by activating mitogenic signals. In chronic phase CML, Bcr-abl$^+$ cells do not seem to demonstrate an increased proliferative rate but undergo additional cell divisions and have longer survival time than normal myeloid cells. Bcr-abl may mediate mitogenic signals that inhibit cell cycle exit that would normally occur during differentiation[202].

1.11 Genomic Instability in Chronic Myelogenous Leukemia

It is well accepted that the accumulation of genetic changes is the underlying cause of neoplasia[203]. Chronic myelogenous leukemia is a model disease for multi-step progression of cancer[204]. The chronic phase of the disease is characterized by the over-production of myeloid cells which remain growth factor dependent and retain the ability to differentiate. Invariantly the disease enters a terminal blast crisis which is characterized by the predominance of hyper-proliferative immature blast cells[1]. CML patients entering the blast crisis exhibit an increase in the rate of acquiring additional mutations[205]. Genomic instability in such patients is supported by cytogenetic findings such as the duplication of Bcr-abl, +8, i(17)q, +19, +21, -Y, and +Y [40; 205] . Genomic instability is also observed in cell line as well as mouse models expressing Bcr-abl. Mice transgenic for a DNA construct that produces the P190Bcr-abl succumb to both myeloid and lymphoid acute leukemia [124]. Normal karyotypes

in the initial stages of Bcr-abl expression in the mice were observed while later stages of the disease is characterized by nonrandom numerical changes of mouse chromosomes that have a genetic analogy to human chromosomes affected in leukemia[206]. The chromosome changes most prominent were the acquisition of extra copies of chromosomes 10, 12, 14, and 17 either alone or in combination. Chromosomal abnormalities, in addition to the Philadelphia chromosome, seem to be variable in ALL. Hence, the nonrandom chromosome changes and clonal evolution of P190Bcr-abl mice are more comparable with the nonrandom chromosome changes of human blast-phase CML[206]. The observation that the expression of Bcr-abl precedes the development of chromosomal abnormalities in those mice suggests that the activated kinase may directly or indirectly result in cytogenetic and genetic instability which enable cells to accumulate additional mutations and progress to later stages of the disease.

Retroviral expression of P210Bcr-abl in murine IL-3 dependent myeloid cell line 32D C13(G)' cells resulted in karyotypic instability and proliferative advantage *in vitro* over time, mimicking the changes associated with the transition from chronic phase CML to the acute leukemic phase[127]. P210Bcr-abl expression in those cells induced structural as well as numerical chromosomal abnormalities. Similar to lymphoblastic leukemia/lymphoma cells from P190Bcr-abl transgenic mice, a preference for the development of chromosomal abnormalities involving chromosomes 12 and 17 was detected in 32D C13(G)' cells transformed by retroviral expression of P210Bcr-abl, suggesting that *in vitro* evolution of karyotype abnormalities due to Bcr-abl expression might be very comparable to that obtained *in vivo*[127].

Other genetic alterations in CML have been described and may be caused by the underlying genomic instability characteristic of disease progression. Recently six of six patients with advanced CML showed loss of imprinting (LOI) of the insulin-like growth factor-II (IGF2) gene [207]. On the other hand, only one of six patients in the chronic phase exhibited LOI suggesting that disease progression in CML is associated with LOI. Genomic imprinting entails the monoallelic expression of either the paternal or the maternal allele and is important for the development of cancer as loss of heterozygosity (LOH) in cancer usually involves one of the parental chromosomes preferentially [208]. Loss of imprinting has been reported in human cancer and results in the loss of parental-origin-specific differential allele expression [208]. Some imprinted genes, such as IGF2, are directly involved in cell growth. IGF2 is normally imprinted and is expressed only from the paternal allele[210].

Another important genetic alteration in CML progression is *de novo* methylation. Two studies document the abnormal methylation of the calcitonin (CT) gene during the progression of CML. In the first,[209] abnormal methylation pattern was found in the peripheral blood of 2 out of 31 patients in chronic phase compared to 11 of 12 patients in blast crisis. In the other study, [210] 20 patients in the chronic phase of the disease out of 27, had a normal methylation pattern while 5 of 6 patients in blast crisis had a hypermethylated CT gene[217]. These findings indicate that abnormal methylation of the CT gene is a useful marker of disease progression in CML and probably reflects a more wide spread process that could contribute to genetic instability events characteristic of the progression of the disease[209].

The multistep progression in cancer is best exemplified in colorectal cancers which develop over decades and require at least seven genetic events for completion[211]. However,

a marked predisposition to colorectal cancer can result by the inheritance of a single altered gene as observed in Hereditary Nonpolyposis Colorectal Cancer (HNPCC)[211]. Recently, three independent observations helped in understanding the molecular pathogenesis of HNPCC. First, linkage studies using microsatellite markers in individual HNPCC families concluded a tight linkage to either chromosome 2p16 or 3p21 providing strong evidence that HNPCC is a hereditary disease [212]. The second observation was documented following attempts to demonstrate allelic losses within the 2p16 microsatellite marker linked to HNPCC susceptibility[213]. Surprisingly, new microsatellite alleles not found in normal cells of HNPCC patients were observed rather than allelic loss. These new alleles were observed in all dinucleotide and trinucleotide repeats examined, suggesting a general state of instability of the replication and/or repair of simple repeated sequences in the whole genome[213]. A third landmark discovery came from the observation of similarities between the microsatellite instability observed in tumors such as HNPCC and those observed in bacteria harboring mutations in mismatch repair (MMR) genes such as *mutS* and *mutL* [214]. Similar experiments in yeast also suggested that microsatellites observed to be unstable in HNPCC patients were also unstable in yeast with defective MMR genes suggesting that HNPCC was caused by hereditary mutations in human homologs of mutS and mutL [214]. A search for human homologs of mismatch repair genes resulted in the discovery of at least five such genes[215]. A human homolog of *mutS* (*hMSH2*) was located on chromosome 2p suggesting that it can be involved in the form of HNPCC linked to chromosome 2p[216]. Direct evidence of the involvement of an MMR gene in this disease came from studies showing germline mutations of *hMSH2* in HNPCC kindreds[217]. This observation was followed by the identification of

mutations of mutL homologs in HNPCC[218] and it is believed that mutations in three human MMR genes (*hMSH2*, *hMLH1*, and *hPMS2*) account for most mutations in HNPCC kindreds[211].

The MMR gene pathway in human cells is responsible for the repair of single base mismatches that may result from misincorporation by polymerases or larger mismatches resulting from strand slippage. This resulting mismatch is recognized by *mutS* homologs where optimal mismatch recognition is thought to require at least two *mutS* homologs, *MSH2* and GTBP (G/T binding protein). *MutL* homologs are then recruited to the complex and the mismatch is repaired by a process that in bacteria involves an exonuclease, helicaseII, DNA polymerase III, single-stranded binding protein, and DNA ligase[219].

An important question to be addressed in multi-step cancer progression is whether tumor cells have intrinsically high rates of mutations sufficient to account for this accumulation of mutations[211]. Studies in HNPCC have shown that mutation rates in tumor cells with MMR deficiency are two to three orders of magnitude higher than in normal cells[220]. HNPCC accounts for only 2-3% of total colorectal cancer cases while sporadic tumors exhibiting microsatellite instability constitute 13% of total colorectal cancers. In those cases, the underlying instability is not well defined and may be caused by mutations in genes involved in MMR repair, such as GTBP, MSH3, or the proof reading domain of polymerase δ [211]. In 85% of colorectal cancers that do not demonstrate microsatellite instability, elevated rates of mutations were not observed using standard assays[221]. Those tumors, however, have a large number of genetic alterations characterized by the loss of large parts of chromosomes, as a consequence of mitotic recombination or aberrant mitotic

segregation of chromosomes, resulting in aneuploidy[213]. Tumors, therefore, accumulate multiple genetic alterations required for malignancy, either by subtle alterations due to the mismatch repair deficiency or through gross chromosomal alterations [211]. Studies on HNPCC, therefore, demonstrate the association of genomic instability with cancer development.

1.12 Project Rational

Cell culture systems have proven difficult to examine the suspected inherent genomic instability associated with the expression of Bcr-abl. As discussed previously, cell-cycle control defects, as well as inhibition of apoptosis, both fundamental in the preservation of genomic stability, have been reported in cell culture systems expressing Bcr-abl[127;176;222;223]. However, such systems do not appear to adequately reproduce primary effects of Bcr-abl expression *in vivo*. For example, induction of apoptosis as a result of growth factor deprivation or γ-irradiation in proliferating primary hematopoietic $CD34^+$ CML cells was not different than the response seen in the same cells from normal individuals[224]. These conflicting results suggest that Bcr-abl effect on apoptosis strongly depends on the cells used. The observation that CML cells derived from newly diagnosed CML patients undergo apoptosis as a result of growth factor withdrawl or γ-irradiation suggests that Bcr-abl expression is not necessary to induce resistance to apoptosis in those cells[224]. Cell lines fail to accurately reflect the primary in vivo effect of Bcr-abl expression probably because such lines are established from patients in the acute phase of the disease and are growth factor independent and resistant to apoptosis[225]. Alternatively, differences in the relative levels of

Bcr-abl might explain such discrepancy. Acute phase cells carry karyotypic changes such as the duplication of the Ph chromosome and an enhanced expression of *bcr-abl* mRNA resulting in an increase in the relative levels of Bcr-abl[226].

A marked difference in the biologic consequences of high and low levels of Bcr-abl expression was observed in immortalised myeloid and lymphoid cell lines[227]. Low levels of Bcr-abl expression were sufficient to remove cytokine requirements and render cells tumorigenic, while high levels were necessary for protection against apoptosis. The observation that resistance to apoptosis critically depended upon the level of Bcr-abl could help explain the sensitivity of primary CML cells to the induction of apoptosis compared to cell lines expressing Bcr-abl[227]. In the same study, and utilizing a dominant negative Ras mutant, it was shown that an activated Ras did not offer the same degree of protection as high levels of Bcr-abl expression. The activation of the Ras/MAPK pathway is suggested to be important for Bcr-abl-mediated transformation and anti-apoptotic function as a result of IL-3 withdrawal and other stimuli[69; 180; 228]. Low levels of Bcr-abl were observed to stimulate the same Ras-dependent survival signals as IL-3, but there were additional anti-apoptotic pathways only activated by high levels of Bcr-abl. Likely candidates include phosphatidyl inositol-3 kinase pathway which is activated by Bcr-abl and may play a role in anti-apoptotic activity[227].

Such discrepancies have lead us to consider *in vivo* models for Bcr-abl induced leukemia in investigating Bcr-abl induced genomic instability. Several Bcr-abl induced leukemic mouse models have been described[124; 229-231]. I have chosen the model developed by Heisterkamp *et al* [124] for its availability and the well established data derived from it

concerning the expression of Bcr-abl in different tissue and disease pathogenesis. In those transgenic mice, the P190Bcr-abl human fusion gene was used as a transgene[124]. P190Bcr-abl transgenic mice succumb to pre-B cell acute lymphoblastic leukemia on an average of 100 days post natal[124]. Although these mice express the form of Bcr-abl associated with ALL, this model offers an opportunity to investigate the primary biologic consequences of Bcr-abl expression in the presence of intact cellular and physiological properties. A mouse colony from the 623 transgenic line which has been bred for more than 4 generations and, within each generation, animals developed lymphoblastic leukemia/ lymphoma[232] Before the development of disease the transgene is expressed in most tissues tested[124; 232], and during this period the consequence of Bcr-abl expression on biological processes controlling genomic stability can be addressed. I tested, *in vivo*, the integrity of the cell cycle response to DNA damage in bone marrow and spleen cells from irradiated P190Bcr-abl pre-leukemic transgenic mice. In both cell types no defects were observed in cell-cycle regulation following the induction of DNA damage. Concomitantly, I show that the G_1 cyclin-dependent kinase inhibitor $p21^{WAF1/CIP}$ is normally induced in kidney, spleen, and lung cells in irradiated transgenic mice. I also demonstrate, quantitatively and morphologically, that bone marrow and spleen cells from irradiated pre-leukemic P190Bcr-abl mice exhibit the same degree of apoptosis as cells from irradiated normal mice. My data suggests that the expression of Bcr-abl at a level which induces leukemia in an animal model does not interfere with the two biological processes regarded as the primary safeguards against the accumulation of mutations and preserving genomic stability.

I concluded that contrary to results obtained in cell lines expressing Bcr-abl[73; 127; 222]

loss of sensitivity to DNA damage might define a late event in Bcr-abl oncogenesis. I then proposed to directly investigate genomic instability in pre-leukemic P190Bcr-abl by measuring mutation rates as a result of the expression of the Bcr-abl kinase. The Big Blue™ transgenic mouse system is a reliable mean to determine mutation frequencies *in vivo* in the context of normal physiological, metabolic, and DNA repair mechanisms [233]. Every cell in the Big Blue mouse contains multiple copies of the a chromosomally-integrated λ bacteriophage shuttle vector (λLacI-αlacZ). This vector contains the bacterial *lacI* gene which encodes the LacI repressor protein that controls β-galactosidase activity. *lacI*, therefore, serves as a target for mutation and αLacZ as a reporter gene[234] (Fig. 6).

Big Blue™ /P190Bcr-abl double heterozygotes were generated allowing the expression of Bcr-abl in the presence of the Big Blue mutation detection system. DNA extracted from normal as well as from leukemic tissue was used to recover the λ phage vector. This is done by shuttling the phage vector, from its origin in mammalian DNA, to a bacterial *E. coli* host. Using a Transpack™ lambda packaging extract (Stratagene), the λ shuttle vector was excised from mammalian DNA and packaged into virulent lambda bacteriophage. This viable bacteriophage was then used to infect *E. coli* cells to produce bacteriophage plaques on a bacterial lawn.

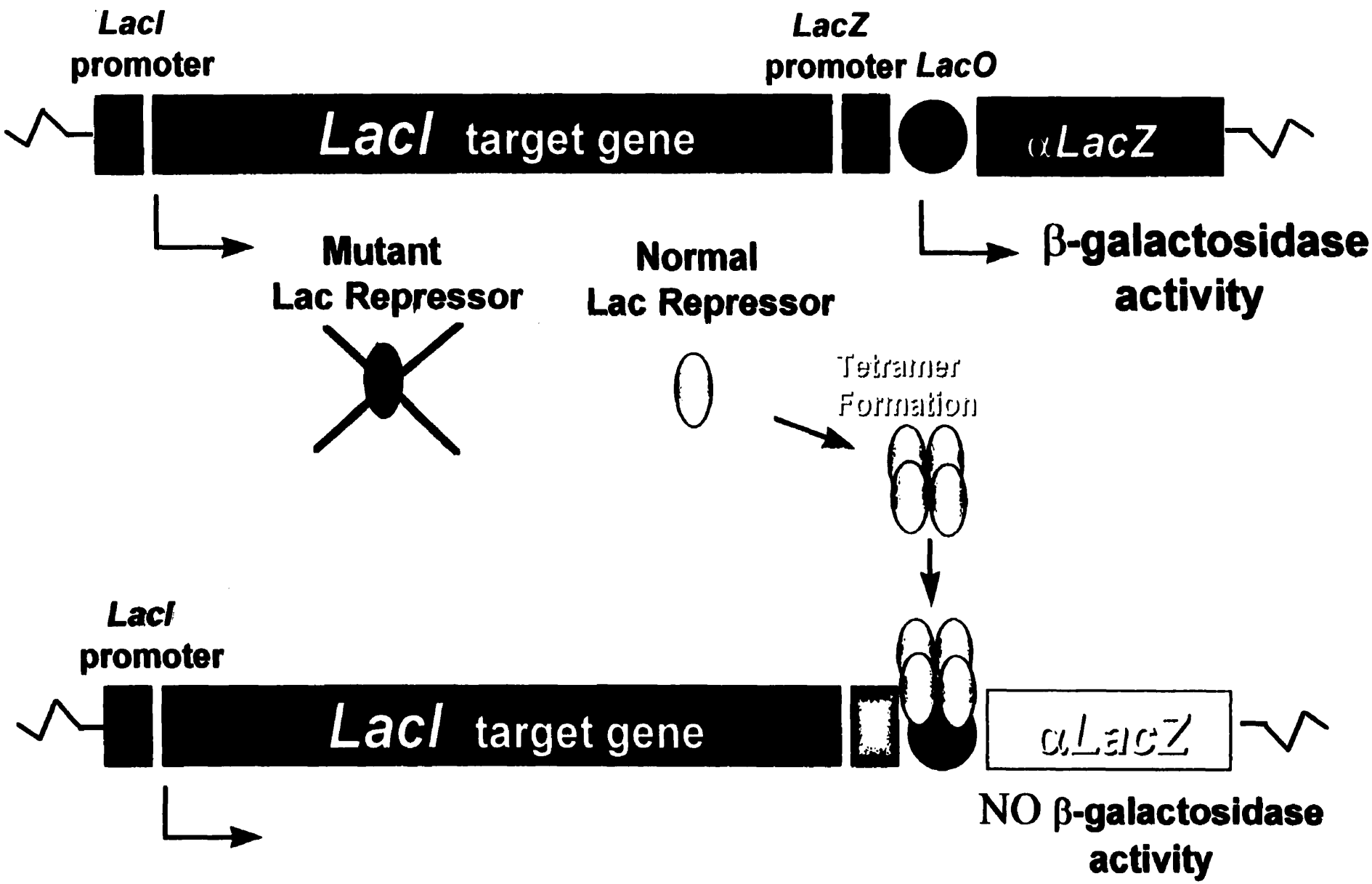
Bacteriophages carrying mutations in *lacI* will express β-galactosidase and will produce a mutant blue plaque when used to infect *E. coli* growing in the presence of the chromogenic analog 5-bromo-4-chloro-3-indol-β-galactopyranoside (X-gal) [234]. The mutation spectrum from mutant plaques was determined by sequencing the whole *lacI* gene and the LacI promoter (1.2 Kb) from the shuttle vector. I observed a significant increase in

mutant frequency in the kidney and spleen of PL Big Blue/P190Bcr-abl mice and in two out of four tumors obtained from leukemic mice. Bcr-abl expression may, therefore, confer a mutator phenotype rendering cells susceptible to the accumulation of further mutations.

Figure 6 *In vivo* measurement of mutation frequencies in Bcr-abl transgenic mice.

The Big Blue™ transgenic mouse system was employed to determine the mutation frequency and spectrum induced by Bcr-abl expression in mice tissue. Big Blue mice were first crossed to P190Bcr-abl mice. Big Blue mice contain chromosomally integrated λ bacteriophage vector (LIZ) at a rate of 40 copies/cell in heterozygote mice. DNA extracted from P190Bcr-abl/Big Blue mice can be used to generate viable λ phage particles (see Materials and Methods). Bcr-abl induced mutations in LacI will result in a defective LacI repressor allowing the expression of LacZ. λ phage particles that express LacZ produce blue colonies when used to infect *E coli* lawns grown in the presence of X-gal. Mutation spectrums induced by Bcr-abl can then be determined by sequencing the *lacI* gene.

Color Screening for Mutations



II-Materials and Methods

2.1 Animals

P190Bcr-abl transgenic mice were generated by utilizing a construct coding for the human P190Bcr-abl fusion protein under the control of the mouse metallothionine-1 promoter[124] (Fig. 7). Founder animals were the offspring of mating between C57BL/CBA F_1, while transgenic progeny was the result of mating between transgenics and C57BLxCBA F_1 mice. A homozygote colony of P190Bcr-abl mice was established through breeding. $p53^{-/-}$ null mutant mice contain a PolII-neo expression cassette interrupting exon 5 of the p53 gene (Fig. 7), resulting in the absence of p53 protein expression in all tissues of the animal[235]. p53 homozygote null ($p53^{-/-}$), as well as heterozygote ($p53^{+/-}$), mutants were established under a breeding licence from Taconic Farms Inc (NY, USA). Mice from a similar genetic background to P190Bcr-abl transgenics, (F_1 C57BLxCBA) (Jackson, USA), were used as controls. Where indicated, genotoxic stress by ionizing radiation was induced by subjecting mice to whole body irradiation at different doses (2, 6, 8, and 10 Gy) at a rate of 107cGray/min from a ^{60}Co (Cobalt) source. P190Bcr-abl in the pre-leukemic phase were confirmed to be disease free by studying the morphology of blood and bone marrow smears for the presence of immature blast cells. Differential cell blood counts (CBC) were performed to detect elevations in different hematopoietic cell populations especially lymphocytes which increase dramatically in leukemic mice .

Homozygote Big Blue^{TM} (LacI) transgenic mice [234] were purchased from Stratagene

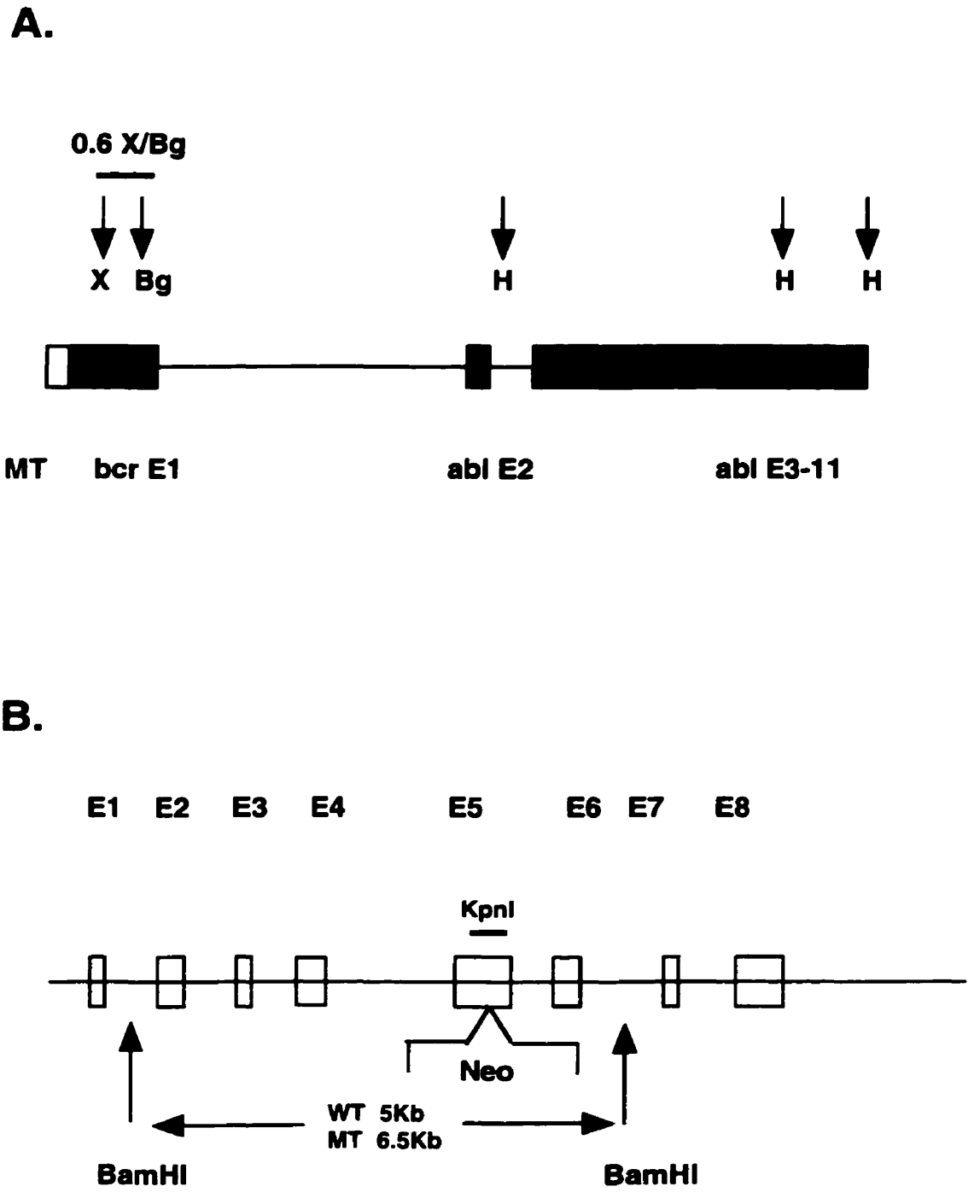


Figure 7 P190Bcr-abl and p53$^{-/-}$ genotyping.

The P190Bcr-abl - producing construct is shown (A). The metallothionin (MT) promoter and the position of coding sequences from both *bcr* and *abl* are represented by boxed areas. An 600 bp XhoI (X) / BglII (Bg) fragment from *bcr* exon (E)1 used as a probe in tail blots is indicated. Tail DNA were digested with XhoI and HindIII(H).

The p53 gene is disrupted at exon 5 by the insertion of a neomycin (Neo) expression vector (B). Tail DNA digested with BamHI and probed with a KpnI fragment of Exon 5 will yield a larger (6.5 Kb) fragment indicating the presence of a disrupted p53 allele.

(La Jolla, Ca) and licensed to be bred in the Royal Victoria Hospital Research Institute animal facility. P190Bcr-abl/Big Blue double heterozygotes were generated by crossing young homozygotes from each mouse group and were further confirmed for the presence of the Bcr-abl trangene by southern blot analysis as described later. I was interested in determining the mutation frequency of disease free pre-leukemic (PL) P190Bcr-abl/Big Blue mice. Health profiles for each sacrificed PL P190Bcr-abl/Big Blue mouse were, therefore, confirmed by examining peripheral blood and bone marrow smears for the presence of leukemic blast cells, as well as performing differential cell blood counts (CBC), to determine any elevations of the different blood cell types. Peripheral blood and bone marrow smears from leukemic mice revealed the presence of immature blast cells. Tumor tissue from leukemic P190Bcr-abl/Big Blue mice were collected upon autopsy and processed in parallel with tissue from PL P190Bcr-abl/Big Blue mice. Mutation rates were also determined for normal control mice generated by crossing homozygote Big Blue mice to the genetic background of P190Bcr-abl (F_1 C57BLxCBA).

2.2 Expansion of Replicating Cell Pool *In Vivo*

Mice were sub-cutaneously injected with human granulocyte colony-stimulating factor (G-CSF) (Amgen, Ont.) for three days at a dose of 5ng/g mouse body weight. This resulted in a 4-5 fold increase of granulocyte precursor cells in the S-phase of the cell cycle in bone marrow. Similarly, mice were injected intra-peritoneally with 10,000 cetus units of human IL-2 (Amgen, Ont.) for three days to expand lymphoid precursor cell population in the bone marrow.

In addition, I was able to increase lymphocyte counts in the spleen by injecting mice intra- peritoneally with 0.1μg/g mouse body weight of anti-CD3 (anti-mouse T3 CD3ϵ complex) antibody (CedarLane, Ont.) for three days and rat Interleukin 2 (IL-2) (CedarLane, Ont.) at a dose of 10,000 cetus unit/mouse. CBCs of blood samples from induced mice were performed to obtain a profile of the different blood lineages and to confirm the increase of targeted populations in peripheral blood.

2.3 Southern Blot Analysis

DNA for mice genotyping was obtained by over-night incubation at 55°C of tail biopsies in 1 ml digestion buffer (100mM Tris pH 8.0, 5mM EDTA, 0.2% SDS, and 200 mM NaCl) containing 0.1μg/μl of proteinase K. DNA in supernatants of over-night digests were precipitated by isoproponal. 15μg of DNA was digested, ran on a 1% agarose gel, and transferred onto hybond N+ nylon membranes (Amersham, Ont.). For P190Bcr-abl genotyping, membranes were hybridized with a 600 bp BglII/XhoI fragment of the *bcr* exon 1 sequences in the transgene (Fig. 7). $P53^{-/-}$ mice were genotyped using a 605 bp KpnI probe that can hybridize to exon 5 of the p53 gene [235]. Blots were pre-hybridized at 42°C for 4 hours in pre-hybridization solution (6x SSC, 5x denhardt's, 0.5% SDS) containing 50μg/ml of salmon sperm DNA. Blots were incubated in hybridization solution (50% formamide, 6x SSC, 5% dextran sulfate, 1% SDS, and 100μg/ml salmon sperm DNA) containing appropriate probes prepared to high specific activity with [α-^{32}P] dCTP (3000Ci/mmol) (DuPont/NEN, Boston-Ma) using multiprime DNA labeling system (Amersham, Ont.). Blots were washed at room temperature (1x SSC, 0.1% SDS) and at 65°C before being exposed

to all purpose hyper sensitive film (Amersham, Ont.).

