MOUSE MODELS IN LEUKEMIA

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1. Bcr negatively regulates superoxide production in polymorphonuclear leukocytes and its absence results in oxidative tissue damage and multiple organ system failure during Gram negative septicemia.

this thesis

2. Expression of the chimaeric oncoprotein P190 *BCR/ABL* in transgenic mice causes genomic instability in pre B-lymphoid cells, which ultimately results in tumor progression.

this thesis

3. P190 Bcr/Abl and P210 Bcr/Abl cause clinically distinct leukemias in transgenic mice and provide evidence for specific biochemical differences between these two chimaeric oncoproteins.

this thesis

4. The effects of *BCR/ABL* expression in cell lines other than those of hematopoietic origin are doubtlessly valid *in vitro*, but have little bearing on the *in vivo* situation.

this thesis

5. The inability of some *grown-ups* to draw a mouse as anatomically correct as a masterful five year old does, is direct evidence that some of us indeed never fully lose the *child* within us.

this thesis

6. The prefix *c-* to *c-BCR*, used by Reuther et al., incorrectly implies that the human *BCR* gene has a viral counterpart, *v-bcr*, and that *BCR* is an oncogene.

Reuther GW, et al. Science 266:129, 1994

7. The conclusion of Daley et al., that both chronic phase and blastic transition could be mimicked by serial transplantation of *BCR/ABL* expressing bone marrow cells to recipient mice, is not justified.

Daley GQ, et al. Proc Natl Acad Sci USA 88:11335, 1991

8. Research carried out in cultured cells, should be referred to as *'in vitro'* or *'in whole cells'*, and not as *'in vivo'* research.

9. Without animal models, the different biological functions of small GTP-binding proteins $p21^{Rac1/2}$, $p21^{CDC42}$ and $p21^{Rho}$ will be very difficult to ascertain.

10. The claim by Letterio et al., that maternal rescue explains the phenotypic differences between TGF-β1 null-mutant pups born from heterozygous mothers, versus those born from mothers homozygous for allelic TGF-β1 inactivation has not been convincingly validated.

Letterio JJ, et al. Science 264: 1936, 1994

11. The ambivalence in statistical analysis of the oxygen isotope signature of phosphate in fossilized bones of *Tyrannosaurus rex* indicates, that we will probably never know whether this large theropod was endothermic or ectothermic.

Millard AR. Science 267:1666, 1995; Barrick RE, et al. Science 267:1667, 1995

12. Conducting science in a place where tectonic plates collide, gives additional meaning to the notion "earth-shocking discoveries".

13. "The mind is like a TV set - when it goes blank, it's a good idea to turn off the sound."

Propositions belonging to the dissertation:

'Mouse Models in Leukemia'

by Jan Willem Voncken, June 1995

Contents

'Mouse'. By Rogier (1½ years old)

CHAPTER 1

Introduction

1.1. Hematopoietic development; a brief overview

Hematopoiesis in higher vertebrates is a complex and accurately controlled process. Blood cells originate from a population of totipotent hematopoietic stem cells, which retain proliferative and regenerative capacity, and underlie the continuously renewing hematopoietic system (Broxmeyer, 1991; Tavassoli, 1991).

A myriad of cytokines, acting either alone or in concert, are capable of stimulating proliferation of hematopoietic stem cells and progenitor cells and/or promoting their subsequent differentiation *in vitro* and *in vivo*. These factors are at the basis of a dynamically regulated hematopoietic homeostasis, which allows an organism to adequately respond to altered demands (Sachs, 1987; Metcalf, 1989; Harmening, 1992).

1.2. Leukemia

Leukemia is a blood disease characterized by a great increase in numbers of abnormal white blood cells. Uncontrolled production of leukocytes is caused by cancerous mutation of a myelogenous or a lymphogenous cell. Usually, the more undifferentiated the cell type, the more acute the leukemia is, often leading to death within a few months if left untreated (Guyton, 1991).

Leukemia is accompanied by metastatic growth of leukemic cells in abnormal areas of the body. Leukemic cells of the bone marrow may be produced in such great numbers that they invade surrounding bone, causing pain and a tendency to easy fracture. Almost all leukemias spread to the spleen, lymph nodes and other well vascularized regions, regardless of whether the origin of the leukemia is in the bone marrow or in the lymph nodes. The rapidly growing cells invade the surrounding tissue, depleting these tissues of metabolic elements and consequently causing tissue destruction and metabolic wasting of the organism.

Increased susceptibility to infection, as a result of immune system failure, severe anemia and bleeding tendency caused by thrombocytopenia (lack of platelets) are all very common effects in leukemia and result mainly from displacement of the bone marrow by non-functional leukemic cells.