2.4 Northern Blot Analysis

Total RNA was isolated from 100mg mice tissue by homogenizing in 1ml of Trizol™ (Gibco BRL, Ont.) according to manufacturer's protocol. Twenty μg of total RNA was denatured and electrophoretically seperated on 1.2% agarose gel containing 1% MOPS and 16% formaldehyde. The separated RNA was then transferred onto hybond N+ nylon membrane (Amersham, Ont.) and WAF-1 ($p21^{CIP}$) messages were detected by probing the membrane with 50 ng of an EcoRI fragment of the mouse waf-1 cDNA in BlueScript SK^{236} (generous gift from Dr. Bert Vogelstein), labeled to a high specific activity with [α-^{32}P] dCTP (3000 Ci/mmol) (Dupont/NEN, Boston-Ma) using a multiprime DNA labeling system (Amersham,UK). Blots were pre-hybridized (6xSSC, 5x denhardt's, 0.5% SDS containing 50 μg/ml of salmon sperm DNA) at 42°C for 4 hours and hybridized (50% formamide, 6x SSC, 5% dextran sulfate, 1% SDS, containing 100μg/ml salmon sperm DNA) over-night at 42°C. Blots were washed at room temperature (1x SSC, 0.1% SDS) and then at 62°C (0.2x SSC, 0.1% SDS) before being exposed to all purpose hyper sensitive film (Amersham, Ont.).

2.5 Western Blot Analysis of P190Bcr-abl Expression

Forty grams of spleen and kidney tissue from normal controls as well as from PL P190Bcr-abl/Big Blue mice were homogenized and lysed in cold lysis buffer [50mM HEPES-KOH, pH 7.9, containing 0.4 M KCL, 0.1% NP-40, 4mM NaF, 4mM $NaVO_4$, 0.2 mM EGTA, 0.2 mM EDTA, 10% glycerol (v/v), 1mM DTT, 0.5 mM

phenylmethylsulfonylfluoride (PMSF), 1 μg/mL pepstastin, 1 μg/mL leupeptin, and 1 μg/mL, benzamidine]. Alternatively tissue were homogenized in 1% SDS and then incubated at 100°C for 2 minutes. The homogenized cell extracts were centrifuged at 13,000*g* for 20 minutes at 4°C. Total protein concentrations, in the supernatant, were assayed using Micro Bicinchonic Acid (BCA) protein assay kit (Pierce, IL). Fifty μg of total protein was separated on a 6% sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis and transferred onto BioTraco polyvinylidene fluoride (PVDF) blotting membrane (Gelman Sciences, Que). Membranes were blotted with the mouse monoclonal antibody to Abl [c-abl (Ab-3), Oncogene Science, Calbiochem, Ont.]. P190Bcr-abl was detected using a horseradish peroxidase-conjugated antibody to mouse IgG using an enhanced chemiluminescence system (ECL, Ilinois).

2.6 Determination of Cell-cycle Distribution by BrdU and Propidium Iodide labeling

Cells from mice labeled *in vivo* with bromodeoxyuridine (BrdU) (Sigma, Ont.) were analyzed for cell cycle distribution by using fluorescein conjugated anti-BrdU antibody (Becton Dikinson, Que.) following a protocol supplied by the manufacturer (Fig. 8). Replicating cells in irradiated (2, 4, 6, and 8 Gy) and control (0 Gy) mice, treated with G-CSF or αCD3 anti-bodies for 3 days, were labeled *in vivo* by injecting mice intra-peritoneally with BrdU at a dose of 50 μg/g body weight. BrdU is an analog of uridine but is incorporated specifically into DNA in place of thymidine. Fluorescein conjugated anti (α)-BrdU antibodies can then be used to identify those cells undergoing replication. The .

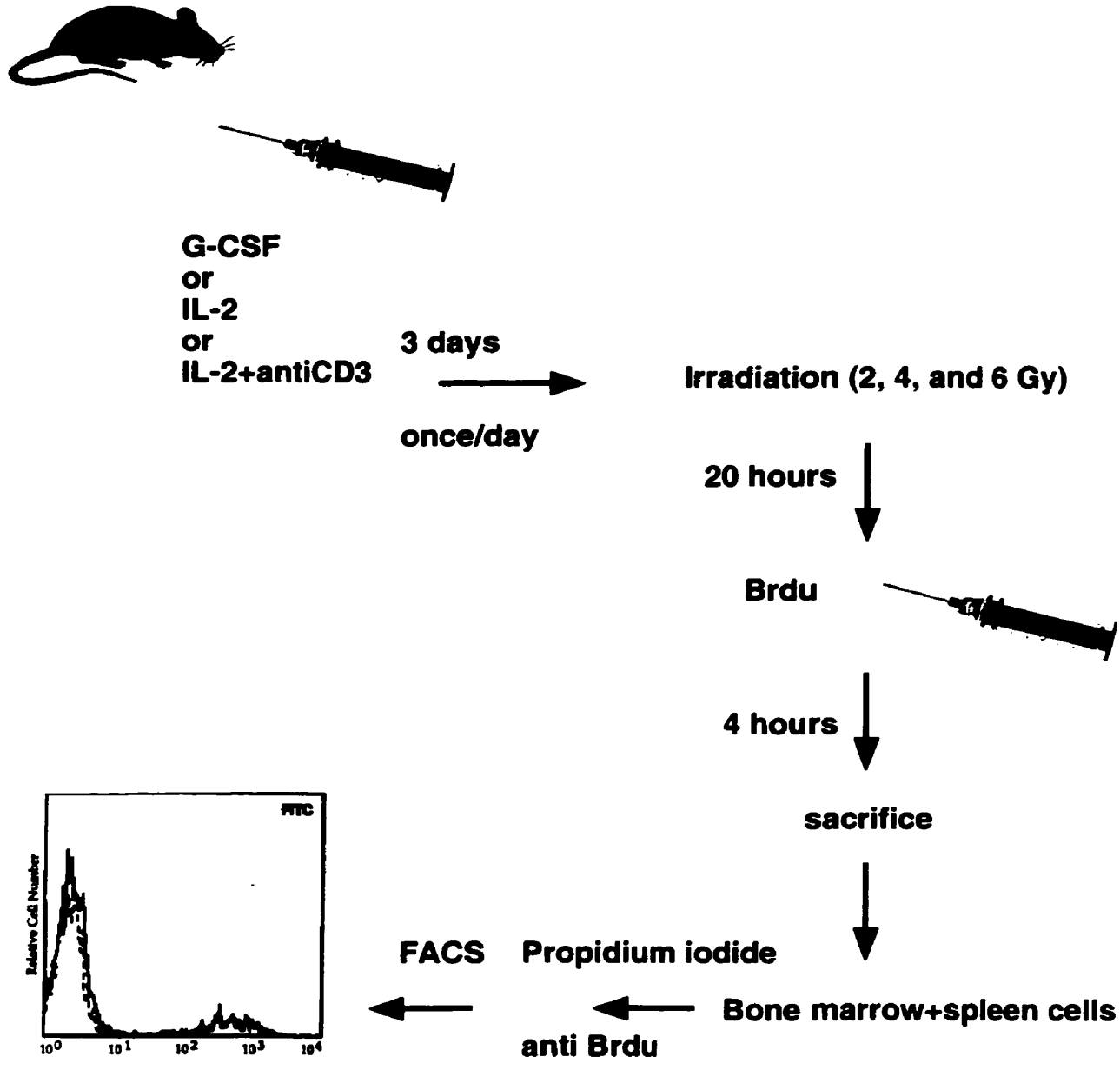


Figure 8 Summary of cell cycle regulation experiments.

PLP190Bcr-abl, $p53^{-/-}$, and normal mice were injected with either G-CSF, IL-2, or a combination of IL-2 and anti-CD3 antibody for three days. Half of the mice are irradiated at the third day and 20 hours later both groups are injected with BrdU for four hours. Mice are then sacrificed and bone marrow as well as spleen cells, are stained with propidium iodide and FITC anti-BrdU antibody. Cell cycle profiles are then determined utilizing flow cytometry.

proportion of cells in the S phase of the cell cycle can be quantitated using flow cytometric analysis. Replicating cells were labeled for four hours, subsequently mice were sacrificed by cervical dislocation. Bone marrow cells were flushed out of the femur with αMEM media (Gibco BRL, Ont.), while spleens were removed and crushed manually by passing through a sieve. Cells were washed twice with phosphate-buffered saline (PBS) (Gibco BRL, Ont.), fixed in ice cold 70% ethanol, and then incubated for 30 minutes at 4°C. Fixed cells were then denatured in 2N HCL with 0.5% (v/v) Triton X-100 (BioRad, Ont.) for 1 hour at room temperature, and subsequently neutralized in 0.1M sodium borate. Approximately 10^6 cells of each sample were permeabilized by re-suspending in 50µl of 0.5% (v/v) Tween-20 (BioRad, Ont.) and 1% (w/v) bovine serum albumin (BSA) (Sigma) in PBS. Twenty µl of anti-BrdU anti-body (Becton Dickinson) were added to each sample which were then incubated for 30 minutes in the dark at room temperature. Cells were then re-suspended in 1 ml PBS containing 5 µg of propidium iodide (PI) (Sigma, Ont.). PI is a fluorescent stain of nucleic acids. The cell-cycle profile of each sample was determined by dual-color flow cytometric analysis for DNA content (PI) and cells in S-phase of the cell cycle ($FITC^+$) using a FACScan (Becton Dickinson) flow cytometer. Data from flow cytometry was analyzed using Modfit and Winlist (Verity Software House, Mu, USA) software to determine the proportion of cells in different phases of the cell-cycle.

2.7 Quantitative Assay and Morphological Determination of Apoptosis

The level of apoptosis in irradiated (6 and 8 Gy) and control unirradiated mice was determined using the In Situ Cell Death Detection Kit, Fluorescein (Boehringer Mannheim,

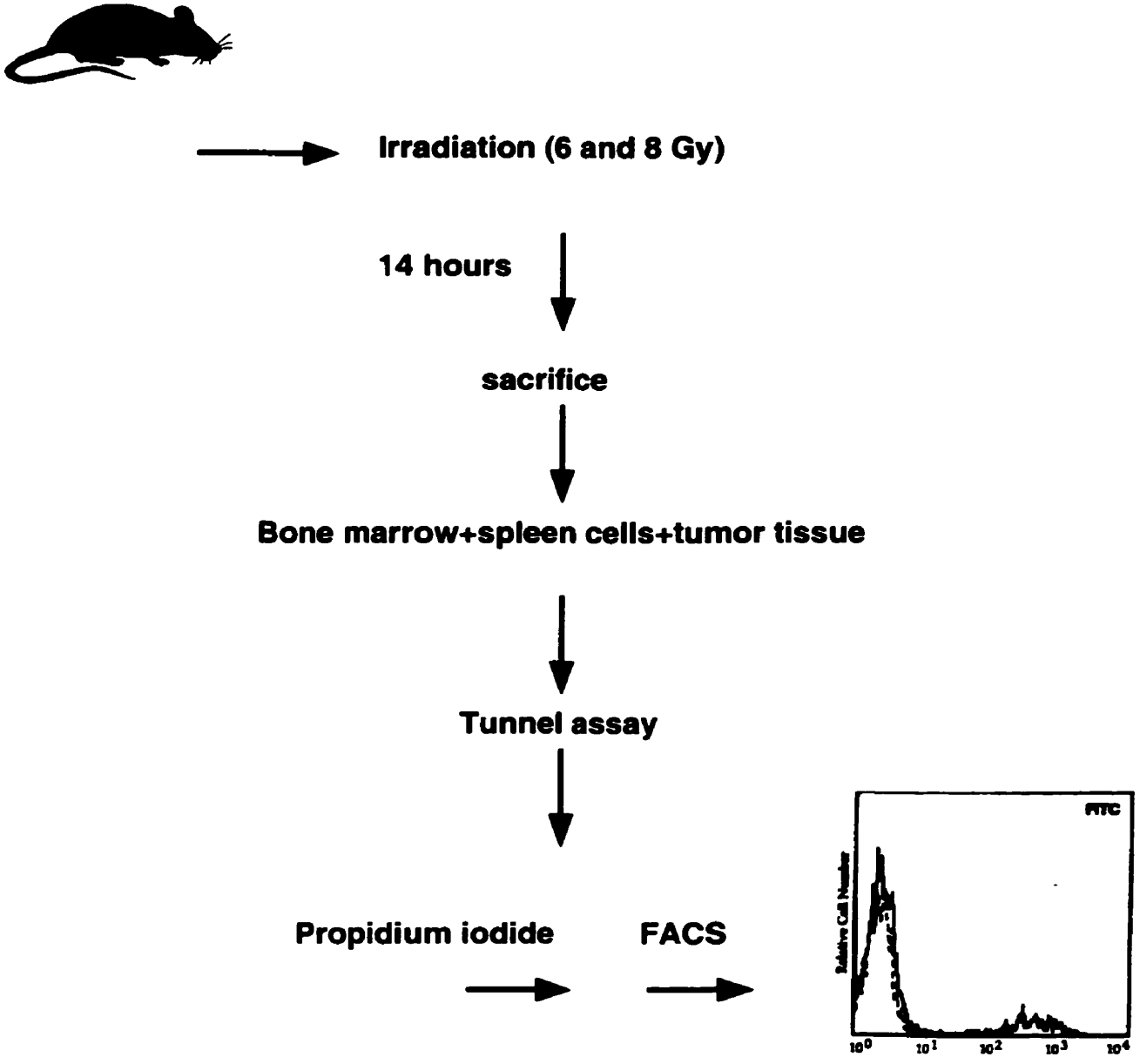


Figure 9 Summary of apoptosis experiments.

Pre-leukemic(PL) P190Bcr-abl, leukemic (L) P190Bcr-abl, and $p53^{-/-}$ mice were irradiated and 14 hours later sacrificed. Apoptosis in bone marrow and spleen cells was quantitated by the tunnel assay. The cells were then resuspended in propidium iodide and analyzed using flow cytometry.

Ont.) (Fig. 9). The hallmark of apoptotic cells is DNA degradation which can be double-stranded as well as single-stranded DNA breaks (nicks). Both types of breaks can be detected by labeling the free 3'-OH termini with modified nucleotides such as fluorescein-dUTP. This labeling reaction can be accomplished by using terminal deoxynucleotidyl transferase (TdT). TdT catalyzes the template independent polymerization of deoxyribonucleotides to the 3' end of single- and double-stranded DNA. Fourteen hours post-irradiation, mice were sacrificed and bone marrow cells were flushed out of femur. Spleen and tumor tissue were surgically removed and homogenized manually by passing through a fine sieve. Cells were then washed in PBS and fixed in a 4% paraformaldehyde solution in PBS for 30 minutes at room temperature. Cells were washed again in PBS and then permeabilized by re-suspending in 0.1% Triton X-100 (BioRad, Ont.) in a 0.1% sodium citrate solution. DNA strand breaks resulting from apoptosis were then labeled in the fixed, permeabilized cell. This was achieved by washing cells with PBS and then re-suspending in a reaction mixture containing terminal deoxynucleotidyl transferase (TdT) and fluorescein dUTP. DNA strand breaks were labeled by incubating for 60 minutes at 37°C in a humidified atmosphere in the dark. Cells were then washed twice in PBS and re-suspended in 1ml of PBS containing 5µg of propidium iodide. Cells were then analyzed for apoptosis (fluorescein- dUTP) and cell cycle status (PI) using a FACScan (Becton Dickinson) and then analyzed with Modfit and Winlist computer software (Verity Software House, Mu, USA)

Typical apoptotic morphology was shown by mounting spleen and bone marrow cells from irradiated mice stained with 5µg/µl acridine orange (Sigma, Ont.) in PBS on microscopic slides and observing under a fluorescent microscope.

2.8 DNA Extraction and Mutagenesis Assay

Genomic DNA was extracted from spleen (n=4) and kidney (n=3) of PL P190Bcr-abl/Big Blue and normal control as well as from four tumor tissue dissected from two leukemic P190Bcr-abl/Big Blue mice. DNA was extracted using RecoverEase™ DNA isolation kit (Stratagene, La Jolla, California), following the manufacturer's protocol. Fifty mg of tissue were homogenized and nuclei were pelleted by centrifugation at 1100*g* for 15 minutes, and subsequently digested with proteinase K for 45 minutes at 50°C. DNA from digested nuclei was hydrated and purified by dialyzing in TE (10mM Tris-HCL pH7.5 and 1mM EDTA) buffer for 16 hours.

The λ bacteriophage shuttle vector was recovered from the mouse DNA by *in vitro* packaging of viable phage particles. Eight μl of genomic DNA was added to Transpack™ packaging extract (Stratagene, La Jolla, California) which automatically excises the λ vector target and packages it into viable λ particles (Fig. 10). The titer of the packaged phage was estimated by infecting SCS-8 *E. coli* cells with 1μl of the packaged reaction. The estimated value was used to plate large agar plates (25cm x 25cm) at a density of 15,000-20,000 plaque forming units (pfu). Phage infected SCS-8 *E coli* cells were plated in the presence of 5-bromo-4-chloro-3-indol-β-galactopyranoside (X-Gal)(1.5mg/ml). Phage particles carrying a mutation in *lacI* coding sequences or promoter region of the shuttle vector will express αLacZ from the shuttle vector, which will combine with the remaining part of LacZ in SCS-8 cells to give a complete LacZ enzyme capable of cleaving X-gal to produce a blue color. A plaque reading enhancer (Stratagene, La Jolla, California) was used to aid in selecting mutant

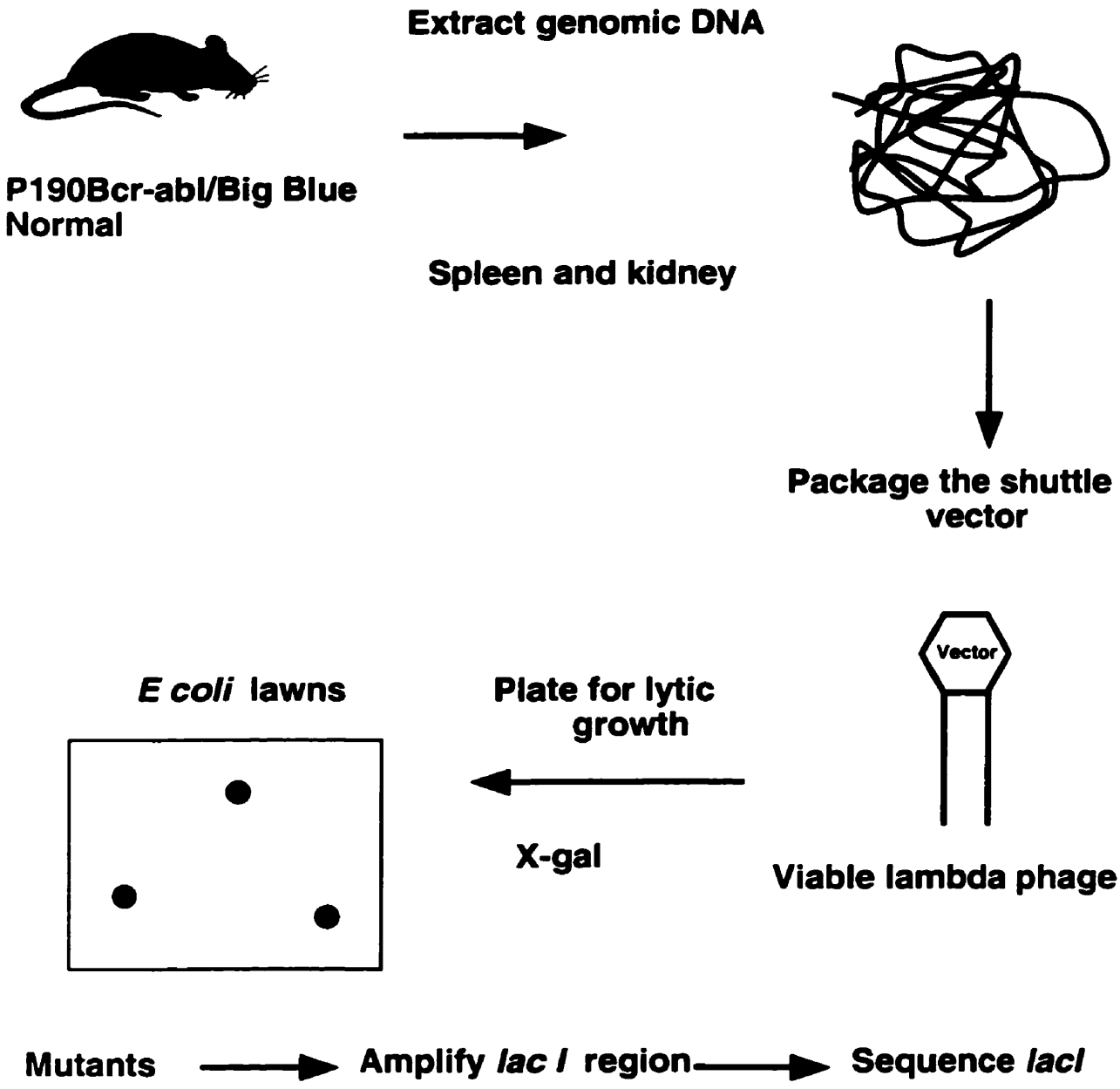


Figure 10 Summary of mutant frequency experiments.

Genomic DNA was extracted from P190Bcr-abl/Big Blue and normal mice. The Big Blue lambda-LIZ vector was rescued from the extracted DNA and packaged into viable phage particles which were then used to infect *E coli* bacterial lawns grown in the presence of X-gal. Phage particles with a mutation in *lacI* will result in a blue mutant plaque. The *lacI* region in mutants was subsequently amplified and sequenced.

blue plaques which were picked into 500µl of SM buffer (100mM NaCl, 8mM $MgSO_4.7H_2O$, 50mM Tris- HCL pH 7.5, and 0.01% w/v gelatin) and plated two more times at lower density to confirm their identity and obtain a pure plaque. Mutation frequencies were determined for each sample by calculating the ratio of blue mutant plaques to the total number of plaques counted.

2.9 DNA Sequence Analysis and Mutational Spectrum

Twenty two representative mutants from spleen and kidney PL P190Bcr-abl/Big Blue and from the two tumors with high mutation rates were grown and sequenced. Mutant bacteriophage were grown using the Wizard™ Lambda Preps DNA purification System (Promega, Madison-WI), following the manufacturer's protocol. One µl of the grown phage DNA was used as a template to amplify a 1338 bp region containing the *lacI* gene and the *lacZ* operator[237]. Two upstream and four downstream primers were used to circumvent failed amplifications due to mutations in the primer region.

PCR-F(Stratagene) 5'-3' GTA TTA CCG CCA TGC ATA CTA G

PCR-R(Stratagene) 3'-5' AAC AGC TAT GAC CAT GAT TAC G

-1280D[238] 5'-3'ACA CCA CGA TGC CTG TAG CAA

-226D[238] 5'-3' GTG ATG CTC GTC AGG

-55D[238] 5'-3' GTA CCC GAC ACC ATC GAA TG

1283U[238] 5'-3' GAG TCA CGA CGT TGT A

Enumerated primers correspond to base position on the *LacI* gene and are either upstream (U) or downstream (D). PCR reactions were performed using the following conditions: 2.5mM $MgCl_2$, 0.2 mM dNTPs, 0.4μM of each primer, and 0.02 Units/μl of *Taq* DNA polymerase (Gibco BRL) in a total of 60μl of reaction volume. The reaction mixture was subjected for initial denaturation at 95°C for 5 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 52°C for 90 seconds, and elongation at 72°C for 2 minutes followed by a final extension at 72°C for 10 minutes (DNA Thermo-cycler, Perkin Elmer). PCR products were purified using the QIAquik PCR Purification Kit (Qiagen). The entire *lacI* gene and the *lacZ* operator were sequenced by cycle sequencing using ABI automated sequencer (373A Applied Biosystems, Perkin Elmer). Five primers reading in the 5'-3' direction (1A, 3, 4, 6, and 7) (Fig. 11) (Big Blue guide p.26, Stratagene) were initially used with Dye Terminator Cycle Sequencing kit (Perkin Elmer). Mutations were then confirmed by sequencing in the opposite direction with one of the 3'-5' primers (14, 13A, 11A, and 9A) (Big Blue guide p.26, Stratagene) (Fig. 11). Readable chromatograms were typically 300-350 bps. in length per each primer run. Different chromatograms of the same mutant were combined, aligned, and compared to wild type *lacI* sequences [239] using the MacVector sequence analysis software. The mutational spectrum in PL P190Bcr-abl/Big Blue and in tumors from leukemic P190Bcr-abl/Big Blue mice was grouped into four categories: transitions at CpG, transitions not at CpG, transversions at CpG, and transversions not at CpG. The obtained mutation profile was compared to a list of reported mutations in the Big Blue *lacI* gene compiled in *lacI* database and software (MutaBase Software, Neal Cariello) to determine mutations specifically induced by Bcr-abl.

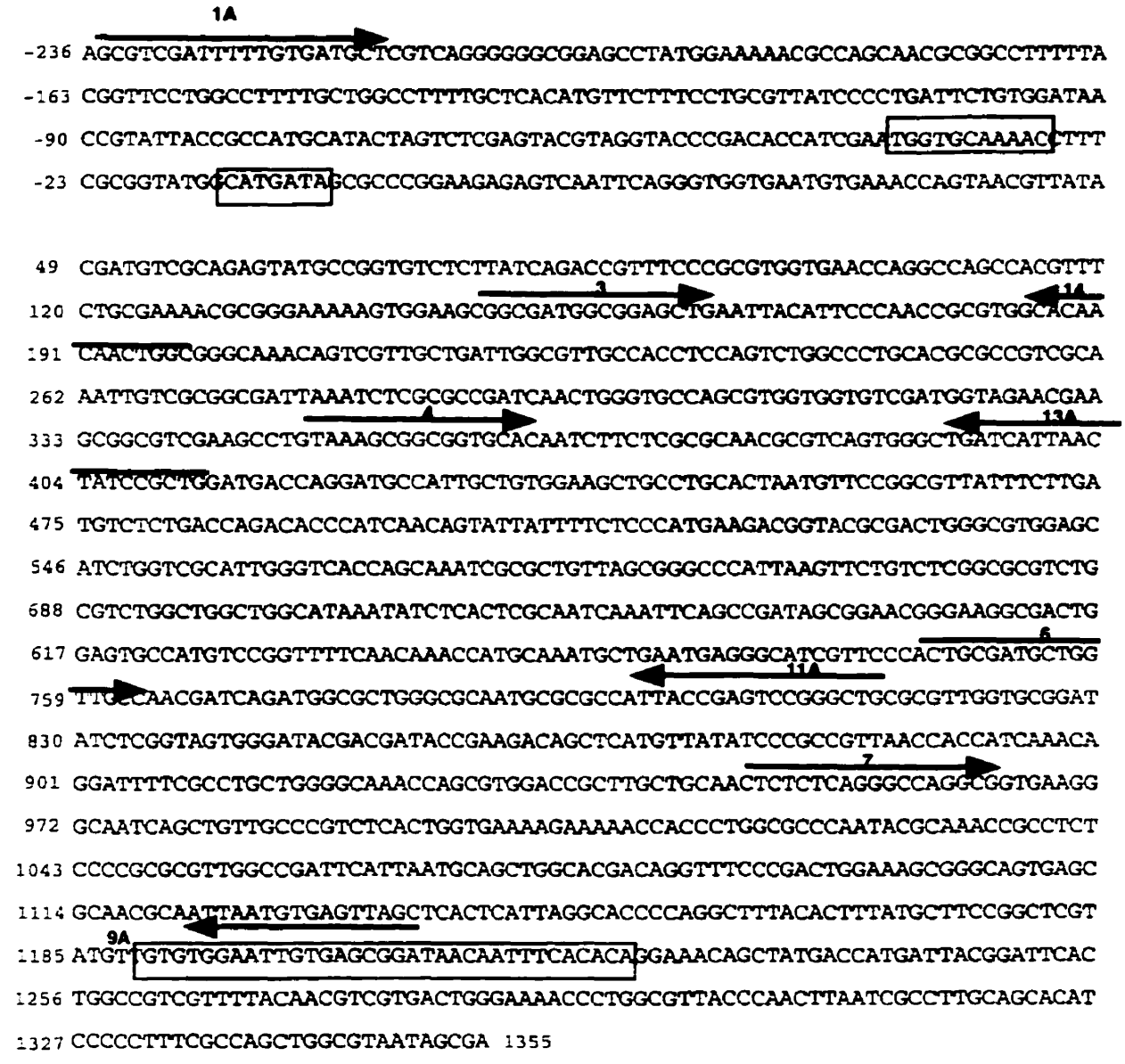


Figure 11 Primers utilized to investigate the presence of mutations in *lacI* from blue mutant plaques.

Five primers reading in the 5' to 3' direction (1A, 3, 4, 6, and 7) were used to sequence the entire *lacI* gene including the two promoter regions (boxed upstream) and the *lacZ* operator region (boxed downstream). Mutations were confirmed by sequencing in the 3' to 5' direction using one of four primers (14, 13A, 11A, and9A). Primer positions are indicated by arrows above the coding seqeunces.

2.10 Statistical Analysis

In cell cycle and apoptosis experiments, changes in the fraction of cells in different cell cycle stages or undergoing apoptosis as a result of irradiation were compared to non-irradiated samples by determining *P*-values using the student t-test (Sigmastat).

Mutant frequencies in spleens and kidneys of PL P190Bcr-abl mice were compared to those in normal mice by determining *P*-values also calculated using student t-test (Sigmastat). The mutation spectrum observed in P190Bcr-abl/Big Blue mice was compared to the *lacI* mutations in the Big Blue system compiled in *lacI* database and software (MutaBase Software, Neal Cariello) using a statistical program[298] especially designed for such tasks and provided in the same software package. This program is an extension of Fisher's exact hypergeometric test in its computer intensive Monte Carlo implementation.

III- Results

3.1 Mice Genotyping

3.1.1 P190Bcr-abl and P190Bcr-abl/Big Blue Mice

The protocol used to identify transgenic P190Bcr-abl mice was described previously[124].Tail DNA were cut with XhoI/HindIII and the digested DNA was ran on a 1% agarose gel and southern blotted. The probe used in southern analysis is a fragment from a BglII/XhoI digest of the human *bcr* gene exon 1 which can recognize *bcr* sequences in the P190Bcr-abl translocation used as the transgene[124] (Fig. 7). Three bands were obtained (Fig. 12A and B); the top and bottom bands are present in the digested DNA from both transgenic and normal mice. Those two bands correspond to mouse *bcr* sequences which cross hybridize with the human *bcr* probe. The diagnostic band corresponds to human *bcr* exon 1 sequences fused to human *abl* exon 2 sequences both part of the P190Bcr-abl trangene used to construct the P190Bcr-abl transgenic mice and are not found in normal mice[124] (Fig. 7). For P190Bcr-abl/Big Blue mice, only screening for the presence for the P190Bcr-abl transgene was undertaken as such mice were always the result of homozygote Big Blue™ (obtained from Stratagene, Ca.) and P190Bcr-abl mice.

3.1.2 $p53^{-/-}$ Mice

$p53^{-/-}$ mice were purchased from Taconics Farms Inc.™ and maintained under a licensed agreement. To screen for $p53^{-/-}$ homozygotes, a protocol obtained from Taconics was followed. Tail DNA was digested with BamHI, ran on a 1% agarose gel, and southern

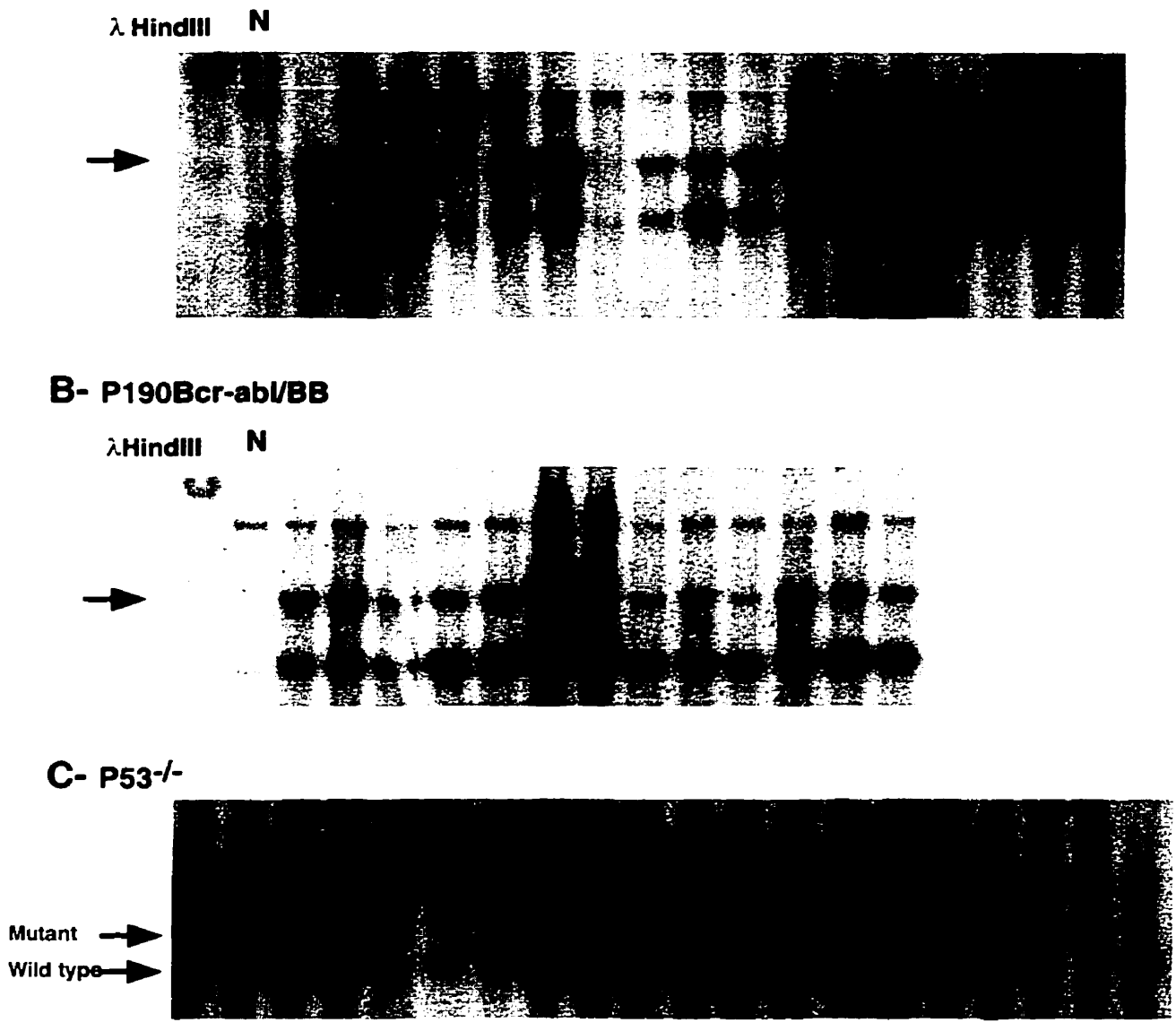


Figure 12 Mice genotyping.

Southern blot analysis was performed on mice tail DNA to determine their identity. The presence of the P190Bcr-abl transgene in P190Bcr-abl (A) and P190Bcr-abl/Big Blue(BB) mice (B) was determined using a 600 bp XhoI/BglII fragment of the *bcr* sequences in the transgene following an XhoI/HindIII digest of tail DNA(arrows point to the diagnostic fragment). $p53^{-/-}$ mice were genotyped using a 605 bp KpnI probe that can detect inactivating insertions in exon 5 of the p53 allele. BamHI digests of $p53^{-/-}$ tail DNA yield a homozyote null phenotype ($p53^{-/-}$) when only the disrupted (larger) allele is observed (first sample in C). All patterns confered to the expected pattern established by the founders of these mice. A DNA sample from normal (N) mice is used in (A) and (B).

blotted. BamHI digestion of tail DNA releases a fragment which contains exons 2-6 of the p53 allele (Fig. 7). This fragment can be detected by a probe prepared using a 605bp exon 5 fragment of a KpnI digest of an LR10 plasmid also obtained from Taconics. This probe can detect two forms of the BamHI 2-6 exon fragment of the p53 allele (Fig. 7)(Fig. 12C). A 5 Kb fragment corresponding to the wild type allele and a larger 6.5 Kb corresponding to the mutated form of the allele[235]. The larger form is due to the insertion of a PolII-neo expression cassette in exon 5[235]. Homozygote $p53^{-/-}$ mice correspond to those with only the mutant copy of the allele present. A larger band present in all types of mice corresponds to a pseudogene.

3.2 Confirming Pre-Leukemic Status of P190Bcr-abl Mice

P190Bcr-abl mice were confirmed to be disease free by analyzing bone marrow and peripheral blood for the presence of leukemic cells. Bone marrow cells were obtained by crushing a piece of the mouse femur between two glass slides. Peripheral blood was mounted directly on a glass slide. Both preparations were stained with giesma stain and analyzed under a light microscope. In leukemic mice, bone marrow cells consist of round immature blast cells. In contrast, bone marrow cells from pre-leukemic mice show normal morphology with the characteristic appearance of neutrophils at different stages of maturity (Fig. 13A). In the peripheral blood from leukemic mice, immature blast cells that have traversed the bone marrow-blood barrier are observed (Fig. 13B). The same procedure was routinely applied to P190Bcr-abl/Big Blue mice to confirm that they are disease free.

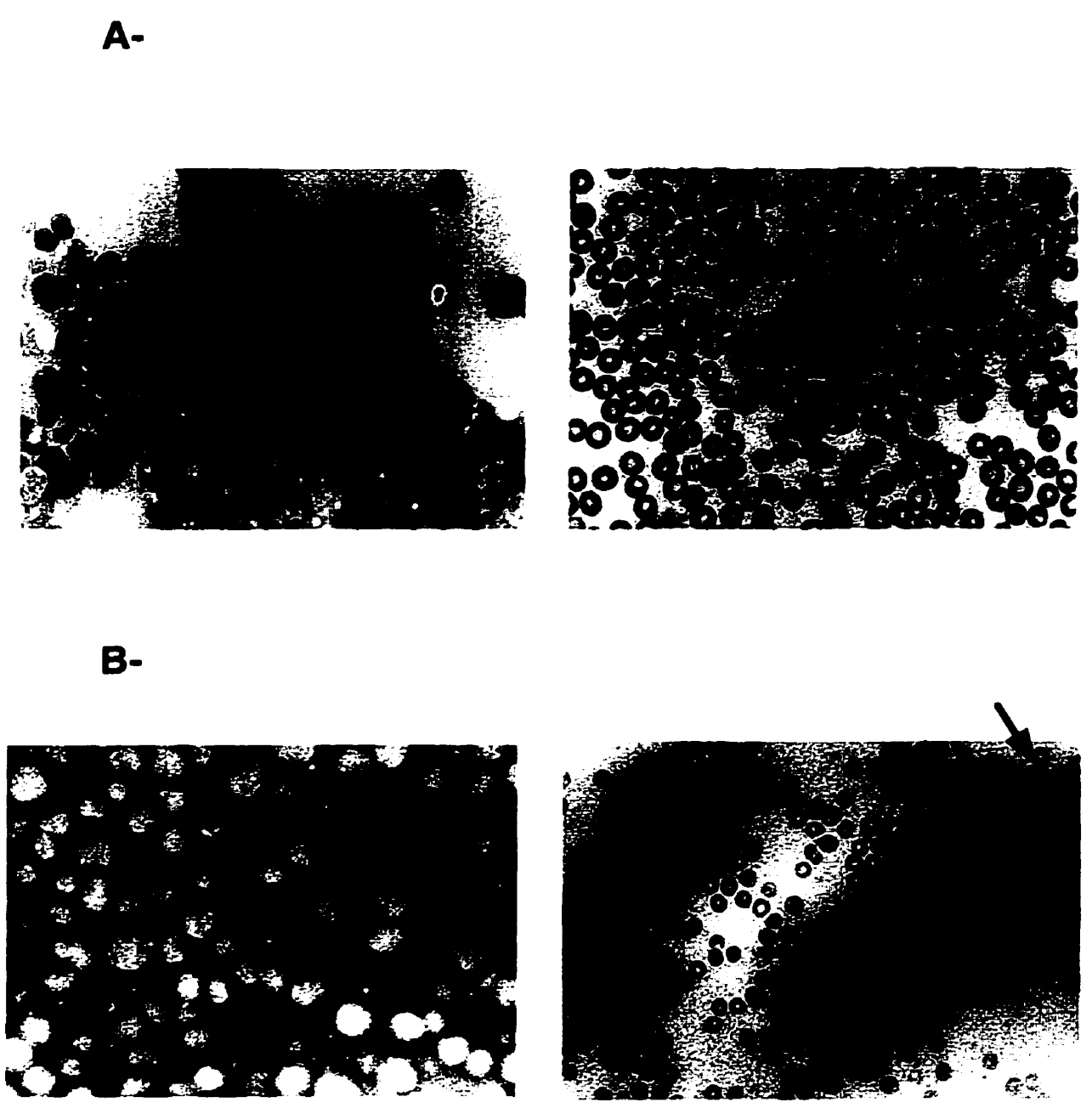


Figure 13 Morphological analysis of bone marrow and peripheral blood smears of P190Bcr-abl mice.

Mice were confirmed to be in the pre-leukemic (PL) stage by studying bone marrow (left panel) and peripheral blood smears (right panel). Bone marrow smears of PL P190Bcr-abl (A) show normal morphology with the apparent predominance of neutrophils at different stages of maturation. In leukemic mice (B), the bone marrow becomes packed with immature blast cells which can also be detected in circulation (arrows).

3.3 Cell-cycle Arrest in Cells from P190Bcr-abl Mice

The principal role of the cell cycle is to ensure the fidelity of DNA replication and chromosomal segregation. In the event of in-born error or exogenous genotoxic stress, cellular machineries have evolved that can impede the progression of the cell cycle and by that circumvent the amplification of such error. Contradictory results have so far been reported addressing the effect of the expression of Bcr-abl on cell cycle checkpoints following the induction of DNA damage in cell lines.

3.3.1 Cell-cycle Regulation in Bone Marrow Cells from G-CSF Treated Mice

First I decided to test whether the expression of the Bcr-abl constitutive tyrosine kinase in mouse tissue interferes with the ability of cells to block cell cycle progression following the induction of DNA damage by γ-irradiation. Normal mice utilized in those and subsequent experiments have the same genetic back-ground (F_1 C57BL 6 x CBA) as P190Bcr-abl mice. Normal, pre-leukemic (PL) P190Bcr-abl, and $p53^{-/-}$ knock- out mice were injected with granulocyte colony stimulating factor (G-CSF) for three days. This resulted in the increase of the percentage of proliferating cells in the bone marrow of different animals from 4-5% in non stimulated mice to 18-25% in stimulated mice. Differential analysis of peripheral cell blood counts (CBC) performed on individual mice indicated an increase in monocytes and neutrophils. This allowed me to monitor the decline of cells in the S-phase of the cell cycle following the induction of DNA damage and the activation of the G_1 and G_2 check points. Cells in the S-phase of the cell cycle were labeled *in vivo* by injecting mice with bromodeoxyuridine (BrdU) for four hours prior to their sacrifice. Utilizing an irradiation dose of 2 Gy and studying cell cycle profiles 24 hours post-irradiation, I found

an arrest in cell cycle progression in bone marrow cells from both P190Bcr-abl and normal irradiated mice. Cell cycle profiles were determined by labeling bone marrow cells with fluorescein anti-BrdU antibodies and propidium iodide (PI). Cells were then analyzed by dual flow cytometry for anti-BrdU (y-axis) and PI (x-axis) (Fig. 14). Beneath each dual flow cytometric analysis, the cell cycle distribution of the same sample is represented by PI staining alone. The arrest in cell cycle was reflected in a decrease in the amount of cells in the S-phase of the cell cycle (Fig. 14). Bone marrow cells from irradiated normal mice (n=4) demonstrated a 35% decrease in cells in the S-phase of the cell cycle compared to bone marrow cells from unirradiated mice (n=4) (Fig. 15). This difference was statistically significant (P=0.011). This was paralleled by nearly an identical decrease in bone marrow cells from irradiated pre-leukemic P190Bcr-abl (35.1%) (n=6) compared to non-irradiated mice (n=5) (P<0.001). Bone marrow cells from irradiated $p53^{-/-}$ mice (n=4) showed only a 15.5% decrease in cells in the S-phase of the cell cycle compared to non-irradiated mice (n=4) (P=0.07). The arrest was mainly at the G_1 phase of the cell cycle as the decrease in cells in S phase was accompanied by an increase of cells accumulating at G_1 (Fig. 15).

The G_2/M cell cycle check-point also responded normally in cells treated with G-CSF but irradiated at 6 Gy and analyzed at an earlier time point (12 hours) (Fig. 16). Irradiation resulted in a decrease in the fraction of bone marrow cells in S-phase from both P190Bcr-abl mice (n=4, P<0.001) and normal controls (n=4, P=0.002) (Fig. 17). This was accompanied by an increase in the fraction of cells at G_2/M in bone marrow cells from P190Bcr-abl mice (P<0.001) as well as normal mice (P=0.029) (Fig. 17).

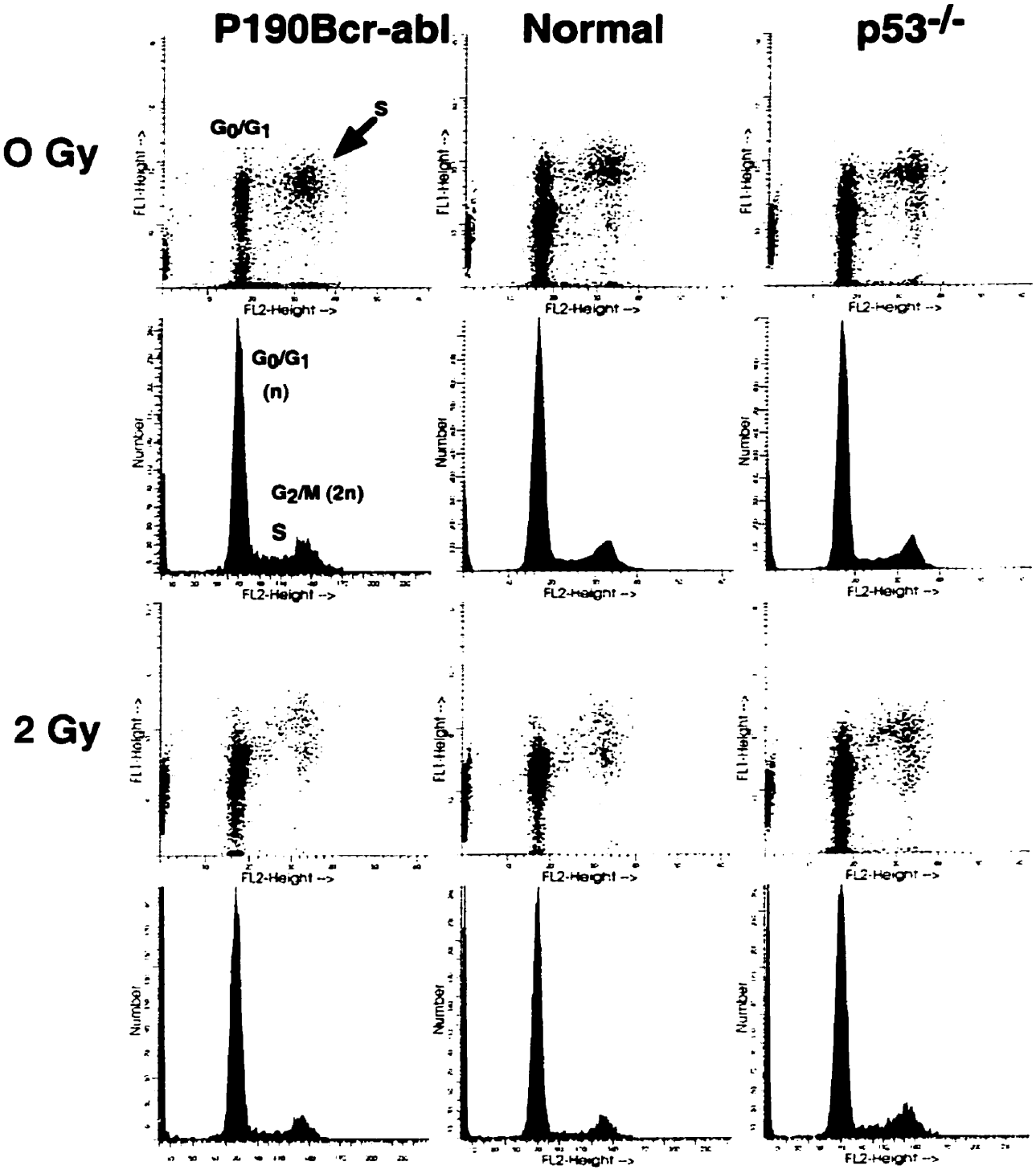


Figure 14 Flow cytometric analysis of cell cycle response in bone marrow cells from mice treated with G-CSF.

Mice were stimulated with G-CSF for three days after which they were irradiated at 2 Gy. Twenty hours later mice were injected with BrdU to label replicating cells. Four hours later, mice were sacrificed and bone marrow cells were removed from femurs and labelled with FITC-conjugated αBrdU anti-body (y-axis) and propidium iodide (PI) (x-axis). Cells were then analyzed using flow cytometry. Cells in S-phase are labelled with a-BrdU and score high on the y-axis. The x-axis (PI) labels total DNA and therefore facilitates the segregation of cells that underwent replication (2n) and those which have not (n).

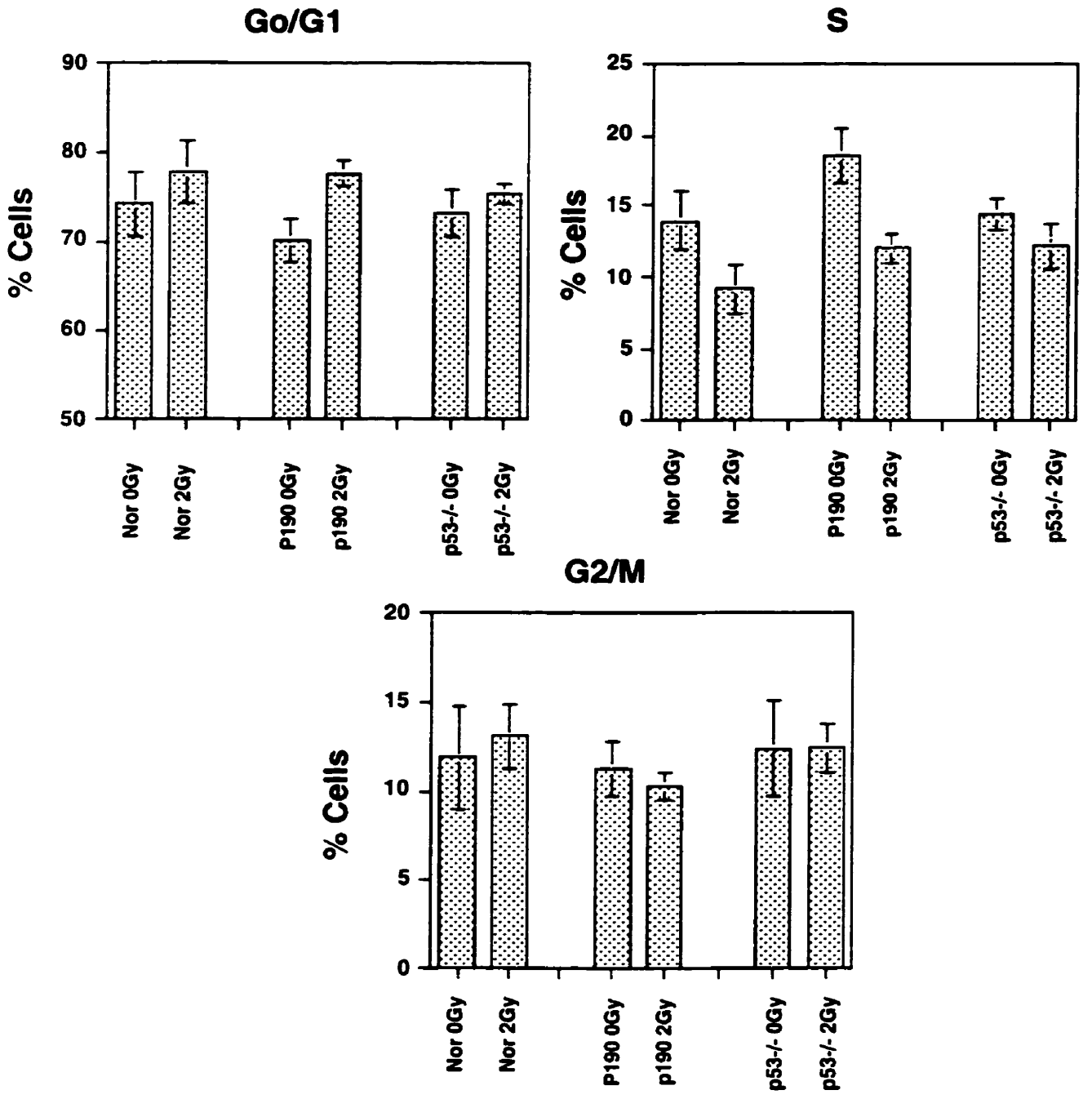


Figure 15 Cell cycle regulation in bone marrow cells following irradiation (2 Gy) in G-CSF treated mice.

Normal, PL P190Bcr-abl (P190), and $p53^{-/-}$ mice were treated with G-CSF for three days after which half of the mice were irradiated (2 Gy). Twenty hours later, mice were injected with BrdU for four hours and subsequently sacrificed and the cell cycle profile of bone marrow cells was determined by flow cytometry. Each data point represents four mice (normal, $p53^{-/-}$), five mice (irradiated P190), or six mice (non-irradiated P190).

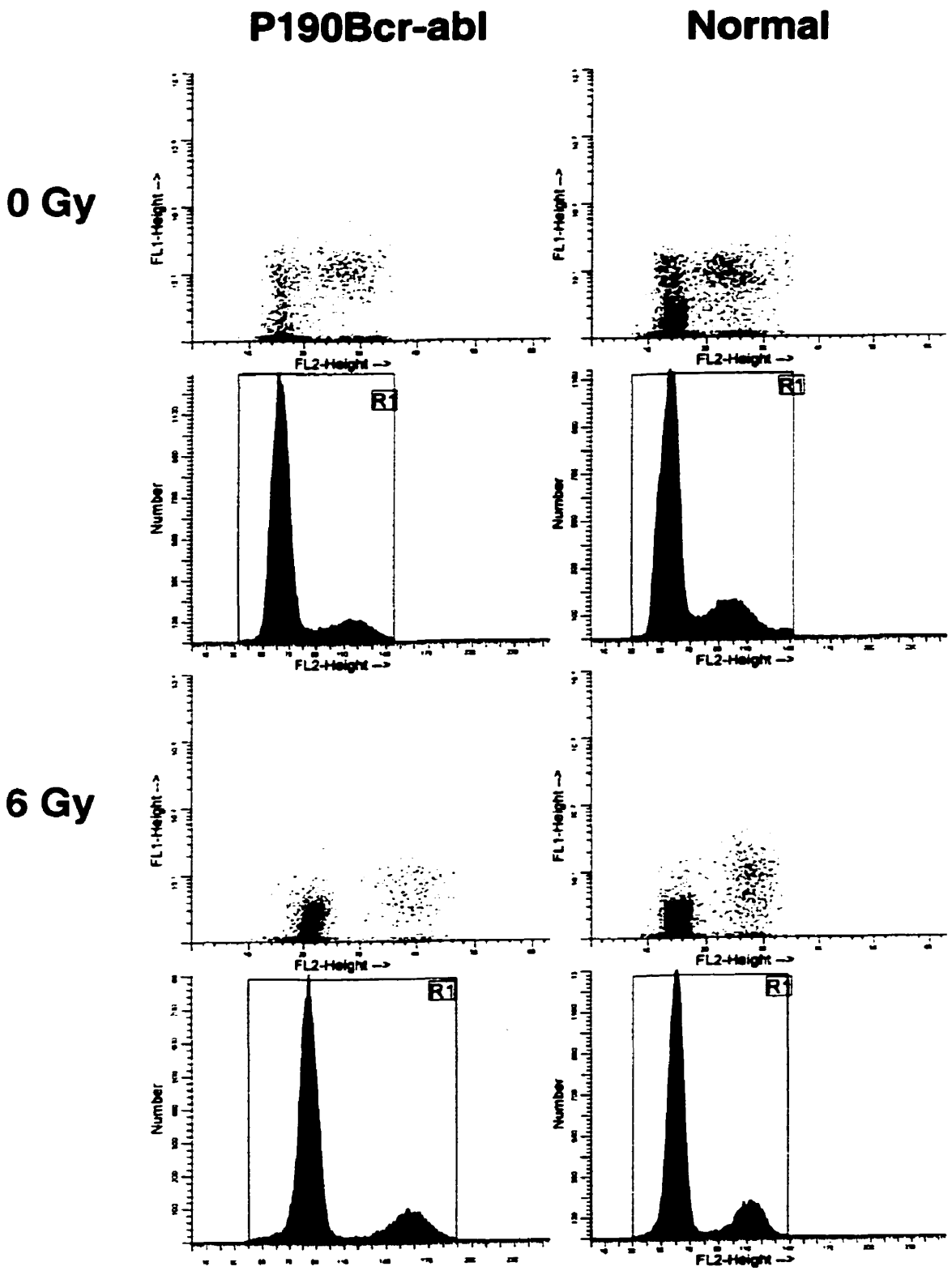


Figure 16 Flow cytometric analysis of bone marrow cells following high dose irradiation in G-CSF treated mice.