1.3. Genetic basis of human cancer

Most cancers in human are of genetic origin: accumulation of multiple mutations within the DNA of a single somatic cell cause the cell to lose growth control (Hopkins, 1987). The concept of *somatic mutation* of cancers was proposed early this century (Boveri, 1929) and later specified (Nowell, 1970; Knudson, 1975). Karyotypically visible abnormalities have been known to occur in neoplasias (Von Hansemann, 1890; Boveri, 1914) since the turn of the last century. For many years it had been suspected, that such abnormalities might cause cancer by alteration of specific genes or their expression. Only in the last decades, due to introduction and continuous improvement of cytogenetic and molecular techniques, has the significance of chromosomal aberrations in the onset and progression of cancers become clear: analysis of recurrent cytogenetic abnormalities in cancer has led to the identification of many *oncogenes*, genes involved in the development of cancer (Bishop, 1991; Rabbitts, 1994).

• Cellular oncogenes in cancer; viral origin

A significant part of current conceptual understanding of carcinogenesis in vertebrates is derived from virologic research: cellular oncogenes were first discovered as cellular counterparts of transforming oncogenes found in retroviruses. At the beginning of this century it was shown that some avian leukemias and sarcomas were transmissible in cell-free extracts (Ellerman and Bang, 1908; Rous, 1910). This pointed to an infectious nature of certain cancers. Following the isolation of mammalian tumor viruses in the early 60's, interest in oncogenic viruses was greatly enhanced. At present, viruses that can cause cancer

and transform cells in culture include several DNA tumor viruses (families: Papovaviridae, Adenoviridae and Herpetoviridae) and RNA containing viruses (family: Retroviridae). All oncogenic viruses of the latter family belong to the subfamily Oncovirinae. Among these oncornaviruses, C-type virus particles are equipped with the ability to transduce genes of cellular origin onto their own genome. These so-called viral oncogenes (v-*onc*) are, due to altered expression and/or acquired genetic mutation(s) of the cellular genes, responsible for initiation and maintenance of malignant cellular transformation. The cellular counterparts of v-*onc*s appear to be evolutionary conserved sequences and are, under normal circumstances, often implicated in growth control. These genes are referred to as cellular (proto-)oncogenes (c-*onc*). Since the first isolation of Rous sarcoma virus from avian sarcomas, many more oncornaviruses have been found in chicken, turkey, mice, rats, cats and monkeys. To date, over 30 different viral oncogenes are known (Varmus, 1989; Bishop, 1991), a number which is still increasing.

While numerous transforming viruses have been isolated, viral carcinogenesis in animals is a relatively rare process and even more so in human. There are several examples where tumor viruses may be implicated in human cancer, such as hepatitis B (and C) in hepatocellular carcinoma, human papilloma virus in anogenital cancers, Epstein-Barr virus in nasopharyngeal carcinoma and Burkitt's lymphoma, human T-cell leukemia/lymphoma viruses in adult T-cell leukemia/lymphoma. These are nonetheless rare cases, and the mechanism(s) by which the viruses cause the disease are often not understood (Hopkins, 1987). Since the discovery of proto-oncogenes in the mammalian genome, however, it has become clear that, analogous to the role of v-*oncs* in viral carcinogenesis, these c-*oncs* play a key role in human carcinogenesis as well (reviewed by: Varmus 1989; Bishop, 1991). The development of a fully malignant tumor involves the activation or altered expression of oncogenes, or the inactivation of tumor suppressor genes that control normal cellular development. It generally is accepted that approximately 80% of all cancers have an environmental component (Higginson, 1993): the majority of human cancers arises by chance, as a result of (accumulation of) mutation(s) through exposure to various occupational or environmental genotoxins (e.g. tobacco, asbestos and radon (lung cancer), UV light (melanoma), ionizing radiation (leukemia)). Only in a few instances are inherited predisposing mutations known to be responsible for tumorigenesis (e.g. Wilms' tumor; retinoblastoma).

- **Mechanisms of oncogenic activation**

Among cytogenetic changes known to be at the basis of oncogenic transformation, are: point mutations (such as those in *RAS*, *P53*, *Rb* genes in various cancers; reviewed by Bishop, 1991), deletions (often resulting in loss of tumor suppressor genes, like the *Rb* gene in retinoblastoma; reviewed by Marshal, 1991), gene amplifications (among the best characterized are the *MYC* gene amplifications in neuroblastomas and *RAS* gene amplifications in primary lung, bladder, colon and rectum carcinoma; reviewed by Bishop, 1991), and gene inversions and translocations (reviewed by Rabbitts, 1994). Among the latter group, mutations consistently found in certain tumor types (specific), and those found in isolated cases (idiopathic) are distinguished.