P190Bcr-abl mice and normal mice were treated with G-CSF for three days afterwhich they were irradiated at a dose of 6 Gy. Eight hours later mice were injected with BrdU for four hours, afterwhich they were sacrificed. Flow cytometry analysis revealed a normal G_2/M check-point response.

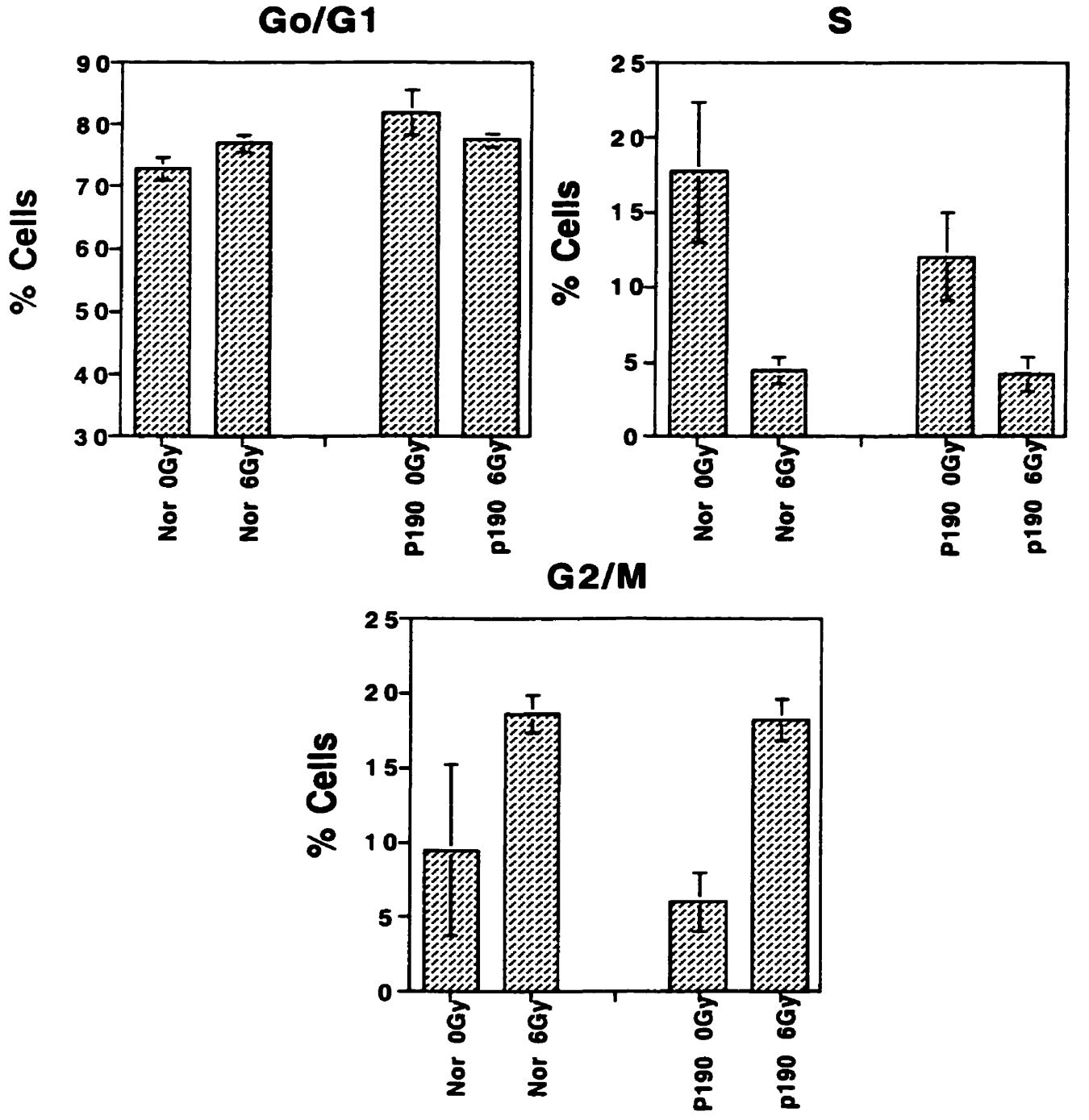


Figure 17 Cell cycle regulation in bone marrow cells following a high dose of irradiation (6Gy) in G-CSF treated mice.

Normal, and PL P190Bcr-abl (P190Bcr-abl) mice were treated with G-CSF for three days after which half of the mice were irrradiated at a high dose of irradiation (6 Gy). Eight hours later, mice were injected with BrdU for four hours after which mice were sacrificed and cell cycle profiles of bone marrow cells was determined by flow cytometry. Each data point represents the average of four different mice.

3.3.2 Cell-cycle Regulation in Bone Marrow Cells from IL-2 Treated Mice

G-CSF mainly stimulates the proliferation of granulocytes of the myeloid lineage. Whereas the expression of Bcr-abl messages was detected in most tissues [232], P190Bcr-abl mice usually succumb to lymphomas[124; 232]. This lead me to investigate whether the expression of Bcr-abl in lymphoid cell progenitors affects cell cycle regulation following the induction of DNA damage in a manner different than that observed in myeloid cell lineage in the bone marrow .

Mice were injected with IL2 for three days to expand both the T- and B- lymphocytes cell pools. CBC differential analysis indicated the increase in lymphocytes in peripheral blood samples. IL2-treated normal (n=4), pre-leukemic P190Bcr-abl (n=4), and $P53^{-/-}$ (n=3) mice were irradiated at a dose of 6 Gy. Twenty- four hours post-irradiation, the cell cycle profiles of bone marrow cells from both irradiated and non-irradiated mice (P190Bcr-abl and normal mice n=4, $p53^{-/-}$ n=4) were determined. In normal mice, a 48 % decrease in cells in the S-phase of the cell cycle was observed following irradiation, compared to non irradiated mice ($P<0.001$). Also, a 61% decrease in bone marrow cells in the S-phase of cell cycle was observed in similarly treated P190Bcr-abl mice ($P<0.001$), while only an 18.8% decrease in the same cells from $p53^{-/-}$ mice was detected (Fig. 18 and 19). Cells from normal and P190Bcr-abl mice demonstrated a significant ($P<0.001$) increase in cells arrested at G_1 after irradiation. A significant difference, however, in the kinetics of G_2/M arrest was observed. In irradiated P190Bcr-abl mice a 30% increase ($P=0.012$) in the cell population in the G_2/M phase of the cell cycle was observed compared to only 12% in irradiated normal mice

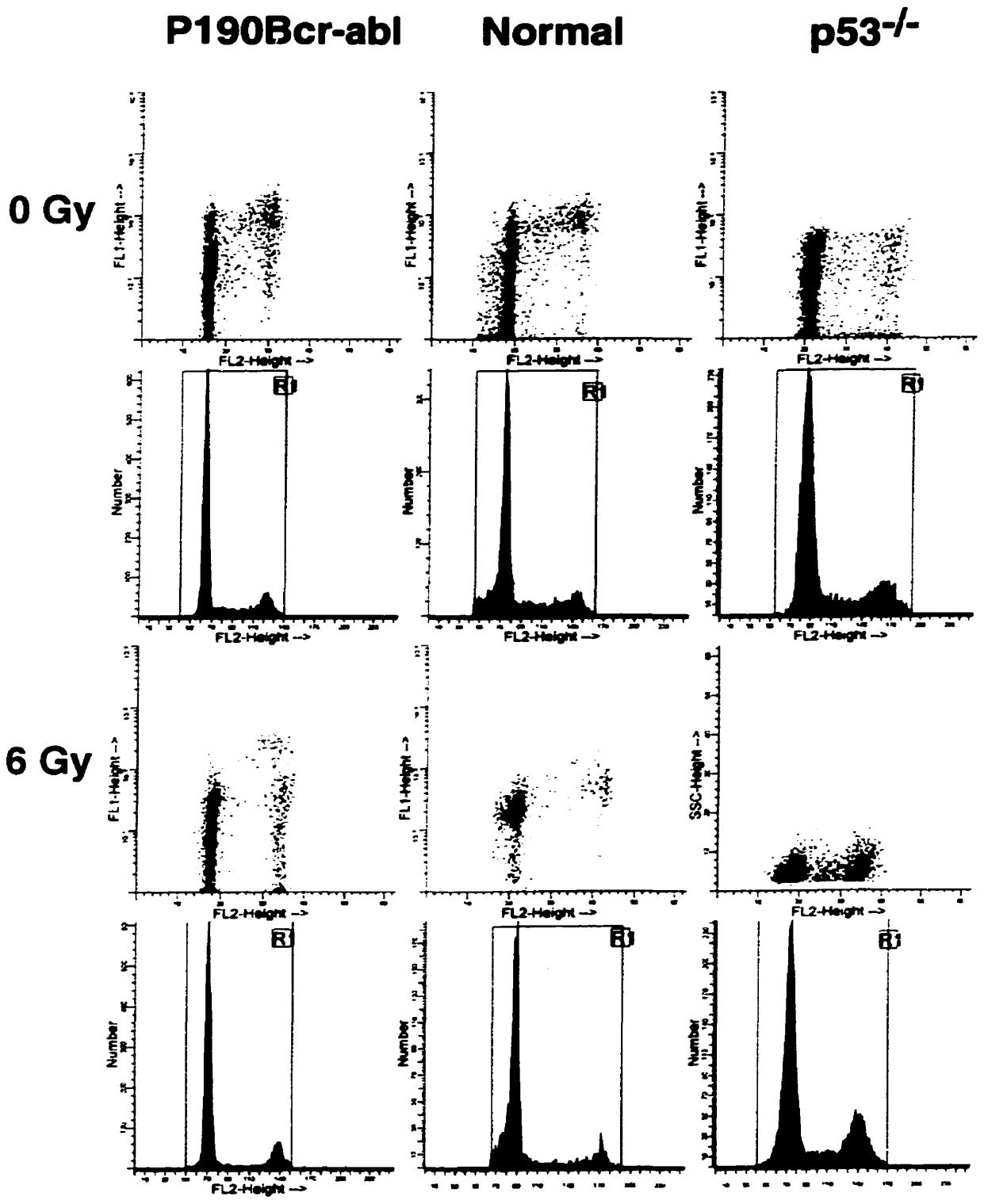


Figure 18 Flow cytometric analysis of bone marrow cells after irradiation in mice stimulated with IL-2.

Mice were treated with IL-2 for three days after which they were irradiated (6 Gy). Twenty hours later replicating cells were labelled by injecting mice with BrdU for four hours. Flow cytometric analysis of bone marrow cells labelled with FITC-conjugated αBrdU and propidium iodide revealed a normal cell cycle response in this fraction of stimulated bone marrow cells. The analysis of the cell cycle profiles of irradiated $p53^{-/-}$ mice relied on DNA content values (PI labelling).

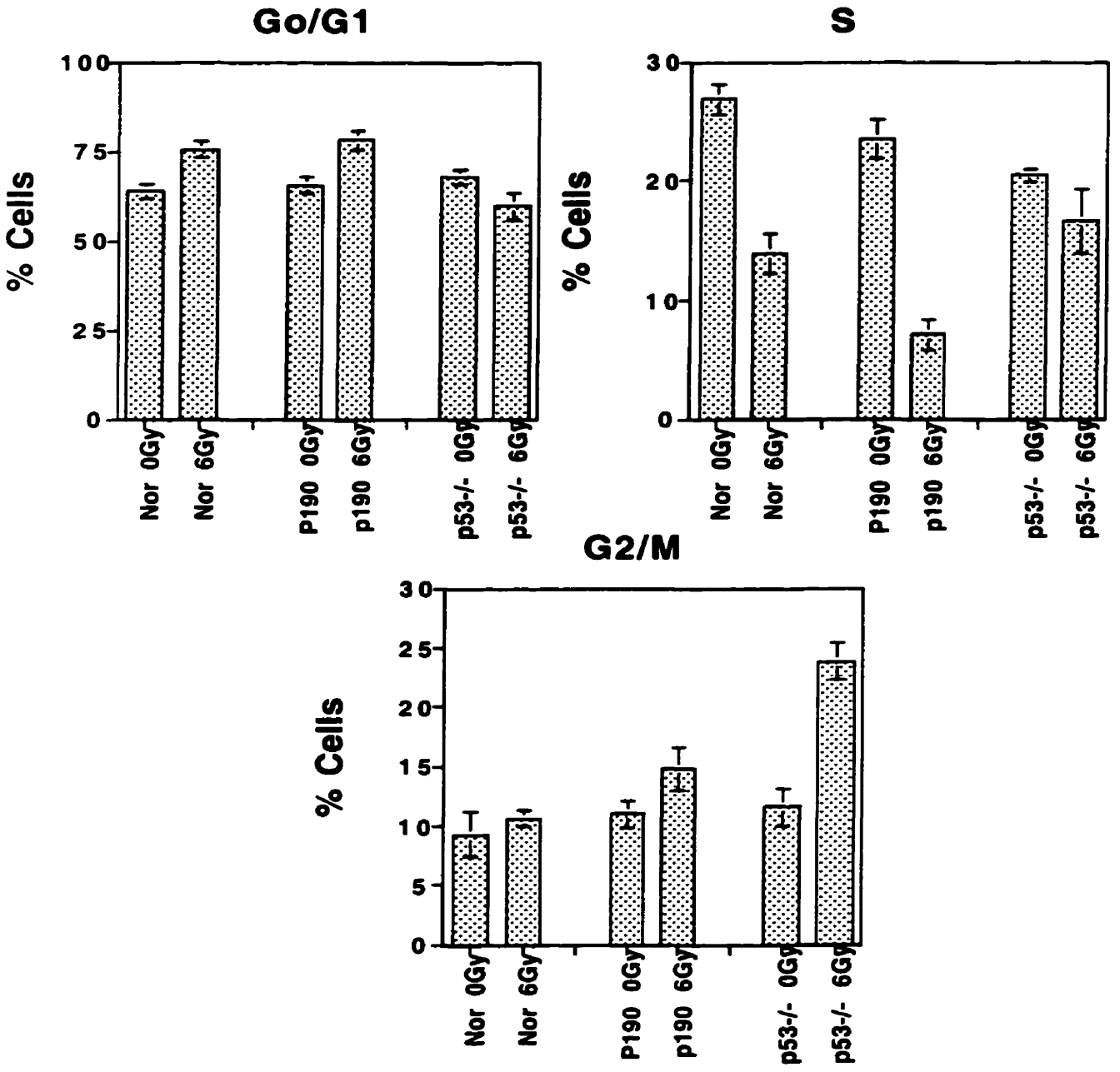


Figure 19 Cell cycle regulation in bone marrow cells following irradiation of IL2 treated mice.

Normal, PL P190Bcr-abl (P190), and $p53^{-/-}$ mice were treated with IL-2 for three days after which half of the mice were irradiated (6 Gy). Twenty hours later, mice were injected with BrdU for four hours after which they were sacrificed and the cell cycle profiles of bone marrow cells determined by flow cytometry. Each data point represents three different mice.

(P=0.222), while in $p53^{-/-}$ mice a two fold increase in cells arrested at G_2/M was noted (Fig. 18 and 19). These results demonstrate that normal cell cycle responses to irradiation exist in lymphocytes from the bone marrow of P190Bcr-abl mice.

3.3.3 Cell-cycle Regulation in Spleen Cells from IL-2 and αCD3 Treated Mice

I was interested in finding out whether lymphocyte in other hematopoietic tissue from P190Bcr-abl mice demonstrate a normal cell cycle arrest following irradiation. Lymphocytes in spleens of normal, P190Bcr-abl, and $p53^{-/-}$ mice were stimulated by administering αCD3 antibody and IL-2 for three days. Following irradiation (6 Gy), I noticed a decrease in the S-phase population of spleen cells from irradiated normal (66%) (P=0.009) and pre-leukemic P190Bcr-abl (61%) (P=0.01) mice (n=3)(Fig. 20 and 21) compared to matched non-irradiated mice (n=3). This accompanied by a cell cycle arrest at the G_0/G_1 phase in both normal (P=0.003) and P190Bcr-abl (P=0.006) mice. In $p53^{-/-}$ (n=3) mice, no statistically significant change in S phase was observed and although a slight increase in cells arresting at the G_2/M phase of the cell cycle was detected it was of no statistical significance (P=0.591) (Fig. 20 and 21).

3.4 $p21^{WAF1/CIP1}$ Induction

Cells exposed to genotoxic stress block cell cycle progression at the G_1/S stage of the cell-cycle, avoiding by that the replication of damaged DNA, and allowing time for repair. The failure of such mechanism results in the amplification of genetic insults and gives rise to genomically unstable clones. $P21^{WAF-1/CIP1}$ plays an important role in regulating the G_1/S

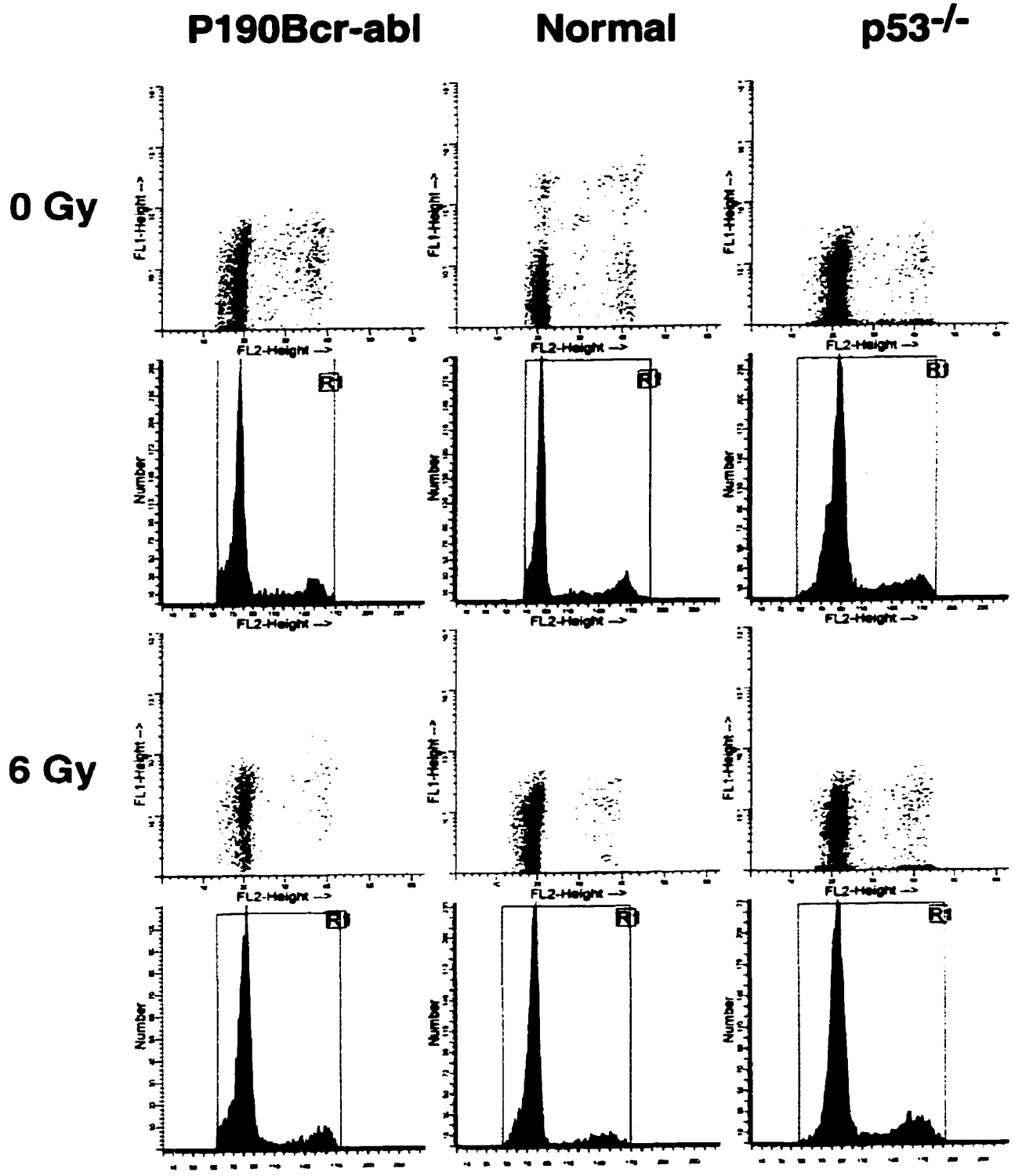


Figure 20 Flow cytometric analysis of spleen cells from IL-2 and αCD3 treated mice.

Mice were treated with a combination of IL-2 and aCD3 for three days to induce the proliferation of spleen cells. Half of the mice were then irradiated and twenty hours later both groups were treated with BrdU for four hours, after which mice were sacrificed. Flow cytometric analysis of spleen cells labelled with αBrdU (y-axis) and propidium iodide (x-axis) revealed a normal function of cell cycle check-points in normal and pre-leukemic (PL) P190Bcr-abl mice.

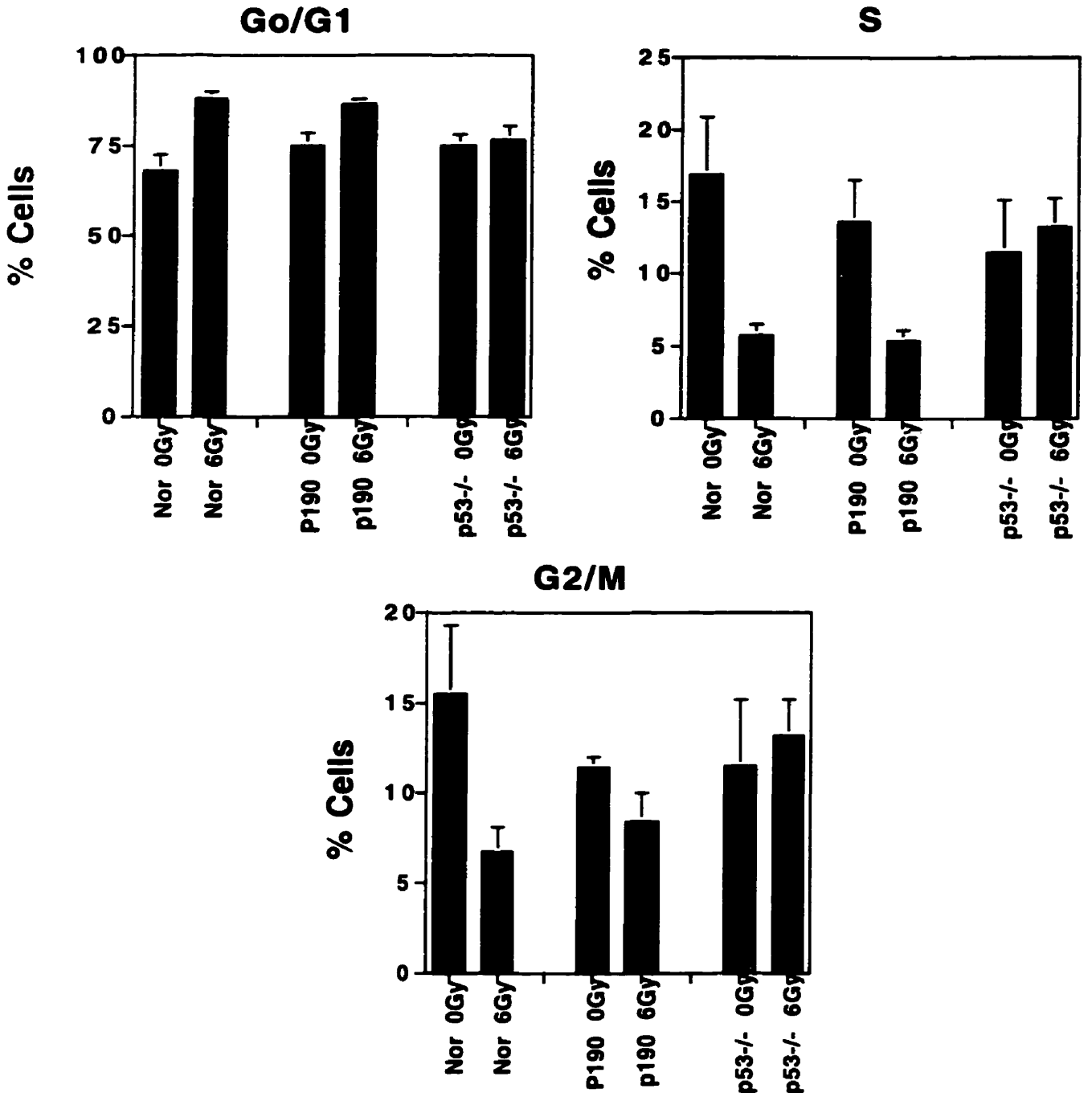


Figure 21 Cell cycle regulation in spleen cells following irradiation of IL-2 and αCD3 treated mice.

Normal, PL P190Bcr-abl (P190), and $p53^{-/-}$ mice were treated with a combination of IL-2 and anti-CD3 antibody for three days to stimulate spleen cells. Subsequently, mice were irradiated at 6 Gy and twenty hours later treated with Brdu for four hours. Cell cycle profiles were then determined by flow cytometry. Each data point is an average of three mice.

checkpoint. Following DNA damage WAF-1 is transcriptionally activated by p53 and directly inactivates multiple cyclin-cyclin-dependent kinase complexes necessary for progression through G_1/S. I investigated whether the expression of P190Bcr-abl in pre-leukemic mice has any effect on the induction of WAF-1 following exposure to γ-irradiation. I compared the effect in hematopoietic and non-hematopoietic tissue, both expressing the activated kinase. Three hours following whole body irradiation (10 Gy), WAF-1 induction was observed by northern blot analysis of total RNA from irradiated spleen, lungs (Fig 22) and kidney (Fig. 23) cells in both normal and pre-leukemic P190Bcr-abl, but not in $p53^{-/-}$ mice. An increase in WAF-1 level was also detected in spleen, lung (Fig. 22) and kidney (Fig. 23) tissue dissected from leukemic P190Bcr-abl mice (LP190Bcr-abl). I speculated that perhaps the induction of WAF-1 will be lost as a result of cells undergoing transformation. Tumor tissue from leukemic mice were, therefore, dissected following irradiation and WAF-1 levels were investigated at the same time point described above. Tumor tissue (Tumor-1 and 2 Fig. 23) from two different mice showed a normal increase in WAF-1 levels following irradiation. These experiments confirm that the induction of $p21^{WAF1/CIP1}$ is intact in spleen, kidney, and lung tissue from pre-leukemic and leukemic P190Bcr-abl mice as well as in tumor tissue dissected from leukemic mice.

3.5 Apoptosis in Pre-Leukemic P190Bcr-abl Mice

Inhibition of apoptosis by Bcr-abl is thought to play a fundamental role in leukemogenesis by selectively increasing the survival of Bcr-abl(+) clones[176; 222; 223]. Data from cell lines suggest that Bcr-abl expression protects genotoxically stressed cells from

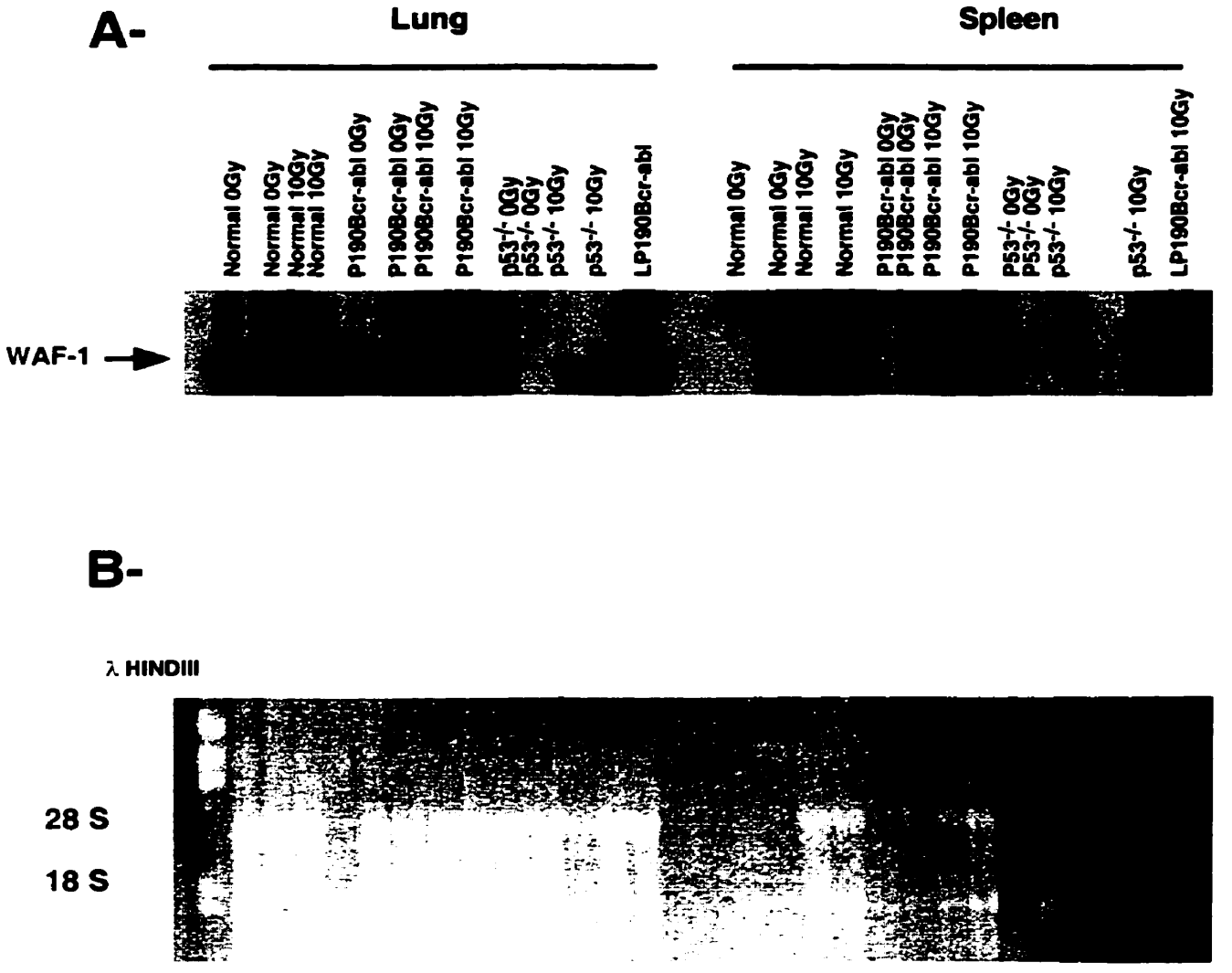


Figure 22 The induction of p21$^{WAF-1/CIP1}$ in lung and spleen.

Total RNA was extracted from non-irradiated (0 Gy) and irradiated (10 Gy) normal, pre-leukemic (PL) P190Bcr-abl, p53$^{-/-}$, and leukemic (L) P190Bcr-abl mice. p21$^{WAF-1/CIP1}$ messages were detected by probing electophoresed RNA samples with a radiolabelled fragment coding for mouse WAF-1 cDNA (A). The same gel is also shown after staining with ethidium bromide to show that comparable amounts of RNA samples are loaded (B).

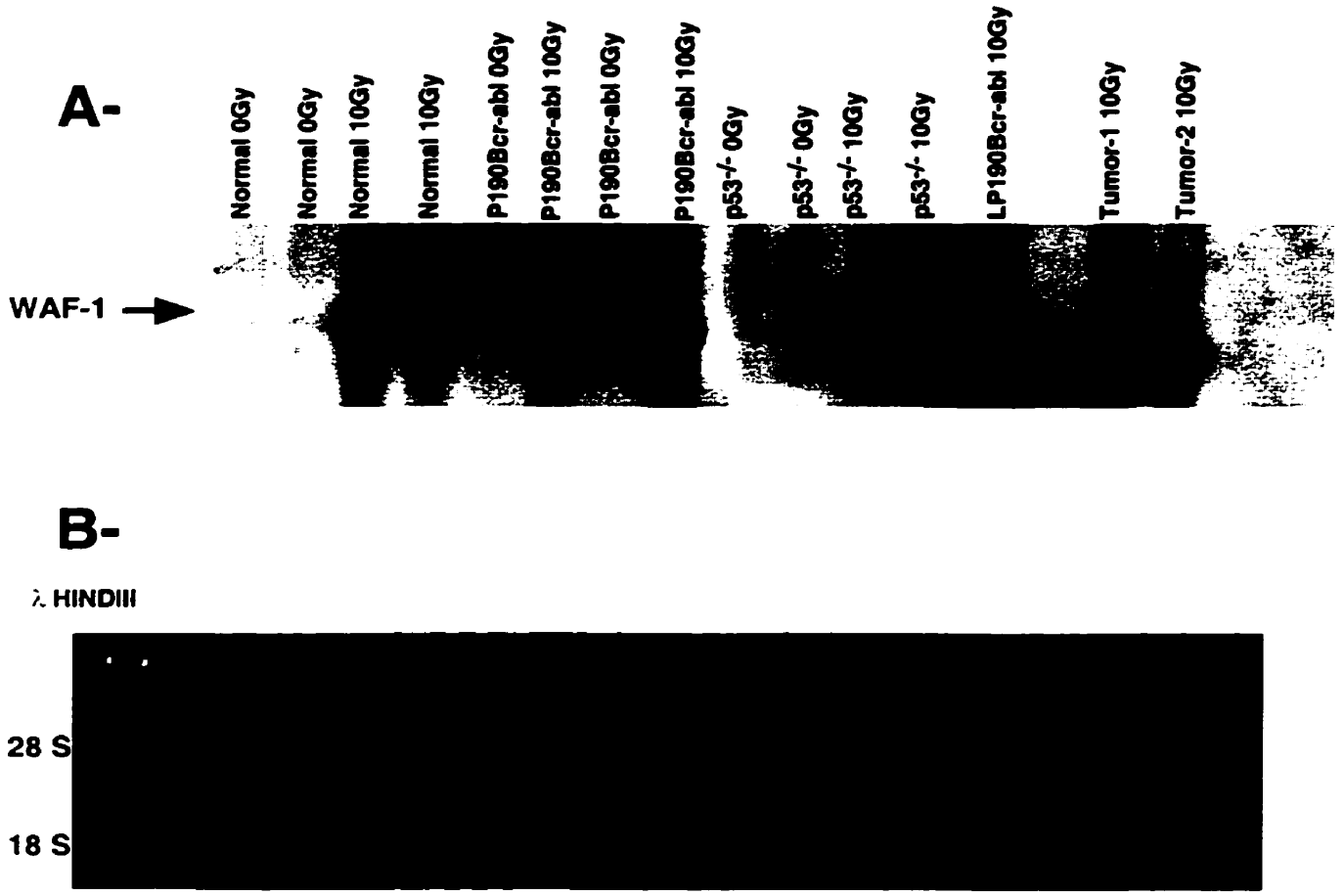


Figure 23 The induction of p21$^{WAF-1/CIP1}$ in kidney.

Total RNA was extracted from non-irradiated (0 Gy) and irradiated (10 Gy) normal, pre-leukemic (PL) P190Bcr-abl, p53$^{-/-}$, and leukemic (L) P190Bcr-abl mice, and from tumor tissue dissected from leukemic mice. p21$^{WAF-1/CIP1}$ messages were detected by probing electrophoresed RNA samples with a radiolabelled fragment coding for mouse WAF-1 cDNA (A). The same gel is also shown after staining with ethidium bromide to show that comparable amounts of RNA samples are loaded (B).

apoptosis, independent of the status of the p53 gene. In the pre-B cell line BaF3, the expression of p210Bcr-abl confers resistance to apoptosis[176]. In the same system, an intact G_1/S cell cycle response and induction of $P21^{WAF1/CIP}$ was observed following exposure to ionizing radiation. I was interested in investigating whether the expression of Bcr-abl during the pre-leukemic stage of the mouse model interferes with the regulation of apoptosis. Apoptosis was measured *in vivo* in bone marrow and spleen cells from irradiated (6 Gy) (n=4) and control non-irradiated mice (n=4). Simultaneous determination of cell cycle profile and quantification of apoptosis was achieved by staining with propidium iodide (x-axis) and in situ TdT-labeling of DNA strand breaks (y-axis). Apoptotic cells were scored as those positive for TdT labeling or those with sub-diploid DNA content after dual flow cytometric analysis. Under each dual flow cytometric analysis, a cell cycle profile of the same sample as determined by staining with PI is shown.

In bone marrow cells from irradiated normal mice a three fold increase in cells undergoing apoptosis compared to non-irradiated mice was observed ($P<0.001$) (Fig. 24 and 27). A comparable 3.8 fold increase in cells undergoing apoptosis was observed in spleen cells from the same set of mice ($P<0.001$) (Fig 25 and 27). In irradiated pre-leukemic P190Bcr-abl mice, a 2.8 and a 2.3 fold increase in the quantity of apoptotic cells was observed in spleen ($P=0.001$) (Fig. 25 and 27) and bone marrow cells ($P<0.001$) (Fig. 24 and 27), respectively compared to cells from non-irradiated pre-leukemic mice. The p53 gene product is indispensable for the induction of apoptosis following exposure to radiation-induced DNA damage. $p53^{-/-}$ knock-out mice were used as a negative control for apoptosis. Spleen cells from irradiated $p53^{-/-}$ mice showed no significant change in the level of

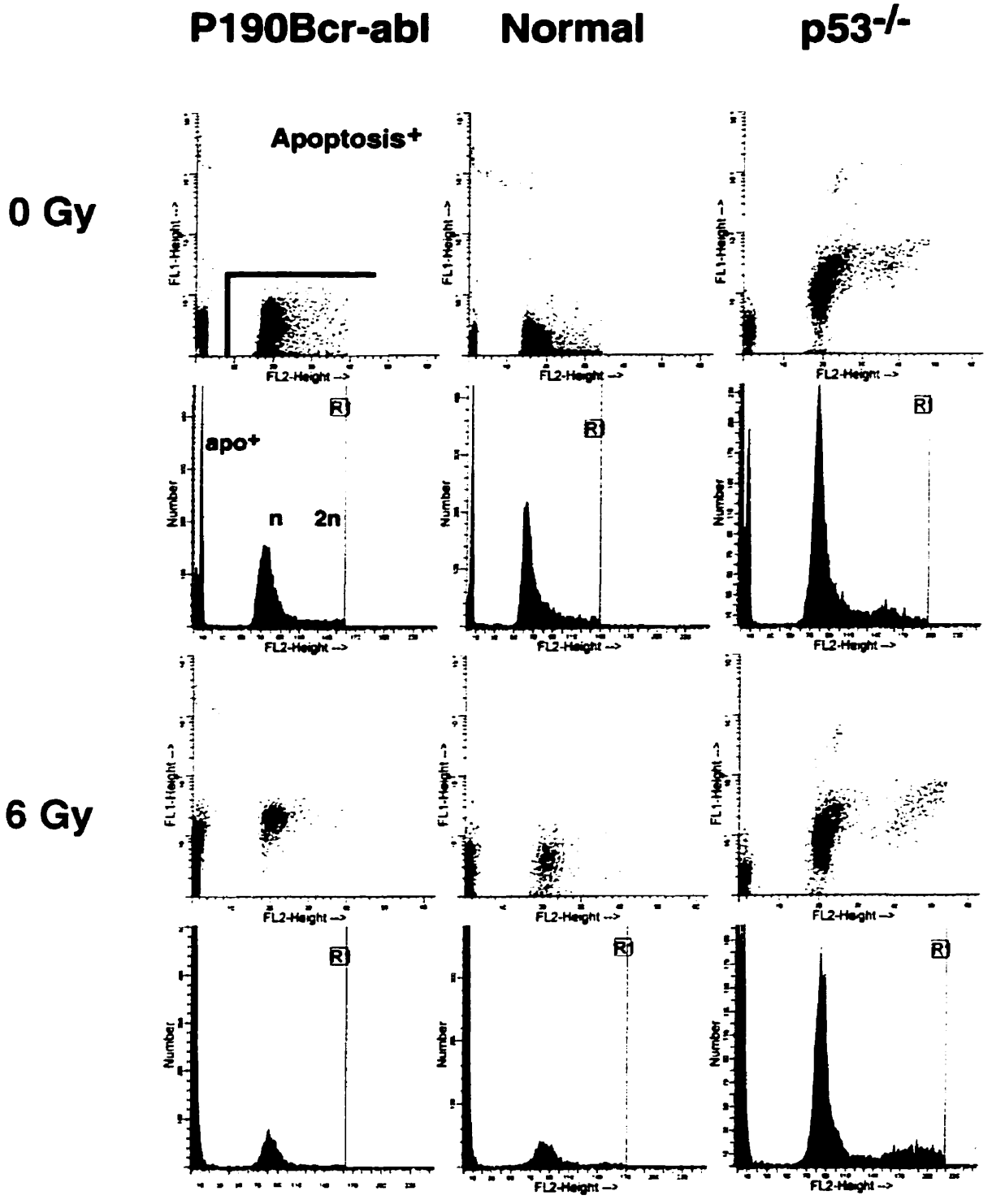


Figure 24 Increase in the level of apoptosis in bone marrow cells following irradiation.

Mice were irradiated at 6 Gy, and sacrificed 14 hours later. The level of apoptosis was determined quantitatively by flow cytometry. Cells were determined to be apoptotic if they either label positively with the tunnel assay (y-axis), or have a sub-diploid DNA content as determined by propidium iodide (x-axis).

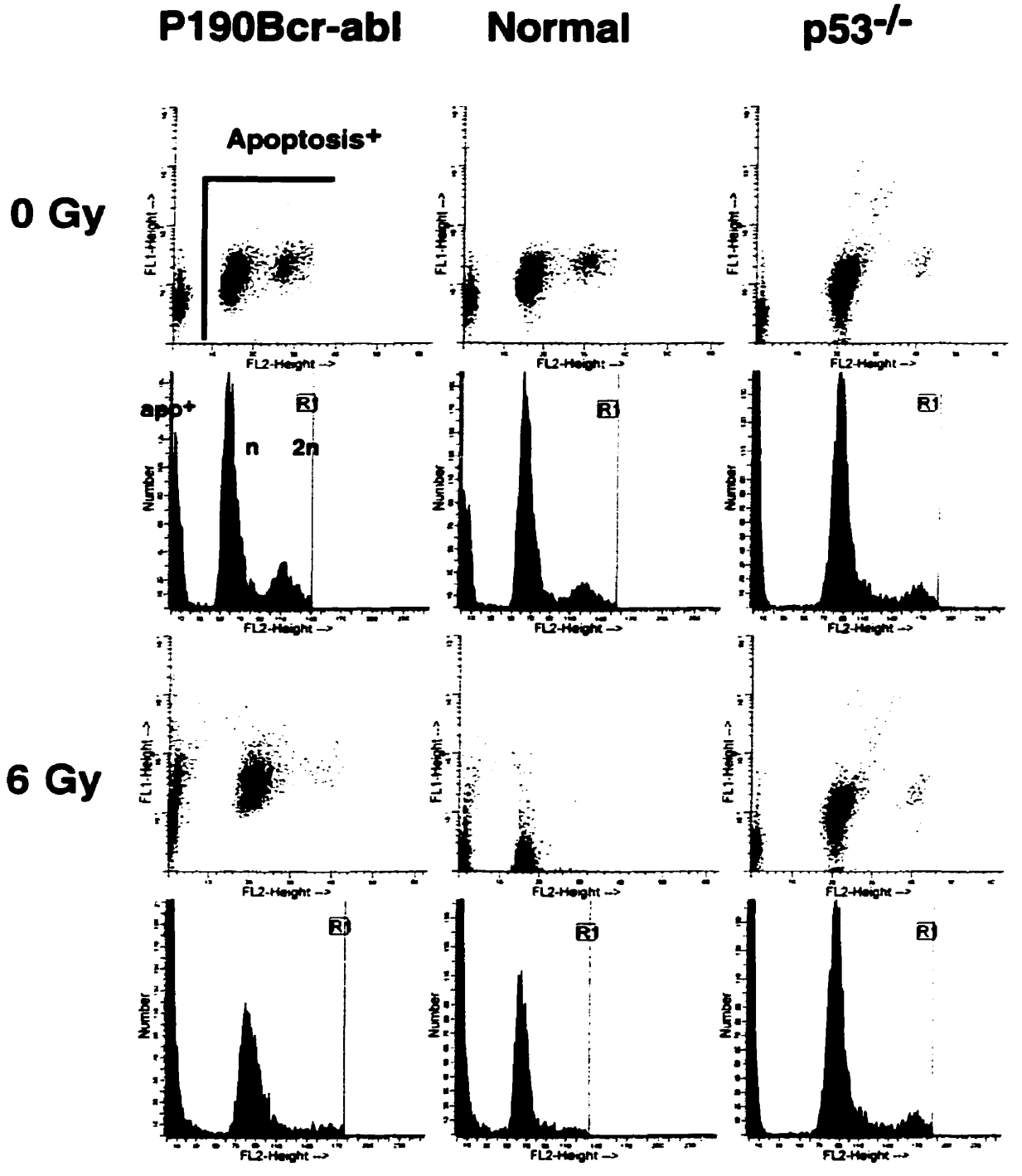


Figure 25 Increase in the level of apoptosis in spleen cells following irradiation.

Mice were irradiated at 6 Gy, and sacrificed 14 hours later. The level of apoptosis was determined by flow cytometry. Cells were determined to be apoptotic if they either label positively with the tunnel assay (y-axis), or have a sub-diploid DNA content as determined by propidium iodide (x-axis).

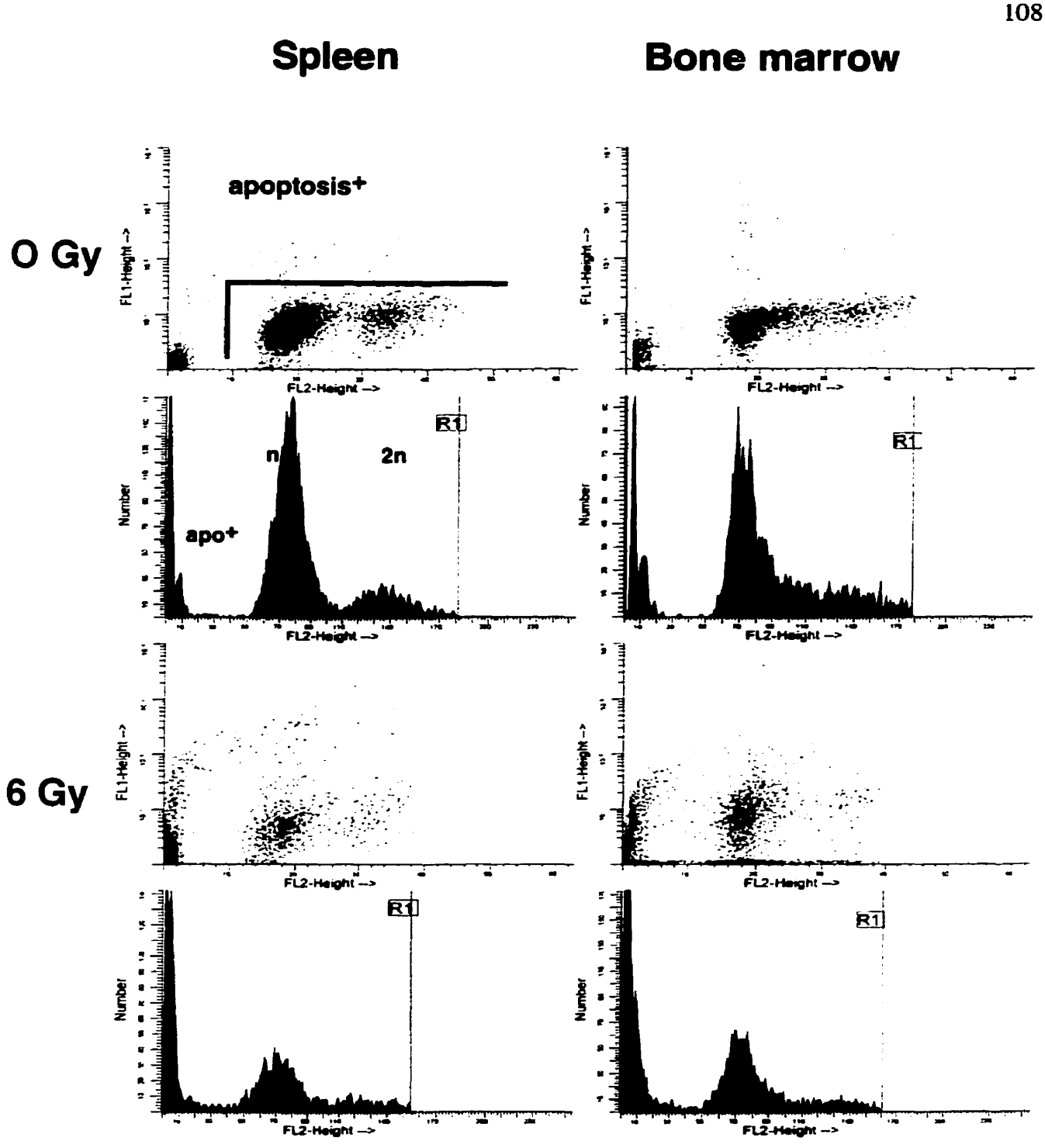


Figure 26 Increase in level of apoptosis in spleen and bone marrow cells from irradiated leukemic P190Bcr-abl mice.

Mice were irradiated at 6 Gy, and sacrificed 14 hours later. The level of apoptosis was determined by flow cytometry. Cells were determined to be apoptotic if they either labelled positive with the tunnel assay (y-axis), or have a sub-diploid DNA content as determined by propidium iodide (x-axis).

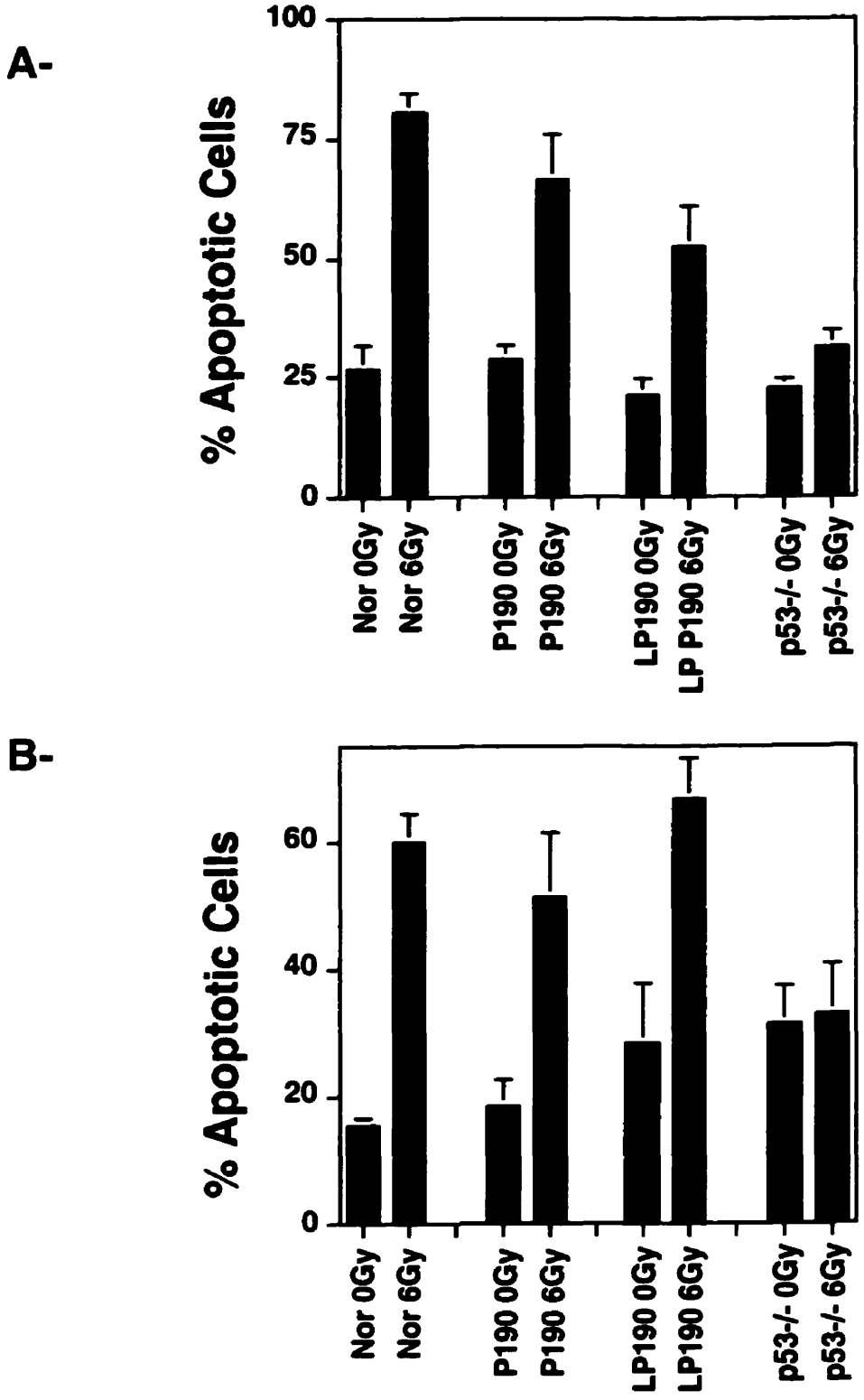


Figure 27 Apoptosis in bone marrow and spleen cells.

Irradiation-induced apopotosis was measured *in vivo* in bone marrow (A) and spleen (B) cells from normal (Nor), pre-leukemic (P190)Bcr-abl, and leukemic (LP190)Bcr-abl mice. Apoptotic levels in irradiated (6 Gy) mice were compared to those in unirradiated counterparts. Each data point represents an average of either four different mice in normal, PL P190Bcr-abl and irradiated L P190Bcr-abl or three animals in $p53^{-/-}$, and non-irradiated LP190Bcr-abl mice.

apoptosis compared to unirradiated $p53^{-/-}$ controls (P=0.855) (Fig. 25 and 27), while in bone marrow cells a 1.4 fold increase was observed as a result of irradiation (Fig. 24 and 27).

The level of apoptosis following irradiation was also determined in bone marrow and spleen cells from mice which have already developed leukemia. This was done to determine whether leukemic transformation might lead to the abrogation of apoptosis suggesting that such an abrogation is a late step in disease progression. A 2.4 fold increase was observed in bone marrow cells from irradiated leukemic (L) P190Bcr-abl (n=4), compared to non-irradiated mice (n=3) (P=0.005) (Fig. 26 and 27). Also a 2.4 increase in cells undergoing apoptosis was observed in spleens from irradiated L P190Bcr-abl (n=5) compared to non-irradiated mice (n=3) (P<0.001) (Fig. 26 and 27). I also observed a three fold increase in cells undergoing apoptosis when tumor tissues from an irradiated leukemic P190Bcr-abl mouse was compared to the same tissue dissected from a leukemic unirradiated P190Bcr-abl mouse. The level of apoptosis was determined by staining cells with propidium iodide and scoring cells with a sub-diploid DNA content as apoptotic (Fig. 28). These results suggest that normal apoptotic responses are present even in tumor tissue arising in those mice where a 100 fold increase in the amount of the Bcr-abl kinase expressed has been reported[236].

Typical apoptotic morphology resulting from DNA, such as chromatin condensation, was observed in cells from irradiated mice by DNA staining with acrydine orange. Acrydine orange labels DNA and therefore make it possible to observe typical DNA fragmentation as a result of apoptosis (Fig. 29 and 30).