Historically, much information has been gathered from studying chromosomal aberrations in leukemias, because of easy access to primary tumor cells. Leukemia often results from specific chromosomal translocations and inversions, that cause activation of proto-oncogene products (through positioning of a T-cell receptor gene or an immunoglobulin gene in close proximity to a proto-oncogene) or, more commonly, creation of tumor specific fusion proteins (chimaeric oncoproteins). Affected genes in both categories often involve transcription factors. In solid tumors, the majority of translocations results in the formation of chimaeric oncoproteins. The combined cytogenetic and molecular biological findings suggest that protein fusion may prove to be a general theme in malignant chromosomal translocation (Rabbitts, 1994).

1.4. The Philadelphia chromosome in leukemia

One chromosomal abnormality in particular has served as a paradigm for investigation of human leukemia and of other forms of malignant disease. Not only was the Philadelphia chromosome (Ph^1) the first recognized cytogenetic aberration consistently associated with a specific form of cancer, cytogenetic and molecular dissection of the breakpoint on chromosomes 22 and 9 and its gene-fusion product have been pivotal in outlining and understanding some of the current mechanistic concepts that underlie oncogenic transformation. The Philadelphia translocation in specific types of leukemia and the genes involved will be discussed in detail below.

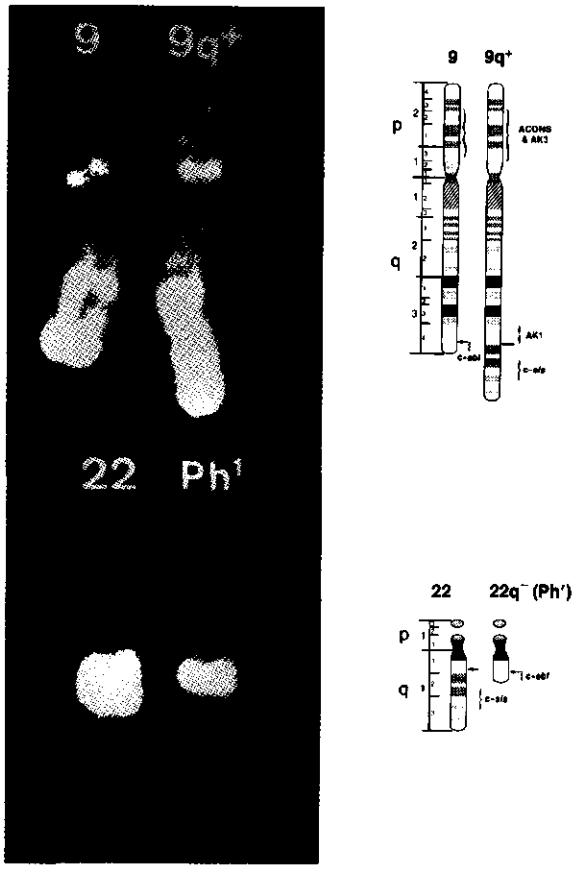

Figure 1. The Philadelphia (Ph) translocation. (Left) The chromosomes involved in the Ph translocation, chromosomes 9 and 22, and the resulting abnormal chromosomes $9q^+$ and $22q^-$. (Right) A schematic drawing of this chromosomal translocation with the approximate locations of the abl and sis oncogenes. Arrows indicate the positions of the chromosomal breakpoints (figure taken from Heisterkamp and Groffen, 1991).

Chronic myelogenous leukemia (CML) was first recognized as a clinical entity around the middle of the last century. In the ensuing years the disease was distinguished from other myeloproliferative disorders on the ground of its distinct clinical and pathological features. The first real clue to its pathogenesis was the landmark discovery of an abnormally small chromosome in 1960 (Nowell and Hungerford, 1960). The *Philadelphia chromosome* (Ph), named in honor of the city in which it was discovered, was the first specific chromosomal abnormality to be consistently associated with one particular form of human cancer.

A decade after the first description of the *Ph*-chromosome, it was identified as a modified chromosome 22 (Caspersson et al. 1970). Subsequently, it was shown that the abnormal chromosome was the product of a translocation, [t(9:22)(q34;q11)], between chromosome 22 and chromosome 9 (Rowley et al., 1973), in which the distal portion of the long arm of chromosome 22 (22q11.21 to qter region) had become joined to chromosome 9 at band q34 (Fig. 1). Despite the general assumption that the translocation was reciprocal, this was not confirmed at the molecular level until 10 years later when it was shown, that a gene (*ABL*) encoded on a small segment of chromosome 9 (q34.1 to qter), moved to chromosome 22 at band q11 (De Klein et al., 1982; Bartram et al., 1983; Groffen et al., 1984).