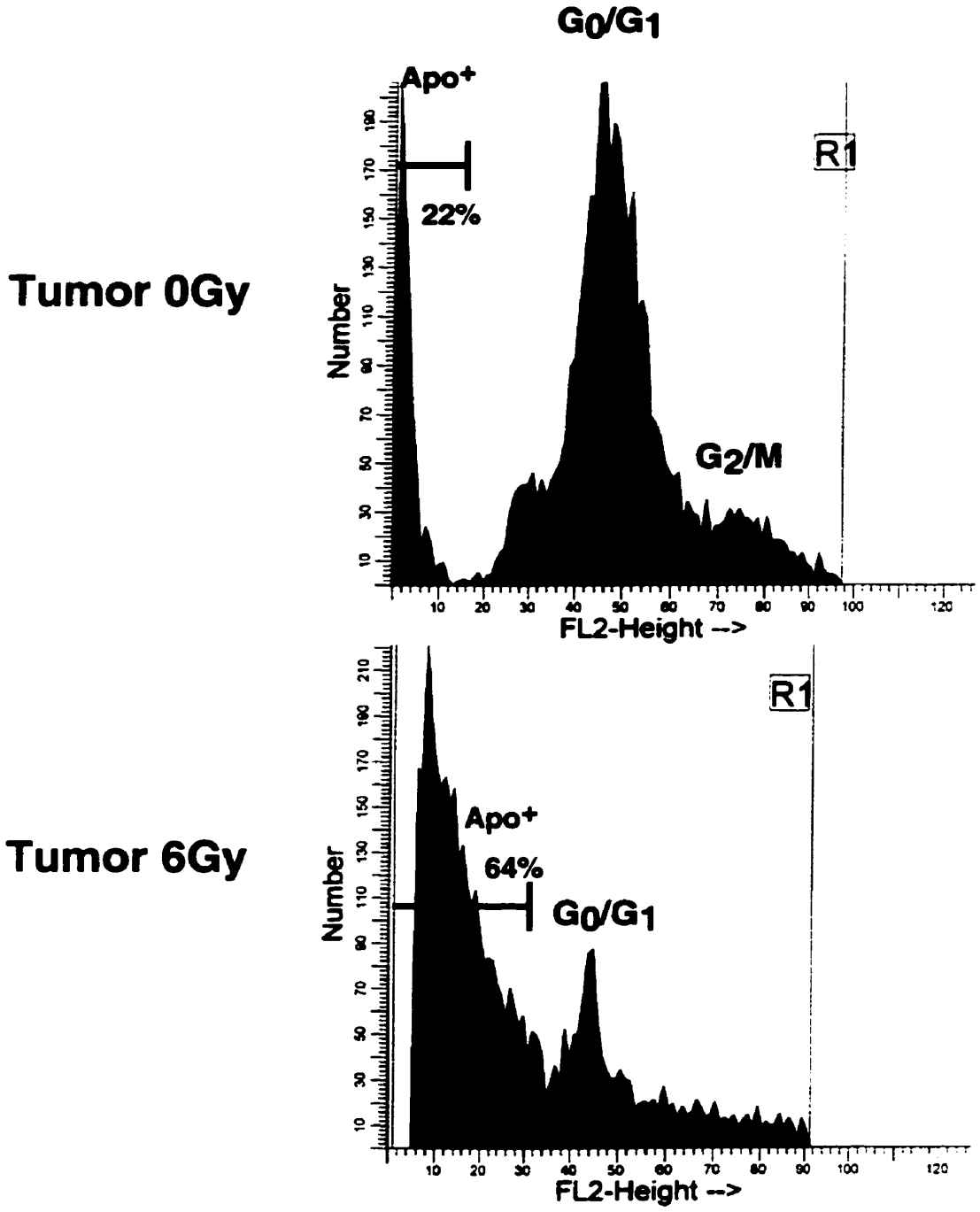


Figure 28 Response to apoptosis following irradiation of tumor tissue from leukemic mice.

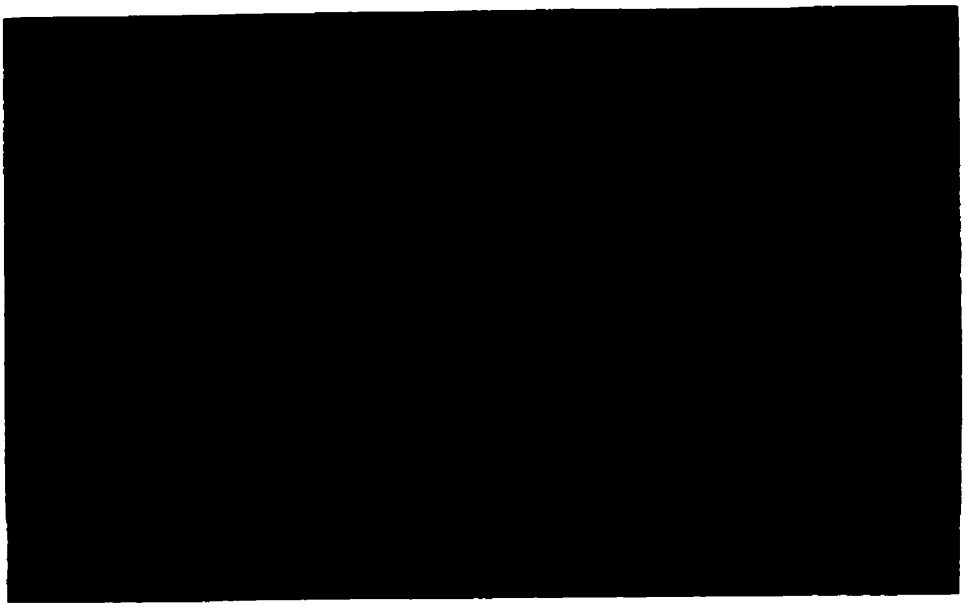
Leukemic mice showing macroscopic growth of tumors were irradiated at 6 Gy and the degree of apoptosis was compared to that in tumors from non-irradiated mice. Tumor cells were processed in parallel 14 hours post-irradiation. DNA content was determined by flow cytometry after cells were labelled with propidium iodide.

Figure 29 Irradiation-induced apoptotic structures in bone marrow cells from normal mice.

Bone marrow cells from irradiated (6 Gy) and non-irradiated normal mice were stained with acrydine orange, mounted on microscopic slides, and observed by fluorescent microscopy. Typical apoptotic bodies consisting of DNA aggregates (arrow), resulting from the endonuclease activity accompanying apoptosis, are observed in irradiated cells.

Normal

0 Gy



8 Gy

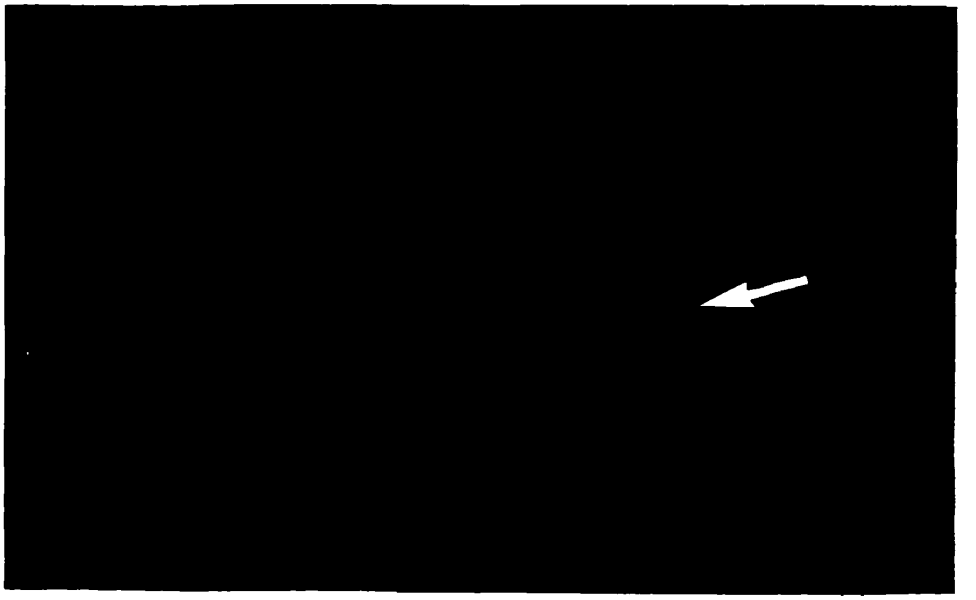
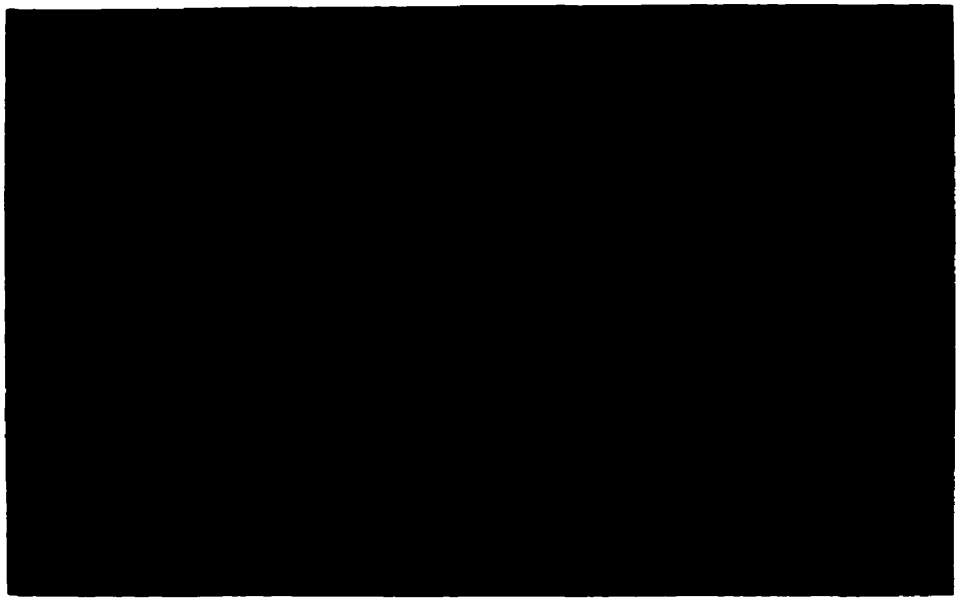


Figure 30 Irradiation-induced apoptotic structures in bone marrow cells from P190Bcr-abl mice.

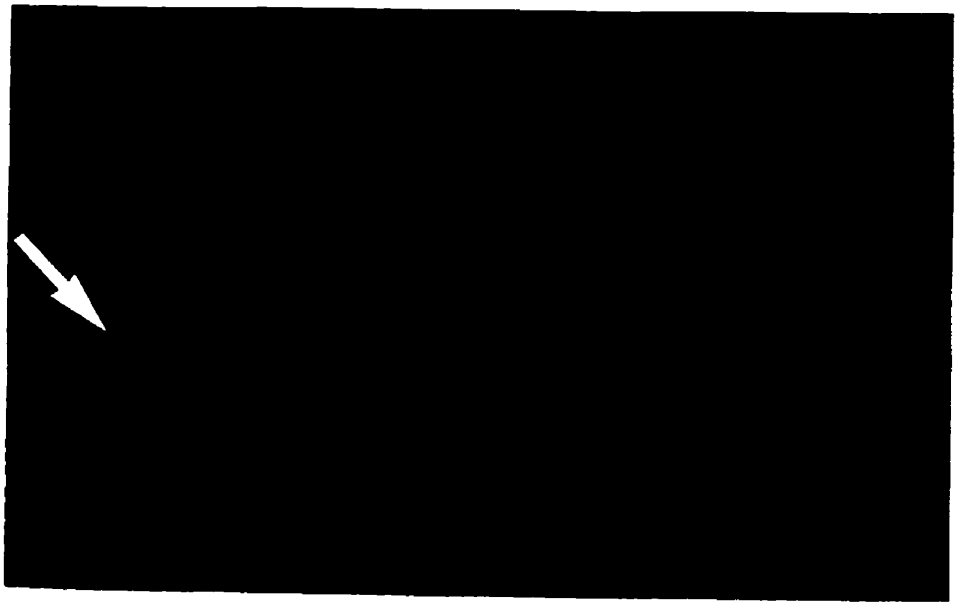
Bone marrow cells from irradiated (6 Gy) and non-irradiated PL P190Bcr-abl mice were stained with acrydine orange, mounted on microscopic slides, and observed by fluorescent microscopy. Typical apoptotic bodies consisting of DNA aggregates (arrow), resulting from the endonuclease activity accompanying apoptosis, are observed in irradiated cells.

P190 Bcr-abl

0 Gy



8 Gy



3.6 Generation of P190Bcr-abl/Big Blue Mice

From the experiments described above it was conclusive that cell cycle arrest and induction of apoptosis following DNA damage were intact in PL P190Bcr-abl mice. The genomic instability associated with the expression of Bcr-abl is, therefore, not due to the loss of these two processes. I therefore decided to look directly at genomic instability resulting from the expression of Bcr-abl by measuring mutation frequencies. This measure can serve as an indicator for the presence of a mutator phenotype which permits the accumulation of mutations and thus the development of malignant clones.

Transgenic mice homozygote for the P190Bcr-abl transgene[124] were mated with Big Blue mice homozygote for the *lacI* gene[234]. The genotype of P190Bcr-abl/Big Blue hybrids was determined by Southern blot analysis (Fig. 12B). The copy number of the P190Bcr-abl transgene has been shown to have no consequence on disease progression or latency[206; 232]. P190Bcr-abl/Big Blue double hemizygotes developed disease at around 90 days after birth. We were interested in examining the causal relationship between the expression of Bcr-abl and an increase in mutation frequencies. The creation of such an environment conducive to the accumulation of genetic mutations may explain the oncogenicity of the activated kinase. It was also imperative to show that an increase in mutation frequencies in those mice was solely due to the expression Bcr-abl prior to the occurrence of overt leukemia. Double hemizygous mice were, therefore, determined to be in the pre-leukemic phase by examining bone marrow peripheral blood smears for the presence of leukemic blast cells as described before (Fig. 13).

Mutation frequencies from P190Bcr-abl mice were compared to those from normal

mice generated from crossing homozygote Big Blue mice to the genetic background of P190Bcr-abl transgenics (F_1 C57Bl6/CBA). This will result in mice with the same amount of λ shuttle vector found in P190Bcr-abl/Big Blue mice.

3.7 Mutant Frequency in Pre-Leukemic P190 Bcr-abl and Normal Mice

Mutant frequencies were compared in spleens and kidneys from PL P190Bcr-abl/Big Blue and normal/Big Blue mice as well as from four tumor tissue obtained from leukemic mice. Mice from both groups were maintained under identical living and diet conditions. Tissue for mutant frequency studies were obtained from young PL P190Bcr-abl and normal mice (6-8 weeks) and were processed in parallel. DNA extracted from spleens and kidneys was used to prepare viable phage particles and infect *E. coli* lawns. A total of 786,880 plaques from six kidneys (3 PL P190Bcr-abl and 3 normal mice) and 1,043,721 from 8 spleens (4 PL P190Bcr-abl and 4 normal mice) were analyzed for the presence of blue mutant plaques (Table 1). A total of 494,434 plaques were also analyzed from four tumor tissues taken from two leukemic mice (Table 2). Blue mutant plaques were confirmed by re-plating twice on SCS-8 bacterial lawns grown in the presence of X-gal. One hundred and forty four blue plaques scored positive after the second plating. The mutant frequencies for PL P190Bcr-abl/Big Blue and normal/Big Blue mice were 9.53×10^{-5} and 4.4×10^{-5} in kidneys, and 7.2×10^{-5} and 3.17×10^{-5} in spleens (Table 1) (Fig. 31). The 2.2 and 2.3 fold increase in mutant frequencies in PLP190Bcr-abl kidneys and spleens compared to tissue from normal mice was of statistical significance(P=0.035 in spleen and P=0.05 in kidneys).

Table 1 Mutant frequencies in *lacI* from spleens and kidneys of PL P190Bcr-abl/Big Blue and normal mice.

Tissue	Mouse	Total pfu $x10^3$	Mutant plaques	Mutant Frequency $x10^{-5}$	Mean $x10^{-5}$ ± SD
Kidney	P190Bcr-abl-1	91.813	9	9.8	
	P190Bcr-abl-54	155.478	18	12.0	
	P190Bcr-abl-73	104.748	7	6.8	**9.5 ± 2.61**
	Normal-4	122.999	8	6.5	
	Normal-43	206.252	6	2.9	
	Normal-30	105.600	4	3.8	**4.4 ± 1.87**
Spleen	P190Bcr-abl-1	146.954	10	6.8	
	P190Bcr-abl-47	105.093	11	10.5	
	P190Bcr-abl-73	144.979	9	6.2	
	P190Bcr-abl-54	150.821	8	5.3	**7.2 ± 2.28**
	Normal-4	83.022	2	2.4	
	Normal-33	136.054	7	5.1	
	Normal-43	112.574	1	0.88	
	Normal-30	164.224	7	4.3	**3.17 ± 1.90**

Pfu, Plaque-forming units.

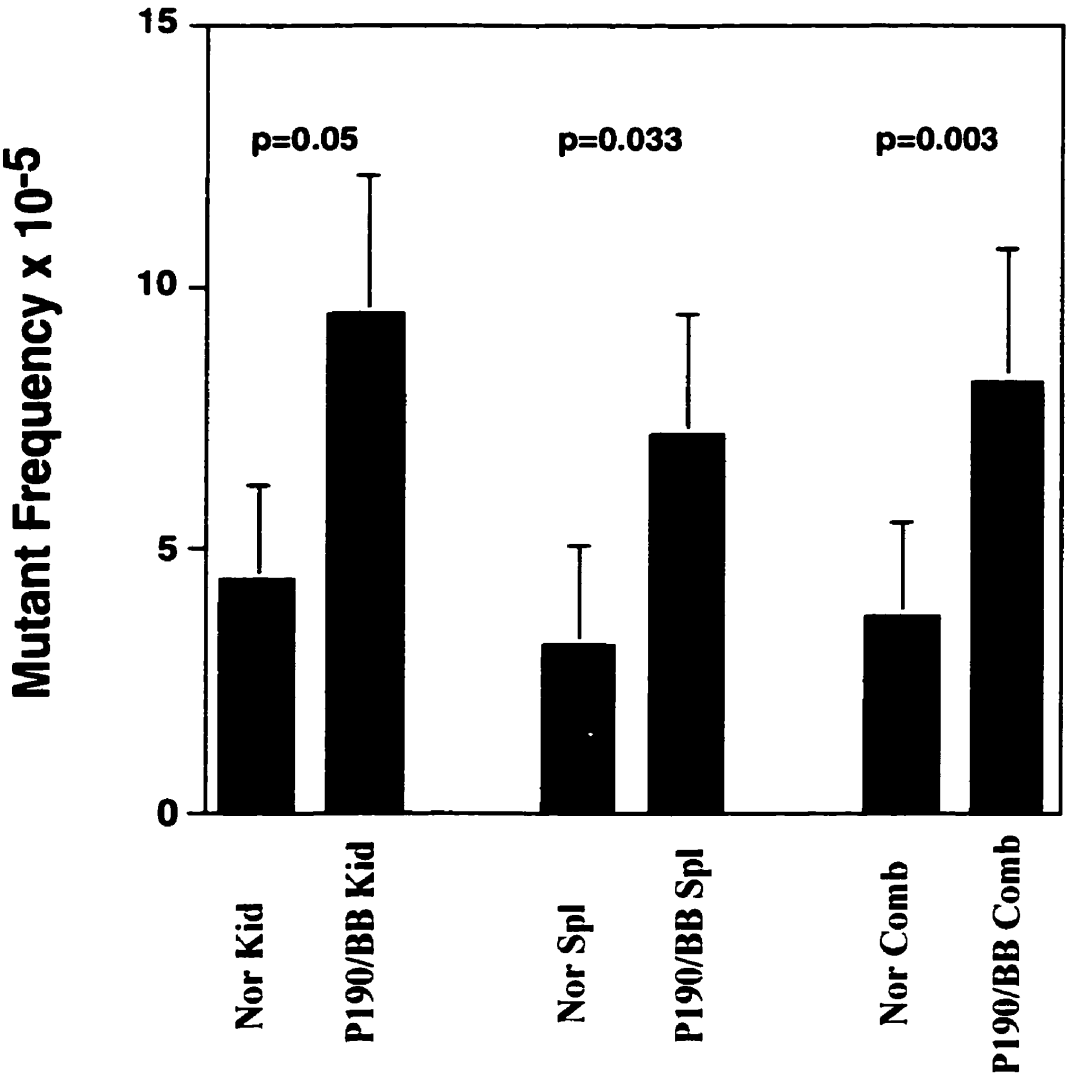


Figure 31 Mutant frequency in kidney and spleen tissues from P190Bcr-abl/Big Blue and Normal/Big Blue mice.

Genomic DNA was extracted from kidneys and spleens of P190Bcr-abl/Big Blue (P190/BB) mice as well as from normal/Big Blue (Nor) mice. Extracted DNA was used to recover the lambda-LIZ shuttle vector and package it into viable phage particles. Packaged lambda phages from each DNA sample are then used to infect *E. coli* lawns in the presence of X-gal. Phages carrying a mutation in *lacI* form a blue mutant plaque. Mutant frequencies are calculated for each sample by enumerating blue mutant plaques relative to total number of plaques plated. Each data point represents an average of three (kidney) or four (spleen) different mice. Also shown are the combined (Comb) spleen and kidney mutant frequencies from both groups of mice. Normal mice were prepared by crossing Big Blue mice to the genetic background of the P190Bcr-abl transgenic mice.

Table 2 Mutant frequencies in tumors from leukemic P190Bcr-abl/Big Blue mice.

Mouse	Total pfu x10^3	Mutant plaques	Mutant frequency x 10^{-5}
P190Bcr-abl-53			
Tumor-1	129.024	3	2.3
Tumor-2	116.229	5	4.3
Tumor-3	130.944	16	12.0
P190Bcr-abl-50	118.237	13	11.0

Pfu, Plaque-forming units.

Table 3 Mutational spectrum in PL P190Bcr-abl spleens and kidneys, and tumor tissues from leukemic mice.

	TS at CpG	TS at non-CpG		TV at CpG			TV at non-CpG	
	GC to AT	GC to AT	AT to GC	GC to CG	GC to TA	AT to TA	GC to CG	GC to TA
PL P190Bcr-abl	12(66.7%)	2(11.1%)	3(16.7%)	-	-	-	1(5.6%)	-
Tumor	1	2	-	-	-	-	-	1
Control	129(45.9%)	41(14.6%)	22(7.8%)	5(1.8%)	34(12.0%)	9(3.2%)	8(2.8%)	33(11.7%)

TS-transition, **TV**-transversion. Data from three PL P190Bcr-abl and two leukemic P190Bcr-abl mice.

Control data is of spontaneous mutants in *lacI* from Big Blue mice compiled in lacI data base and software (MutaBase Software, Neal Cariello™)

The combined mutant rates for PL P190Bcr-abl and normal mice were 8.4 x 10^{-5} and 3.8 x 10^{-5} (Table 1) (Fig. 31). One tumor tissue from leukemic mouse 53 had a high mutant frequency, 12.0 x 10^{-5}, while two other tumor tissues from the same mouse showed low mutant frequencies at 2.3 x 10^{-5} and 4.3 x 10^{-5}. Leukemic mouse 50 had only one tumor which had a high frequency rate at 11 x 10^{-5} (Table 2). I, therefore, observe an increase in the mutant frequencies in spleens and kidneys from PLP190Bcr-abl/Big Blue mice and at least two tumors from LP190Bcr-abl mice.

3.8 Bcr-abl Expression in Pre-Leukemic P190Bcr-abl Mice

I was surprised to find an increase in mutant frequency in non-hematopoietic tissue such as the kidneys, given that P190Bcr-abl mice do not develop kidney-related diseases. The expression of P190Bcr-abl transgenes was previously shown to occur in both haematopoietic, as well as, non-haematopoietic tissue from transgenic mice at the RNA level[124; 232]. Expression at the protein level was only shown in end-stage tumors of P210Bcr-abl mice which usually express a high level of the activated oncoprotein[125]. I was interested in investigating whether there was a difference in the expression of Bcr-abl between the two tissues. Fifty μg of total protein from different tissue of healthy pre-leukemic P190Bcr-abl mice were examined by western blotting for the expression of the oncoprotein. Western blots were probed with an α-Abl antibody, which can detect the human P190Bcr-abl fusion protein as well as the mouse Abl protein (150 kDa). Expression was detected only in kidneys of pre-leukemic P190Bcr-abl and P190Bcr-abl/Big blue mice (Fig. 32) but not in the spleens. This confirms the minimal level of expression of P190Bcr-abl in those mice, as well as, the

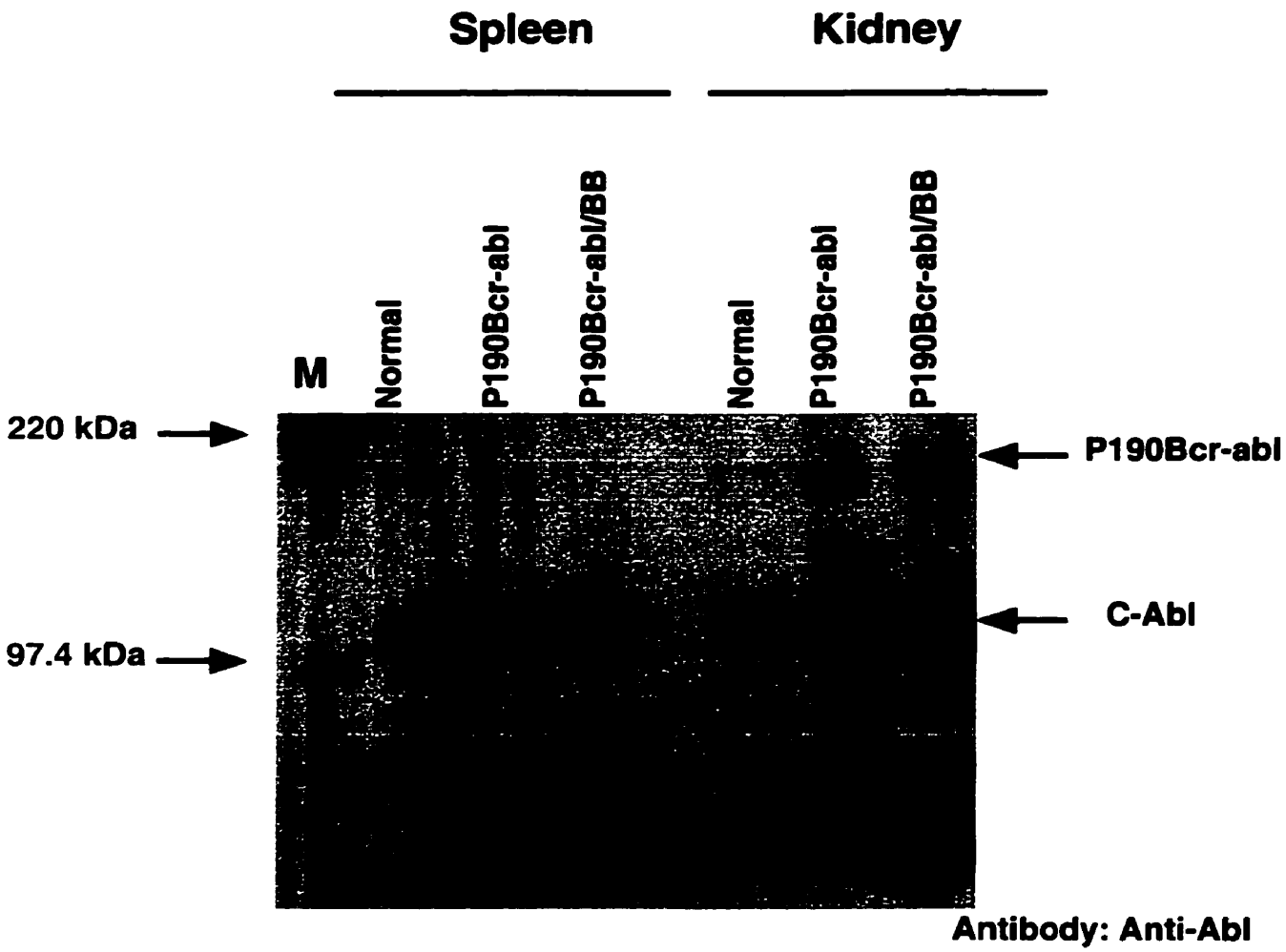


Figure 32 P190Bcr-abl expression in mouse tissue.

Total protein was extracted from the spleens and kidneys of normal, pre-leukemic P190Bcr-abl , and pre-leukemic P190Bcr-abl/Big Blue (BB) mice. P190Bcr-abl expression was detected by exposing western blots prepared from these samples to a mouse monoclonal antibody to Abl. A standard molecular weight protein marker (M) is shown in the first lane from the left.

presence of a higher level of Bcr-abl expression in kidneys.

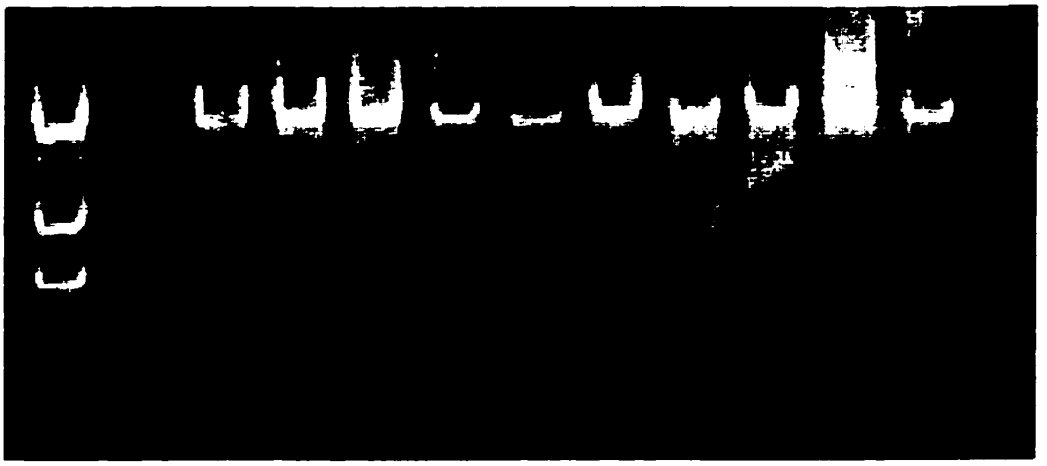
3.9 Mutational Spectrum

The whole *lacI* gene and the *lacZ* operator were sequenced from a samp'e of blue mutant plaques obtained from PL P190Bcr-abl spleens and kidneys as well as the two tumor tissues with high mutant frequencies. Prior to sequencing, individual mutant plaques were grown by infecting several *E. coli* bacterial plates. DNA was then extracted from λ-phage infected bacteria (Fig. 33) and the region corresponding for *lacI* was amplified (Fig. 33). The *lacI* gene, including the two promoter regions and the lacZ operator region was sequenced using 5 primers (1A, 3, 4, 6, and 7) reading in the 5' to 3' direction (Fig. 10). When a mutation was found, it was confirmed by sequencing the same region with one of 4 primers (14, 13A, 11A, and 9A) reading in the 3' to 5' direction (Fig. 10).

Mutations were grouped into one of the following categories: transitions at CpG, transitions not at CpG, transversions at CpG, and transversions not at CpG. The dominant mutation in PL P190Bcr-abl spleens and kidneys were G→A transitions at CpG (Table 3). This profile is similar to spontaneous mutations in Big Blue transgenic mice. A comparison between the mutation spectrum from P190Bcr-abl mice and the mutation spectrum from spontaneous mutation in the Big Blue mouse system (control, Table 3) did not reveal any significant difference (P=0.211). An original mutation was not detected in mutants analyzed from tumor tissue although the data group was small (Table 4). There was no bias towards a specific *lacI* position (Fig. 34), neither was a unique mutation found in all the P190Bcr-abl/Big Blue mutants analyzed as all mutations were previously reported (Table 4).

A-

λ**HindIII**



B-

λ **HindIII**

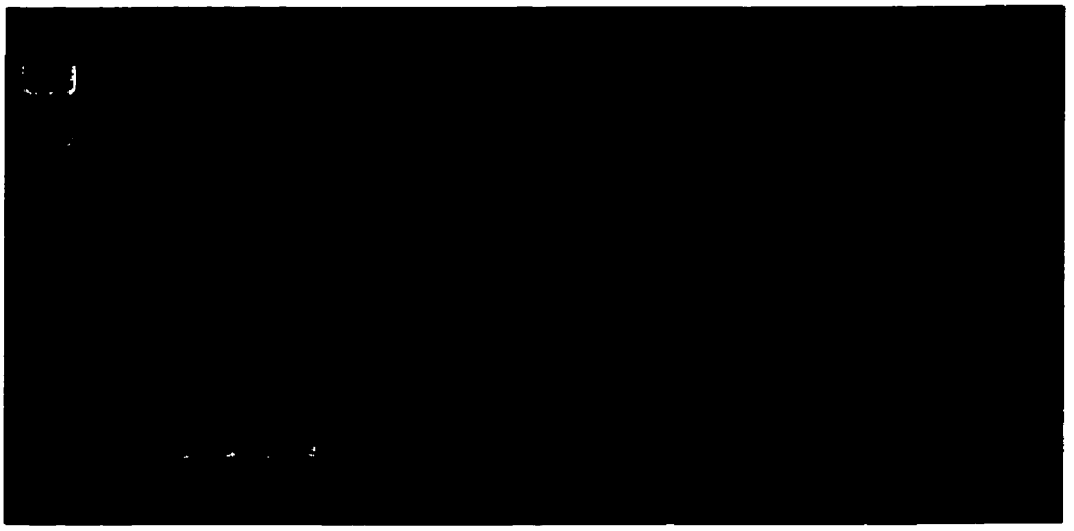


Figure 33 Analysis of mutant bacteriophages.

The entire *LacI* gene and the *LacZ* operator were sequenced in mutant bacteriophages. For that, the bacteriophages were grown (A) and the region to be sequenced was amplified by PCR (B). In this specific excample primers 1283 (upstream) and -55d (downstream) were used.

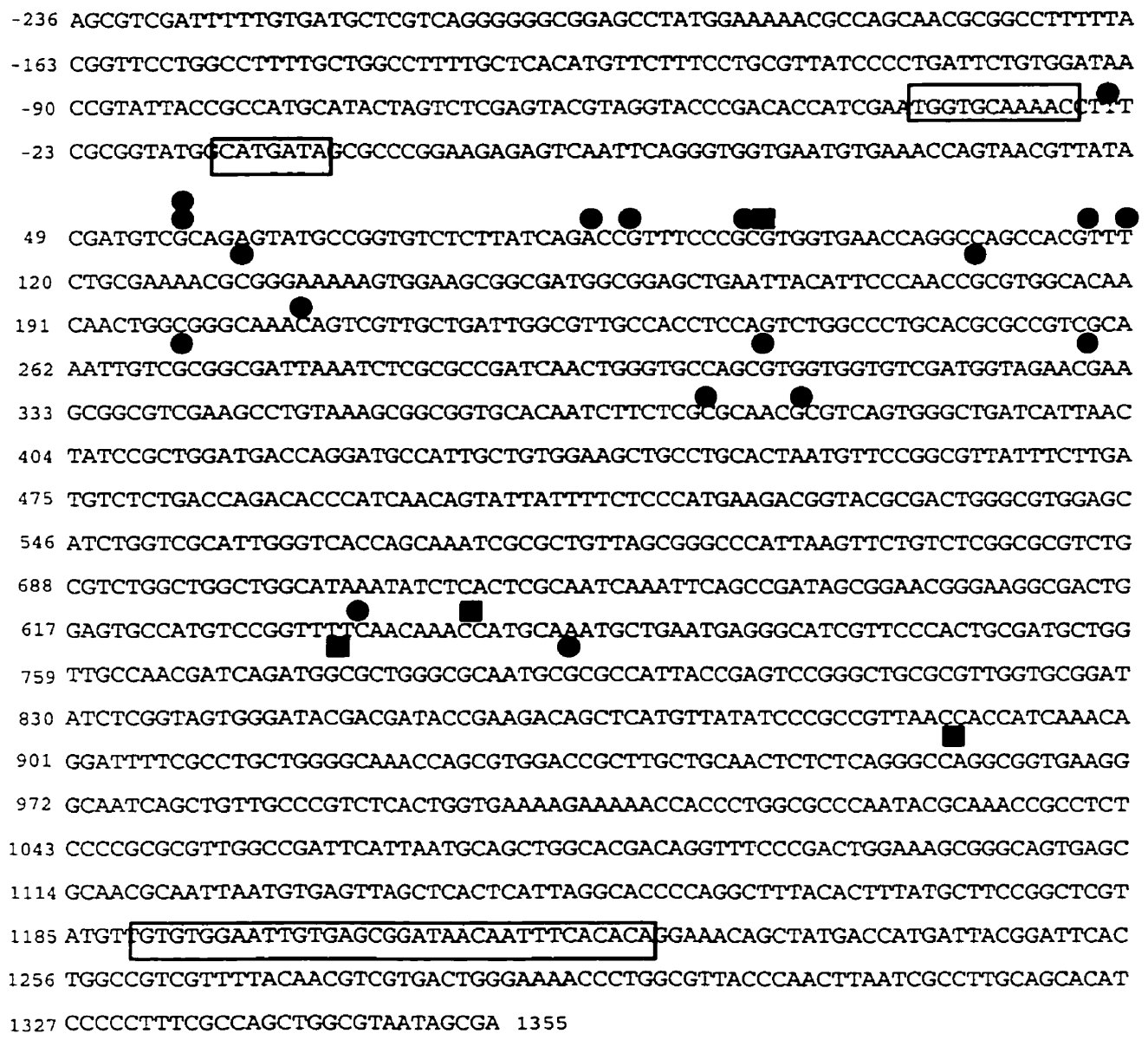


Figure 34 Mutation spectrum in *lacI* from PL P190Bcr-abl and tumor tissue.

Positions of base substitutions in kidney and spleen of PL P190Bcr-abl (black circle) and in tumor tissue (black square) from leukemic P190Bcr-abl mice. The entire *lacI* gene including the two promoter regions (boxed) and the *lacZ* operator region (boxed) is shown.

Table 4 The position of mutations in *lacI*.

Mutant	Tissue	Mutation Type			Position	Unique
P190/BB54-1	Kidney	CG	to	A	56	No
P190/BB1-2	Kidney	CG	to	A	56	No
P190/BB1-1	Kidney	C(G)	to	T	329	No
P190/BB54-6	Kidney	T	to	C	119	No
P190/BB54-10	Kidney	T	to	G	-25	No
P190/BB54-9	Kidney	CG	to	A	792	No
P190/BB54-7	Kidney	C(G)	to	T	375	No
P190/BB54-3	Kidney	CG	to	A	308	No
P190/BB54-2	Kidney	CG	to	A	269	No
P190/BB54-8	Kidney	T	to	C	117	No
P190/BB54-10E	Kidney	AC	to	T	206	No
P190/BB54-5B	Kidney	CG	to	T	86	No
P190/BB54-4A	Kidney	GC	to	T	131	No
P190/BB47-8	Spleen	CG	to	A	180	No
P190/BB47-15	Spleen	CG	to	A	381	No
P190/BB47-11	Spleen	C	to	T	707	No
P190/BB73-4	Spleen	CG	to	A	93	No
P190/BB73-5	Spleen	A	to	G	83	No
Tumor54-4		CG	to	A	95	No
Tumor50-10		C	to	T	959	No
Tumor50-12		C	to	A	714	No
Tumor50-11		C	to	T	777	No

VI- Discussion

Cancer progression is a multi-step phenomenon in which cells acquiring growth advantage accumulate additional mutations giving rise to a malignant clone [240]. Chronic myelogenous leukemia is historically one of the first cancers found to fit such a model for disease progression [204]. The initial expression of the Ph chromosome is sufficient for the induction of granulocytic hyperplasia which is the hallmark of the chronic phase of the disease[40]. The evolution of chronic myelogenous leukemia from an initial benign chronic phase to an acute leukemia phase is associated with genomic instability. As such, transition to the acute terminal phase of the disease is associated with additional accumulation of mutations leading to complete abrogation of hematopoietic maturation [1; 204]. The chromosomal rearrangement resulting in the expression of Bcr-abl is, therefore, believed to be the initiating event in the disease and is followed by secondary mutations assumed to be necessary for disease progression[40].

The expression of Bcr-abl in cell line models results in the inhibition of growth regulatory mechanisms that normally restrict proliferation. Defective cellular adhesion [71; 194] as well as insensitivity to apoptosis [180; 228] observed in these systems may explain the growth advantage acquired by Bcr-abl clones which can trigger the accumulation of more mutations.

Bcr-abl has been shown to abrogate growth factor requirements when expressed in several myeloid and lymphoid cell lines [241; 242]. The myeloid hyperplasia encountered in chronic phase CML can therefore be attributed to the increased survival of Ph positive CML clones presumably by blocking apoptotic signals. This has been shown to be true in both

$CD34^-$ CML progenitor cells as well as in hematopoietic cell lines expressing Bcr-abl [176]. Bedi *et al* have also shown that resistance to apoptosis in Bcr-abl expressing cell lines is accompanied by an altered cell cycle arrest kinetics allowing the repair of cells which otherwise are prone to undergo apoptosis [176]. Also retroviral mediated gene transfer and expression of P210Bcr-abl in IL-3 dependent myeloblastic 32D cl3 (G) cell line resulted in altered cell cycle regulation leading to an increase in the fraction of cells in G_2/M phase of the cell cycle[73]. Other data, however, suggest that proliferating primary $CD34^-$ cells from CML patients do not demonstrate resistence to apoptosis following growth factor withdrawal or irradiation [224]. Also, Ph-positive hematopoietic progenitors as well as chronic phase CML cells do not show altered growth factor requirements or proliferative response [243-245].

Initial work in Dr. Laneuville's laboratory indicated that cumulative changes associated with Bcr-abl expression might be due to an acquired mutator phenotype caused by the loss of key regulatory events controlled by p53. In a 32D cl(3) cell line transduced with a retrovirus expressing P210Bcr-abl, normal p53-dependent G_1 cell-cycle arrest and induction of *gadd45* mRNA following exposure to ionizing radiation was not observed (unpublished data). The expression of P210Bcr-abl in this cell line also resulted in a cell cycle-dependent change of p53 to a mutant conformation, as well as inhibition of transactivation of reporter constructs by ectopically expressed wild type p53 (unpublished data). These results suggested that Bcr-abl expression can functionally inactivate p53-dependent responses producing a mutator phenotype which results in genomic instability and thus influences the rate of disease progression.

Loss of genomic stability represented by the inability of cells to respond to DNA

damage is a central theme in the development of cancer. The tumor suppressor gene, p53, is implicated in the control of key regulatory processes that function in maintaining genomic stability[246]. This is emphasized by the loss of functional p53 in half of all human cancers. In cancers where p53 is not mutated, loss of function may be attributed to the binding and sequestering of the protein as seen in tumors over-expressing the cellular gene Mdm2 [247] or those associated with human DNA tumor viruses such as papilloma viruses [248].

p53 levels increase in response to a number of factors such as damaged DNA, arrest in DNA or RNA synthesis, and nucleotide depletion. A number of cellular proteins are suspected to act as "sensors" which sense these changes and activate p53. DNA-dependent protein kinase (DNA-PK) binds to and is activated by broken ends of DNA, can phosphorylate p53 at residues 15 and 37 [249]. Cells lacking DNA-PK, however, can still undergo cell cycle arrest in a p53-dependent manner raising the possibility of other cellular factors compensating for DNA-PK loss[246]. Poly (ADP-ribose) polymerase (PARP) is involved in increasing the level of p53 in mouse fibroblasts in response to DNA damage and nucleotide depletion[250] although loss of PARP does not affect the activity of p53 in response to such cellular stress [251]. Loss of the product of the *ataxia telangiectasia* gene, ATM, affects the response of p53 to DNA strand breaks caused by γ-irradiation but not to pyrmidine dimers caused by UV irradiation [252; 253]. Deregulated Ras and the mitogen-activated protein (MAP) kinase pathway have been also shown to modulate p53 activity[246; 254; 255]. Over-expression of Ras or activation of the MAP kinase pathway results in growth arrest. Also, cells without a functional p53 and where the MAP kinase pathway is activated exhibit a loss of p53-dependent cell cycle arrest and an increase in genomic instability[254]. p53 mediates a

G_1 cell cycle arrest in response to DNA damage caused by UV or γ-radiation, cytotoxic drugs, or nucleotide deprivation[246]. A key effector of p53-induced G_1 arrest is $p21^{WAF1/CIP1}$ which can bind to and inhibit cyclin-dependent kinases which normally phosphorylate the tumor supressor gene RB. This results in a hypophosphorylated Rb that can maintain the transcription factor E2F in a complex preventing it from favoring a G_1 to S cell-cycle transition[256].

p53 is involved in a mitotic check-point which inhibits the replication of DNA subsequent to mitotic spindle damage[257]. Following the exposure of cells to inhibitors of microtubule assembly, cells with normal p53 arrest with a 4N DNA content, while p53-null cells reach a DNA content of 8 or 16N [257]. p53 in this case is induced after the completion of mitosis and inhibits entry to S-phase. p53, also, blocks entry into mitosis when DNA synthesis is blocked. This is shown in cells treated with hydroxyurea which inhibits dNTP synthesis. In normal cells treated with hydroxyurea, p53 prevents premature entry to mitosis. In p53-null mouse embryo fibrobasts, cells treated with hydroxyurea continously attempt entering mitosis but these attempts are aborted as cells fail to complete cytokinesis[246].

p53 also preserves genomic stability by inducing apoptosis mainly in cells of hematopoietic origin. Apoptosis in those cells can be triggered by DNA damage, growth factor depletion, or the expression of oncoproteins such as Myc and the adenovirus E1A[258; 259]. In some cases the transcriptional activation property of p53 is important in its induction of apoptosis. This is exemplified by p53 transactivation of the pro-apoptotic proteins Bax and Igf-Bp3. The induction of apoptosis or cell cycle arrest by p53 depend on many factors such as the extent of DNA damage and the level of p53 present. Also, the absence of

$p21^{WAF1/CIP1}$ results in cells undergoing apoptosis rather than cell cycle arrest. It is widely believed that loss of p53 function undermine the genomic stability of cells. Gene amplification, a model system to study loss of genomic stability, occurs in cells that lose p53 activity. Embryo fibroblasts from p53-null mice, as well as primary human cells from Li-Fraumeni patients that have lost a single p53 copy, exhibit gene amplification[246].

p53 is mutated at a low frequency in chronic myelogenous leukemia[40] not allowing the deduction of a direct link between the pathogenesis of the disease and the loss of normal p53 expression. However, and as mentioned above, in cell lines expressing Bcr-abl p53-dependent mechanisms such as cell cycle arrest and induction of apoptosis following genotoxic stress were found to be compromised.

In this study, I have decided to utilize an *in vivo* leukemia mouse model transgenic for P190Bcr-abl to determine the consequences of Bcr-abl expression on mechanisms safeguarding genomic stability. The first part of this work was to extend findings in cell lines expressing Bcr-abl to a Bcr-abl leukemic mouse model in an attempt to utilize this model in studies aimed at reversing the effect of Bcr-abl expression. Bcr-abl specific kinase inhibitors, for example, can be used to test whether the effects of Bcr-abl expression on genomic stability can be reversed. This in turn would have presented a unique opportunity for the treatment of CML patients as the expression of Bcr-abl is the sole consistent risk factor associated with the progression of the disease.

Here I show that in both bone marrow and spleen cells from pre-leukemic (PL)Bcr-abl transgenic mice, genotoxic stimuli as a result of irradiation does not result in aberrant response. Cell cycle arrest and induction of apoptosis was comparable to that observed in

normal mice following irradiation. This was accompanied by an elevation in the level of $p21^{WAF-1/CIP-1}$ confirming a normal cell cycle response following genotoxic stress. I also observed an increase in apoptosis in spleen and bone marrow cells from leukemic mice following irradiation. I have decided to perform these experiments *in vivo* in order to assess the primary effect of Bcr-abl expression in the context of intact cellular functions. Mice were confirmed to be disease free by the examination of blood and bone marrow smears for the presence of leukemic cells. Mice were irradiated and cell cycle profiles as well as apoptotic levels were measured from freshly dissected bone marrow and spleen cells. I used $p53^{-/-}$ nullizygous mice as both technical and physiological controls. As expected there was neither a G_1/S cell cycle arrest nor an increase in apoptotic levels in bone marrow and spleen cells derived from $p53^{-/-}$ mice.

An important aspect of the model presented here, which I think must be addressed, is the level of Bcr-abl expression in spleen and bone marrow cells from P190Bcr-abl mice at what we have termed the pre-leukemic (PL) stage. The P190Bcr-abl transgenic colony utilized here is derived from founder 623 [232](C57BLxCBA F_1). This transgenic line has been previously bred for more than four generations with the occurrence of lymphoblastic leukemia/lymphoma in each generation [232]. Prolonged expression in bone marrow was confirmed in that samples from younger mice (19 days, 5-6 days, day1, and between 14-16 days of gestation) were positive for Bcr-abl expression by RT-PCR[232]. Prolonged expression of Bcr-abl was also detected in progenies of line 623 in the brain, liver, kidney, spleen, and muscle [232]. Expression in tissue other than the bone marrow did not appear to be due to Bcr-abl expressing bone marrow cells escaping from circulation as peripheral blood samples of

the same mice were negative for Bcr-abl expression [232]. Quantitatively, using RNase protection assays, the level of Bcr-abl in 13-16 day old mice were compared in different tissue[232]. Compared to the level of expression in lymphoblastic lymphoma, the relative level of Bcr-abl expression is fifty fold lower in brain and 100 fold lower in spleen and kidney [232]. Real difference in the level of expression might also exist between spleen and kidney cells. I have detected Bcr-abl expression in kidneys but not spleens from P190Bcr-abl mice using a less sensitive but more quantitative method of western blotting (Fig. 32). Given the expression of Bcr-abl, whilst at low level, I think that the evaluation of the biological effect of such expression on cellular mechanisms such as cell cycle arrest and induction of apoptosis is relevant in the understanding of disease pathogenesis in CML. Bcr-abl expression in cell culture systems has been previously shown to modulate both processes following induction of genotoxic stress [176; 225]. This serves as an underlying mechanism to explain Bcr-abl associated genomic instability.

The discrepancy between the results reported here and those reported from experiments in cell line systems can, therefore, be due to differences in the level of Bcr-abl expression. The level of Bcr-abl expression in bone marrow and spleen cells from PL P190Bcr-abl mice is many fold less than in cultured cells transiently or stably expressing Bcr-abl [232]. I decided to look at inhibition of apoptosis in mice tissue which express high levels of Bcr-abl. As mentioned previously, Bcr-abl expression is 100 fold more in tumor tissue than PL spleen and bone marrow[236]. In these tissue a block in irradiation-induced apoptosis would have been expected similar to results obtained with cell lines expressing high levels of Bcr-abl[232; 227]. I was not able to document a decrease in levels of apoptosis as

a result of irradiation in tumor tissue dissected from leukemic mice (Fig. 28) neither did I find an inhibition of $P21^{WAF-1/CIP}$ induction (Fig. 23 Tumors 1 and 2). These results suggest that the concentration of Bcr-abl might be only one of many facets involved in the loss of sensitivity to apoptosis. It is, also, difficult to discern whether the increase of Bcr-abl in tumor tissue from leukemic mice preceded loss of apoptotic sensitivity or whether both phenomena occur concurrently. Also, different parts of the same tumor might manifest different degrees of genomic instability as observed in human gastric carcinomas [260]. It is intriguing that in patients with CML blast crisis as well as in ALL patients, apoptosis can be induced to high levels (80% of leukemic cells in peripheral blood and bone marrow) using a variety of chemotherapeutic drugs[261]. This presents further evidence that inhibition of apoptosis is not an early event in Bcr-abl -induced transformation.

It is noteworthy to point out a similar discrepancy between CML lines which are established mostly from patients in the acute phase of the disease [262] and primary cells from patients in the chronic phase of the disease. While CML cell lines are growth factor independent and resistant to apoptosis, CML progenitor cells from patients in the chronic phase of the disease are growth factor dependent and susceptible to apoptotic death [224; 263]. Several lines of evidence point to an increase in Bcr-abl protein level associated with disease progression in CML. Stable Bcr-abl expression is observed in all stages of CML but does increase in some cases with disease progression [226]. Also, duplication of the Philadelphia chromosome is a frequent karyotypic anomaly in transition from chronic phase to acute phase CML [40]. It is, however, hard to discern whether this increase is responsible of the transition to the acute phase of CML or a result of the transformed phenotype associated with

this transition.

The effect of the level of Bcr-abl expression on biological functions generally attributed to its expression was recently addressed by Cambier *et al* [227]. The biological effects of a high and low Bcr-abl expression vectors were studied in hematopoietic cell lines. The low expression vector was driven by the human *bcr* promoter and was utilized to mimic the low level of Bcr-abl expression typical of the chronic phase of CML. Whereas clones derived from low level Bcr-abl expression vector were responsive to IL-3 addition, cell lines derived from the high level Bcr-abl expression vector were refractory to IL-3 addition and proliferated maximally in its absence [227]. However, clones derived from both expression vectors were tumorigenic when injected into syngenic mice. Similar disease latency and phenotypes as well as hematopoietic organs infiltration were observed after the injection of clones derived from both expression vectors. This suggests that *in vitro* growth factor requirements or their absence is not necessarily an accurate measure of tumorigenicity *in vivo*[227].

A direct correlation between the levels of Bcr-abl expression and resistance to apoptosis was also observed in the same study [227]. Only clones expressing high levels of Bcr-abl were resistant to apoptosis induced by γ-irradiation. This is in contrast to results obtained from immortalized murine cell lines expressing Bcr-abl, where resistance to apoptosis induced by a variety of agents was observed [176; 180; 222; 225; 228]. However, this is in agreement with other observations (see above) in CML progenitor cells where the level of apoptosis induced by different stimuli was found to be comparable to that of normal counterparts [224; 263]. The level of Bcr-abl expression in primary CML cells is believed to be low [227], suggesting

that similar to clones derived from low expressing Bcr-abl vectors [227], normal apoptotic response is probably intact in the initial phase of CML. Evidence of glucocorticoid- induced apoptosis in leukemic cells from patients with lymphoid blast crisis may suggest that this mechanism might be preserved even in later stages of the disease. I think that in the transgenic system I utilized, the pre-leukemic (PL) stage is an ideal point of time to investigate the effects of Bcr-abl. P190Bcr-abl is expressed at low but physiologically relevant levels in that lymphoblastic leukemia results in all of these mice [232].

The different biologic responses observed when disparate levels of Bcr-abl are expressed may represent the activation of different signal transduction pathways[227]. Low levels of Bcr-abl might activate pathways such as the Ras/MAPK pathway which are normally activated by cytokines leading to growth factor independence and immortalization[227]. High concentrations on the other hand might be necessary to activate anti-apoptotic signals such as those leading to Bcl-2 activation. Cambier *et al* have established a direct correlation between the level of Bcr-abl expression, the level of Bcl-2 induction, and the degree of resistance to apoptosis[227]. This is also reflected in bone marrow cells from CML patients in the acute phase of the disease which, when compared to cells from patients in the chronic phase, show an increase in the level of Bcl-2 expression[264]. Taken together our results suggest that loss of sensitivity to apoptosis and cell cycle arrest does not seem to be the primary defect in the chronic phase of CML, but might rather arise in later stages of the disease.

It has been technically difficult to delineate the primary consequences of Bcr-abl expression *in vivo* or *in vitro*. Conditional Bcr-abl expression vectors have shed some insight

in *in vitro* cell culture systems on the nature of such primary events. A temperature sensitive conditional mutant of P210Bcr-abl was used to assess the early consequences of Bcr-abl expression in the IL-3 dependent BaF3 cell line [265]. In this system abrogation of growth factor dependence was not found to be the primary consequence of Bcr-abl expression. Cells required a prolonged exposure to Bcr-abl tyrosine kinase activity in order to survive in the absence of IL-3, suggesting the requirement of additional genetic events to escape growth factor requirement [265]. A moderate suppression of apoptosis following growth factor withdrawal was observed suggesting again that the expansion of the myeloid compartment characteristic of chronic phase CML might be the result of prolonged cell survival rather than the increase in the rate of proliferation [265]. To this effect, there was no activation of Ras-dependent MAP kinase pathway following the induction of the temperature sensitive Bcr-abl tyrosine kinase activity in Baf3 cells[265]. Other work, however, utilizing clonal 32D cell lines inducibly expressing P210Bcr-abl from a glucocorticoid response promoter, argue that Bcr-abl expression cause both the increase in the level of GTP-bound Ras and the hyperphosphorylation of Raf[202]. Induction of Bcr-abl expression in G_0 arrested cells results in DNA synthesis and cells entering the S phase of the cell cycle. This was accompanied by the increase in activity of Cdk2, and cyclin D2 and D3 associated kinases suggesting that the cell cycle entry induced by Bcr-abl expression is accompanied by activation of the cell cycle machinary [202]. The requirement for prolonged exposure to Bcr-abl expression to escape growth factor dependency is compatible with evidence of an underlying genetic instability as a result of Bcr-abl expression. Non-random chromosomal cytogenetical and genetical abnormalities[40] are associated with blast transformation in CML patients[40; 266].

Non random chromosomal changes and karyotypic instability similar to those observed in blast crisis transition in CML are encountered during later stages of leukemia in P190Bcr-abl transgenic mice [206] as well as in a myeloblastic cell line transformed to IL-3 independency by P210Bcr-abl expression[127]. Klucher *et al*[267] have also described a BaF3 cell line with a tetracycline-dependent Bcr-abl expression construct. In the presence of doxycycline, BaF3 cells are viable and proliferate independent of IL-3. Nude mice injected with this cell line developed doxycycline-dependent tumors which regress in the absence of doxycycline. Importantly, and after long latency period, tumors reappear in animals after doxycycline withdrawl or in animals injected with the inducible cell line but never exposed to doxycycline. Characterization of those spontaneously transformed subclones demonstrated in most cases an increase in basal expression of Bcr-abl, with some clones augmenting Bcr-abl expression after doxycycline induction, while others do not. An important implication of these results is the contribution of Bcr-abl expression on genetic instability of BaF3 cells, leading to their conversion into growth factor independence and their ability to cause tumors in mice. The emergence of doxycycline-independent tumorigenic subclones, it was argued, results from the expression of Bcr-abl in BaF3 cells, which might accelerate mutagenic events through the induction of genetic instability similar to observations documented in P190Bcr-abl transgenic mice [206; 268].

In vitro models, however, fall short of correlating Bcr-abl induced oncogenesis and an increase in mutation frequency. In the later part of the work presented here, the effect of Bcr-abl expression on genomic instability was investigated by directly measuring mutation frequencies in PL P190Bcr-abl mice. The Big Blue transgenic mouse system is a powerful

in vivo tool for the detection of spontaneous as well as induced mutations in animals [233]. A good correlation between an increase in mutant frequency and oncogenesis has been previously reported using the Big blue mouse system [269]. Also, and except for long deletions induced by X-irradiation, mutations in the bacterial *lacI* locus of Big Blue mice respond in a similar fashion to host mouse loci when stressed with mutagen[270]. I combined this system for mutation detection with the transgenic mouse model for leukemia described earlier. Those mice, as mentioned before, develop pre-B leukemias and lymphomas after an average of 100 days during which the transgene is expressed in most tested tissue [232]. I think that this disease-free period is ideal for the study of the effect of Bcr-abl expression on the accumulation of mutation.

I observed an increase in mutant frequency in both kidneys and spleens of pre-leukemic (PL) P190Bcr-abl mice as well as in two out of four tumor tissue following the unset of overt leukemia (Tables 1 and 2). I think that the observed increase in mutant frequencies is biologically significant when compared to the effects of known carcinogens. A 20mg/Kg dose of the known carcinogen benzo[a]pyrene results in a mutant frequency value of 7.4×10^{-5} in kidneys of *lacI* mice[269]. I also observe that G:C→A:T transitions at CpG dinucleotides were the dominant mutation in PL P190Bcr-abl/Big Blue mice. This mutation spectrum is similar to spontaneous mutations in *lacI* from Big Blue mice. I also compared the distribution of the positions of those mutations in *lacI* with the positions of spontaneous mutations in Big Blue mice and found no statistical difference ($p=0.211$)(Table 3). A Bcr-abl unique mutation or mutation spectrum was not detected either, as all sequenced mutations recovered from P190Bcr-abl/Big Blue mice were previously reported. The small sample size

of sequenced mutants from tumor tissue did not permit a significant comparison with available mutation spectrum from the Big Blue mouse system. The observation that two tumor tissue did not show an increase in mutant frequency suggests that those tumors may have risen from cells that do not carry a mutation in *lacI* but might have acquired it later when transformed cells undergo rapid cell division. It is important to stress that mutant frequency determined in the spleen and kidney of PL P190Bcr-abl mice reflect a measure acquired from birth and until 6-8 weeks. Mutant frequencies can potentially be a measure of mutations acquired through organ development and in later cell divisions following the maturation of an organ. Different organs are expected to have different rates of proliferation. It is therefore important to compare similar organs at similar ages. Tumor tissues are expected to have a high proliferative potential. If mutant frequencies are a measure of mutations acquired during cell division one could expect a high frequency in every tumor tissue. In thymic lymphomas from $p53^{-/-}$ crossed to Big Blue mice only one in four tumors was observed with an increase in *lacI* mutation[271]. This suggests that *lacI* mutation frequencies in tumors are not simply a function in errors accumulated during cell division. In assessing *lacI* mutations from tumor tissues many factors such as tumor size and even the region of the tumor used for the *ex vivo* preparation of λ-phage should be considered. As mentioned before, I think this is essential after a recent study suggesting the presence of heterogenic degrees of mutator phenotype in the same tumor sample from gastro-intestinal carcinomas[260].

A mutator phenotype in *E coli* is exhibited when a mutation affecting factors that control mismatch repair are present. Mismatch repair in *E coli* involves the mutator genes

mutS, *mutL*, *mutH*, *uvrd*, and *dam*. The isolation and purification of those genes has permitted the delineation of their biochemical function. MutS is a DNA-mismatch binding protein which can bind to mispaired bases as well as small (1-5 bases) single-strand loops. Uvrd acts as a DNA helicase II, unwinding DNA in an ATP-dependent manner. MutH is an endonuclease that can incise transiently unmethylated strands of hemimethylated 5'-GATC-3' sequences. The presence of a hemimethylated 5'-GATC-3' sequence in the parent template permits the discrimination between parental and newly synthesized strands. MutL is believed to couple the mismatch recognition function of MutS with MutH incision at 5'-GATC-3' sequences in an ATP-dependent manner[272].

Multistage carcinogenesis appears to require a mutator phenotype [273]. Such a model can explain the high mutation rates encountered in human cancer cells and which spontaneous mutation rates in normal cells cannot account for [273]. It is estimated that the back ground somatic mutation rate is $1.4x10^{-10}$ mutation/base pair/cell [274] which can account for a maximum of 2 or 3 mutations in each tumors and not the much larger number that is observed [275].

Experimental validation of a mutator phenotype in cancer progression initially came from studies on hereditary nonpolyposis colorectal cancer (HNPCC). Microsatellite instability represented by the expansion in the number of repetitive di, tri, and tetranucleotides was observed when the length of specific CA repeats were studied in HNPCC patients [276]. Concomitantly, in some of those patients, mutations in genes homologous to the bacterial genes MutS and L responsible for DNA mismatch repair were reported [216;277]. The importance of this DNA repair pathway is stressed by demonstrating that

inactivating mutations in homologous genes in humans are the underlying cause of a number of cancers [219]. Most HNPCC cases are caused by a mutation in one of four loci: hmSH2 gene which codes for a product homologous to Mut S, while the other three genes hmLH1, hmPMS1, and hmPMS2 encode distinct products all homologous to MutL. Normal cells from HNPCC patients contain a normal and a mutated copy of the affected repair gene resulting in a low mutation rate. In tumor cells, however, the normal copy is lost leading to a defect in mismatch repair. Loss of a critical DNA repair mechanism is the primary event in the development of HNPCC and defines a mutator phenotype responsible in genetic de-stabilization of the cells leading to the acquiring of more mutations in key regulatory pathways that control cellular proliferation [219].

A mutator phenotype was also observed in hematologic malignancies. Microsatellite instability was observed in a subset of patients with chronic lymphocytic leukemia[278]. Patients were in the early stage of the disease (stage I) and did not show an elevated cell count in peripheral white blood cells suggesting that in those cases the mutator phenotype is an early event [278]. Mutations in genes controlling mismatch repair were not analyzed in order to correlate the observed mutator phenotype with the loss of DNA repair mechanisms. This is important as not all tumors with a mutator phenotype have been shown to lack mismatch repair[279].

Until recently most studies aimed at showing the importance of a mutator phenotype in carcinogenesis have been derived from examining non-coding microsatellite sequences throughout the genome of tumor cells. Evidence, however, is emerging that genetic instability especially in gastrointestinal cancers, targets genes which are involved in growth

regulation. In some gastrointestinal cancers, genetic instability targeted the polyadenylate short tract in the coding region of transforming growth factor β receptor type II (TGF-β RII) gene, creating nonsense codons. The resulting mutation in TGF-β RII alleviated the growth constraints of transforming growth factor β on gastrointestinal epithelial cells, leading to uncontrolled proliferation [280]. Also, microsatellite instability-associated frameshift mutations were detected in repetitive mononucleotide repeat stretches in coding sequences of other genes associated with the control of cellular proliferation such as the insulin-like growth factor II receptor (IGFIIR) [281] and Bax[282]. IGFIIR down regulates the proliferation of cells by the internalizing and the subsequent degradation of insulin-like growth factor II, which is implicated in the maintenance of transformed phenotype and protects cells from apoptosis[283]. Bax, on the other hand, and unlike Bcl-2, promotes apoptosis[284].

The model for the contribution of mutator phenotype in carcinogenesis can, therefore, be viewed as a progressive sequential process leading from an initiating event such as mismatch repair failure. This results in genetic instability detected in the form of microsatellite instability as well as somatic mutations in genes involved in the regulation of proliferation. Chang *et al* [260]dissected the various genetic events in the development of gastric carcinomas by looking at different areas in the same tumor tissue obtained from patients. Individual tumor areas from the same patient demonstrated different mutation frequencies in microsatellite sequences. The heterogenous patterns of microsatellite instability (MSI) in the same tumor tissue indicate that genetically unstable tumor cells have different mutation rates[260]. Cells in tumor sites with low MSI level are, therefore, considered to be in the initial stage of the development of widespread MSI and are derived from a common progenitor

clone with a mutator phenotype or MMR deficiency[260]. In six patients which demonstrated various levels of MSI in the same tumor, frameshift mutations were documented in TGFβIIR (5 out of 6) and Bax (4 out of 6). In TGFβIIR, the mutations were in a tract of 10 deoxyadenosine $A_{(10)}$ while in Bax they were in a tract of eight deoxyguanosine $A_{(8)}$. Mutations in TGFβIIR and Bax were common to all tumor sites regardless of the degree of MSI, indicating that mutations in TGFβIIR and Bax are early events. Frameshift mutations in mononucleotide repeats of mismatch repair genes hMSH3 ($A_{(8)}$) and hMSH6 ($C_{(8)}$) were observed only in restricted areas of three out of six patients. This secondary MMR defect gives rise in turn to frameshift mutation in IGFIIR ($G_{(8)}$) as such mutations were only found in tumor tissue carrying mutations in hMSH3 and hMSH6[260]. From this study the genetic progression of a mutator pathway in gastric carcinoma was postulated. An initial mismatch repair defect targets mononucleotide tracts of TGFβIIR and Bax. Later in tumorigenesis, the primary mismatch repair defect may give rise to a secondary mismatch repair insult in hMSH3 and hMSH6 resulting in further mutation in IGFIIR.

A mutator phenotype which can lead to a generalized increase in susceptibility to the acquisition of somatic mutations was not observed in the breast cancer susceptibility genes BRCA1 and 2-associated breast tumors[285]. BRCA1 and BRCA2 are both implicated in cellular response to DNA damage and may function as caretakers to maintain the genomic stability of cells[286]. Whereas p53 was mutated in 66% of BRCA-associated tumors, this was not part of a general increase in sensitivity to somatic mutations[285]. Mutations were not detected in $p16^{INK4}$, a gene normally not mutated in sporadic breast cancer, nor in β-globin whose mutation does not confer any selective advantage. Also, no frameshift mutations were

present in polypurine tracts in the coding sequences of TGFβIIR or Bax. The authors concluded that the loss of function of BRCA1 and 2 do not confer a mutator phenotype such as that encountered in tumors with MSI[285]. It would be interesting, however, to measure mutation rates as a result of BRCA1 and/or BRCA2 loss using a more sensitive method such as the Big Blue transgenic mutagenesis system. Using transgenic mice carrying a *lacI* based transgenic shuttle-phage mutation detection system similar to Big Blue mice, thymic lymphomas arrising in *Msh2*$^{-/-}$ mice exhibited a large increase in *lacI* gene mutation frequencies[287]. The observed increase in mutation frequency was over the observed increase in normal *Msh2*$^{-/-}$ thymi already detected . *lacI* genes harboring clusters of mutations were observed leading to the suggestion that an additional mutator activity such as an error-prone DNA polymerase can lead to the increased genomic instability in Msh2$^{-/-}$ tumors [287]. It is important, however, to point out that clusters of mutations were also observed, albeit at lower frequency, in normal Msh2$^{-/-}$ thymi.

The relevance of a possible Bcr-abl induced mutator phenotype in the progression of CML is supported by a number of observations. The expression of Bcr-abl seems to be the sole important and consistent cytogenetical event in the chronic phase of CML [40]. Patients in the chronic phase of CML are 10,000 fold more likely to acquire acute leukemia than normal individuals[122]. This suggests that the expression of Bcr-abl results in the accumulation of secondary genetic abnormalities. This is further emphasized in transgenic mice expressing P190Bcr-abl. The presence of the activated oncogene in many lymphoid cells lead to multiple leukemias in the same mouse[206].

A direct role of c-abl in growth arrest [97] and DNA repair as a result of genotoxic

damage has been observed (Fig. 4). In response to irradiation, c-Abl inhibits cell cycle progression by down regulating Cdk2 in a p53 dependent manner[97]. Also, c-Abl directly associates with DNA-PK which is involved in DNA double-strand break (DSB) repair as well as V(D)J recombination [100]. In the presence of damaged DNA, DNA-PK phosphorylates and activates c-Abl and the complex can bind to the Ku antigen which is usually bound to DNA ends. The complex formation between c-Abl and PK-DNA was observed in response to irradiation as well as exposure to a DNA alkylating agent methyl methanosulphonate, suggesting the involvement of c-Abl in DNA repair induced by different agents[100]. c-Abl can phosphorylate the C-terminal repeated domain of RNA polymerase II [81] and an increase in tyrosine phosphorylation of RNA polymerase II is observed after the treatment of cells with methymethane sulfonate or ionizing radiation [288; 289]. This increase in phosphorylation is dependent on both c-Abl as well as ATM [288; 289]. The tyrosine phosphorylation of the C-terminal repeated domain of RNA polymerase II is associated with an increase in transcription suggesting that c-Abl might be involved in DNA damage-induced gene expression. The cytoplasmic pool of c-Abl is not regulated by the cell cycle machinery. Cytoplasmic c-Abl has a number of substrates such as the SH2/SH3 adaptor protein Crk and the Crk-binding protein p130Cas[158; 290]. c-Abl kinase activity increases following adhesion to the extracellular matrix (ECM)[82]. As such the tyrosine phosphorylation of p130Cas is dependent on ECM attachment [291]. Cellular activation of c-Abl involves its translocation from the nucleus to the cytoplasm. This process is controlled by integrin-mediated adhesion to the extracellular matrix protein implicating c-abl in a cell surface-nucleus cross-talk mechanism[82]. Loss of c-Abl tyrosine kinase activity is observed in fibroblasts following

detachment from ECM and activity is restored only after adhesion to fibronectin matrix[82]. Similar to the regulation of kinase activity, adhesion to ECM seems to control the subcellular localization of c-Abl[82]. After re-plating detached cells on fibronectin matrix, c-Abl transiently disappears from the nucleus during the first twenty minutes following re-plating, but then rapidly re-appear in the nucleus. The continuous shuttling of c-Abl between the nucleus and the cytoplasm is probably facilitated by the recently discovered nuclear export signal in c-Abl[76]. In CML, Bcr-abl expressing cells are defective in cell adhesion, even in the presence of normal integrin expression[194]. This has been shown to be the result of direct interaction between Bcr-abl and members of the focal adhesion complex [194; 199], disturbing actin cytoskeletal architecture [71], as well as altering the subset of β1 integrin expressed [188; 189]. Therefore, in a cell expressing Bcr-abl, it is possible that the normal activity of the intact c-Abl copy is annulled due to the lack of cell adhesion necessary for its activation. This can explain the increase in mutant frequency observed in kidneys and spleens of P190Bcr-abl mice reported here assuming that similar mechanisms result in the expression of Bcr-abl in human and mice cells. I think that this is a credible assumption given the similarity of Bcr-abl induced pathologies in mice models to disease encountered in humans [124; 125; 230]. This, however, does not explain the lack of kidney related tumors in those mice. I have shown an increase in expression of P190Bcr-abl in the kidneys of such mice compared to spleen (Fig. 32). It is possible that the mutator phenotype observed in kidney cells is not sufficient for oncogenicity by Bcr-abl. Mice transgenic for P190Bcr-abl die primarily of lymphoblastic leukemia/lymphoma, [232] although in an earlier study two mice with the same construct were diagnosed with myeloid leukemia [124]. Examination of cell-surface markers on leukemic

peripheral blood cells consistently yielded a pre-B phenotype [124; 232]. In leukemic mice, both splenic white and red pulp were extensively involved with neoplastic lymphoblasts positive for the B220 antigen, indicating a pre-B phenotype [124]. I was, therefore, surprised to observe Bcr-abl expression at a higher level in kidneys paralleled by a high mutant frequency when kidney related disease have never been reported before. Two scenarios have been proposed to explain restricted oncogenicity in this mouse model[232]. The first suggested that the difference in the absolute level of Bcr-abl expression in different tissue is responsible of the observed restricted oncogenicity[232]. My data argues against such a model. Alternatively oncogenicity by Bcr-abl might be dependent on the cellular environment where it is expressed [232]. Bone marrow precursor cells might contain cellular factors important in mediating the oncogenic potential of Bcr-abl [232]. The data presented here is in agreement with such model. I have observed a higher mutant frequency in kidneys of PL P190Bcr-abl mice suggesting, in addition, that a mutator phenotype alone independent of an appropriate cellular context is not sufficient for transformation by Bcr-abl. In mice homozygous for a mutation disrupting the *c-abl* gene, a specific and restricted effect in lymphoid cell development was encountered [79]. Those mice exhibited a drastic reduction in pre-B and immature B cells and to a lesser extent a similar reduction in developing T cells[79]. This was surprising given that the *c-abl* gene is expressed in all tissues in mammals. Homozygote mice for *c-abl* gene disruption did not demonstrate any changes in other cell populations in the lymphoid system or cells in the myeloid/macrophage lineages suggesting that pre-B cells might be uniquely sensitive and dependent on the level the Abl kinase[79].

Nevertheless, I have shown, using a sensitive *in vivo* system, an increase in mutant

frequency associated with the expression of Bcr-abl at a level relevant for disease induction in a mouse model. I suggest that this phenotype facilitate the accumulation of somatic mutations and thus the progression of CML towards a fatal transformed disease.

It is unclear whether c-Abl is involved in the repair of spontaneous basal DNA error or whether DNA repair genes are mutated in CML . It can be hypothesized that a Bcr-abl clone with an acquired mitogenic potential and lack of apoptosis can accumulate more than tolerable damage. Also the absence of at least a copy of c-Abl as the result of the translocation can affect the repair potential of the cell.

In addition to loss of c-Abl DNA repair potential, other biological properties of Bcr-abl can contribute to a mutator phenotype. Both P190Bcr-abl and P210Bcr-abl were found to be mitogenic in the murine myeloid progenitor cell line 32D [202]. Bcr-abl was found to constitutively activate the components of the cell cycle machinery that influence the G_1-to-S phase transition. Cells expressing Bcr-abl, therefore, do not require mutations in cell cycle factors that control the G_1 cell cycle check-point such as p53 and $p21^{WAF-1/CIP}$, and can proliferate independent of the genetic integrity of the cell. The mitogenic ability of Bcr-abl is also relevant in explaining the increase in mutant frequency observed in the kidney and spleen of P190Bcr-abl/Big Blue mice. Proliferative advantage can explain the expansion of spontaneous mutations observed as the majority of our reported mutations were GC→AT transitions at CpG dinucleotides similar to spontaneous mutations reported in *lacI* from Big Blue mice [292]. Indeed, studies have shown that cell proliferation is an important catalyst for the induction of mutation by dimethylnitrosamine (DMN)[293]. Doses that do not produce significantly high cell turnover in liver cells do not induce mutations[293]. Higher doses of

DMN resulting in high cell turnover produced an increase in mutant frequency.

Although cell proliferation is an important factor in the induction of mutation by genotoxic agents, there is little evidence that cellular proliferation alone can cause an induction in mutant frequencies by non-genotoxic agents[293]. In this context, the non-genotoxic liver carcinogen carbon tetrachloride (CCl_4) and phenobarbitol (PB) produced an increase in liver cell proliferation but failed to show any induction of mutant frequencies. [269] Those experiments were conducted after a short burst of proliferation induced by priming mice over a period of a week. However, longer priming with non-genotoxic agents that induce cell proliferation may result in the clonal expansion of spontaneous mutations or a clone of cells with a specific hit resulting in an activated oncogene [269; 293]. Along those lines, the exposure of Big Blue mice to the non-genotoxic agent O-anisidine produced mostly G:C→A:T transitions at CpG sites which is the mutation profile associated with spontaneous mutations in this system [292]. In this specific study no increase in the mutant frequency was observed, suggesting that mutants examined are spontaneous and not chemically induced and complicating by that the analysis of these results.

Technical caveats

Complications may arise from the interpretation of sequence analysis using the Blue transgenic mouse system due to mutations arising from *E coli* rather than in the mouse. However, previous studies have shown that 75% of C→T transitions in *lacI* from mouse systems occur at CpG dinucleotides whereas in *E. coli* C→T transitions do not occur at CpG dinucleotides. Also, in *E. coli* 67% of mutations are deletions or insertions at a TGGC tandem repeat but only 1.7% of such mutations in *lacI* from mice occur at this sequence[269;

[292]. The existence of a large data bank for *lacI* mutations in *E. coli* facilitate the task of distinguishing the origin of the *lacI* mutation encountered in Big Blue mice.

A plethora of reported mutational spectra in Big Blue mice or similar systems carrying bacterial genes induced by different chemical carcinogens exists. The mechanisms by which such mutagens act are usually well understood and present a useful tool to compare mutations in *LacI* obtained in hybrid mice carrying deletions in tumor suppressor genes or activated oncogenes. Methylnitrosourea (MNU), for example, acts by producing predominantly O^6-methylguanine adducts and results in G:C→A:T transitions [234]. Mutations from mice treated with benzo[a]pyrene, on the other hand, are predominantly transversions at G:C [294] while dimethylnitrosoamine (DMN) treatment results in C:G→A:T transitions in the liver[295] and 7,12-dimethyl-benzo[a]anthracene (DMBA) produced A:T→T:A transversions in most cases [293]. Also, the availability of transgenic mouse models for the study of cancer progression has extended the utility of the Big Blue system to the investigation of both qualitatively and quantitatively the accompanying changes in mutational profiles by combining such models with Big Blue mice.

To date a number of studies have taken advantage of such a strategy. p53 nullizygous mice are viable but develop predominantly thymic lymphomas at early age [235]. p53 nullizygous/Big Blue mice showed statistically indistinguishable mutant frequencies in liver, spleen, and brain alone or when all three tissues were combined [271]. When the DNA binding region of *lacI* in those mutants was sequenced, transitions at CpG dinucleotides were observed to be the dominant mutation in the three tissues. No significant differences in the type of mutations from wild type and nullizygous p53 mice was observed either. Transitions

and transversions at CpG dinucleotides made up 59% and 58% of all mutations respectively[271]. This pattern, though, was different than *lacI* mutations from *E coli*, where most mutations are transitions or transversions not at CpG dinucleotides [296]. In a follow up study, one out of four thymic lymphomas from $p53^{-/-}$ nullizygous mice showed an increase in mutant frequency with an over representation in A:T→G:C transitions compared to normal thymus from $p53^{-/-}$ mice [238]. The similarity between this mutation spectrum and that obtained from *E coli* carrying mutations in one of the mismatch repair genes Mut H, L, ans S lead the investigators to conclude that a somatic mutation in a DNA repair gene is responsible for the mutator phenotype and the high mutant frequency associated with that specific thymic lymphoma. In another study combining a transgenic mouse model for mammary adenocarcinoma and a λ-based mouse mutagenesis sytem utilizing the λ cII gene as a target of mutation, no significant differences in mutant frequencies from normal mammary epithelium, primary mammary adenocarcinoma, and pulmonary metastases was observed [297]. The predominant mutation in the *cII* gene was G:C→A:T transitions most of which occurred at CpG dinucleotides similar to the mutation spectrum observed in the *lacI* gene of Big Blue.

V- Conclusion

The acquiring of the Philadelphia chromosome (or Bcr-abl translocation) represents a detrimental patho-physiological event in humans. The activated tyrosine kinases which can potentially emerge from this translocation are associated with fatal hematological malignancies. The initial molecular dissection of Bcr-abl has linked the expression of the constitutively activated kinase with enhanced genomic instability. *In vitro* models have suggested that loss of cell-cycle regulation and induction of apoptosis are the underlying biological causes of this acquired genomic instability. It was postulated that the expression of Bcr-abl leads to the loss of those two genomic safe-guards and results in the accumulation of mutations leading to the development of malignant clones.

In this study I utilized a transgenic mouse model which expresses P190Bcr-abl in order to extend those findings to an *in vivo* system. My results demonstrate that the expression of Bcr-abl does not necessarily interfere with cell-cycle arrest or the induction of apoptosis following DNA damage. Bone marrow cells and spleen cells from pre-leukemic P190Bcr-abl mice both demonstrate normal cell-cycle arrest and apoptotic cell death after the induction of DNA damage by irradiation. These observations, which contradict the *in vitro* effects of Bcr-abl expression, lead me to directly evaluate the consequence of Bcr-abl expression on genomic instability. This was achieved by measuring mutation frequencies in P190Bcr-abl mice crossed to the Big Blue *in vivo* mutagenesis mouse system. I found that the expression of Bcr-abl in both spleens and kidneys conferred a mutator phenotype

determined by a statistically significant elevation in mutation frequencies.

I propose that the P190Bcr-abl/Big Blue mouse is a useful model to investigate genomic instability associated with Bcr-abl expression. This mouse model can be beneficial in assaying the potential therapeutic effect of different tyrosine kinase inhibitors in reversing the mutator phenotype observed with Bcr-abl expression. I think that the results obtained using this approach will be beneficial not only in understanding the pathogenesis of CML, but could also be used to assess if the aberrant tyrosine kinase expression associated with more common cancers similarly induce a mutator phenotype.

Claims to originality

The following results presented in this thesis constitute original findings:

1- At physiological levels sufficient to cause leukemia, Bcr-abl expression in the P190Bcr-abl leukemia transgenic mouse model does not result in an inhibition of cell-cycle arrest following the induction of DNA damage in both bone marrow and spleen cells.

2- At physiological levels sufficient to cause leukemia, Bcr-abl expression in the P190Bcr-abl leukemia transgenic mouse model does not interfere with the induction of $p21^{WAF1/CIP1}$ in the spleen, lung, and kidney of such mice.

3- At physiological levels sufficient to cause leukemia, Bcr-abl expression in the P190Bcr-abl leukemia transgenic mouse model does not cause the abrogation of irradiation-induced apoptosis in bone marrow and spleen cells.

4- Tumor tissue from leukemic P190Bcr-abl mice show normal induction of both $p21^{WAF1/CIP1}$ expression and apoptosis following the induction of DNA damage by irradiation.

5- A mutator phenotype represented by an increase in mutation frequency in spleens and kidneys of P190Bcr-abl transgenic mice was found. This is the first report of an *in vivo* increase in mutation frequency, using the Big Blue™ mutagenesis detection system, in tissues from a transgenic mouse expressing an activated tyrosine kinase.

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