The association between the presence of the *Ph*-chromosome and CML is well established: over 95% of all patients have this chromosome in their leukemic cells. Although literature has customarily referred to *Ph*-positive and *Ph*-negative CML, some of the remaining 5% of patients were found to have '*Ph*-translocations', which were often not microscopically visible, but which could be detected using molecular techniques (reviewed by: Mitelman, 1993). In recent years it has been suggested that the remaining class of CML patients lacking this chromosomal translocation altogether do not have true CML but rather suffer from other myeloproliferative disorders (reviewed by: Clarkson and Strife, 1991). The *Ph*-chromosome is also found in a low percentage of *acute myeloid leukemia* (AML; also referred to as acute non-lymphocytic leukemia; ANLL) and in adult and childhood *acute lymphoblastic leukemia* (ALL). The percentage of ALL patients reported to have a *Ph*-chromosome varies from 2-6% in children to 17-25% in adults (Priest et al., 1980; Champlin et al., 1985).

- **Origin and pathogenesis of ALL and CML**

Acute lymphoblastic leukemia (ALL) is the major subtype of pediatric cancer in developed countries. ALL is a biologically diverse disease and is divided into broad subgroups, according to immunological and karyotypic criteria. These subtypes correspond roughly to T and B-cell precursor populations. The target cell for ALL is either a multi-potential stem cell or a lymphoid (T or B) stem cell. Childhood and adult *Ph*-positive ALL are acute in onset and invariably of pre-B lymphoblastic origin. Despite advances in understanding of cell and molecular biology of ALL, the etiology of ALL remains elusive. Some leukemogenic factors have been defined, however, including exposure to high levels of radiation, Down's syndrome and other chromosome fragility disorders (Greaves, 1990).

The distinctive and diverse chromosomal abnormalities associated with ALL subtypes most probably reflect important components of the etiological mechanisms involved. The Philadelphia translocation is the most common chromosomal abnormality in ALL. ALL has been referred to as clinically remarkable in that a substantial portion of cases are curable with relatively modest doses of chemotherapy and prophylactic CNS radiation. However, the cytogenetic *Ph*-marker is associated with a very poor clinical prognosis: patients with Ph^+ ALL are rarely cured (Fletcher et al., 1991; Greaves, 1990).

The incidence of CML in most Western countries is about 1.5 per 100.000 population per year and accounts for about 15 percent of all cases of leukemia. About 3500 new cases are diagnosed in the USA each year. The cause of CML and the mechanisms, which determine its progression, are presently unknown. It is an acquired disease, but there are no clues to its etiology other than an increased incidence

in individuals with exposure to increasing doses of ionizing radiation. This has been noted following chronic exposure in radiologists, who practiced without adequate shielding, in patients submitted to radiation treatment, and in individuals exposed to a high doses of radiation from the detonation of nuclear weapons such as in Japan in 1945.

CML is a clonal myeloproliferative disorder, arising from neoplastic transformation of a hematopoietic progenitor cell and is characterized clinically by clonal expansion of the myeloid compartment, involving neutrophils and their direct precursors. This *chronic phase* is characterized by marked hyperplasia of myeloid cells, that maintain their maturation capacity, and is easily controlled with therapy.

The disease goes through an *accelerated phase* during which the myeloid cells gradually lose their capacity for terminal differentiation. Basophilia, thrombocytosis, and cytogenetic clonal evolutions also appear. Ultimately the disease undergoes a transition to a more aggressive type leukemia *(blastic phase* or *blast crisis*) on average four years after diagnosis (i.e. chronic phase). At this point, *Ph*-positive cells appear to have lost their capacity to differentiate and the rapid accumulation of cells, that retain a 'blast' morphology and are highly resistant to therapy, is directly or indirectly responsible for the invariably fatal outcome of the cancer. Blast crisis can be classified as myeloid, lymphoid or undifferentiated, based on morphologic, cytochemical and immunologic features of the cells (reviewed by: Kantarjian et al., 1991).

Treatment has been attempted for over 60 years. Currently, the most promising protocols are bone marrow transplantation, for which only a select group of patients is eligible, and interferon treatment.

The presence of the *Ph*-chromosome in erythrocyte, granulocyte, monocyte, lymphocyte and megakaryocyte precursors in CML seems to point to the original genetic mutation having occurred in an ancestral cell common to these cell types, but the exact location of the transforming event within the progenitor cell lineages remains unclear. Usually, all nucleated cells in the bone marrow are *Ph*-positive at the time of diagnosis. *Ph*-negative normal cells persist, but their growth is apparently suppressed by the leukemic cells. The *Ph*-chromosome is absent in the majority of mature lymphocytes, although in about 20-25% of patients in chronic phase some B cells contain the *Ph*-marker and early B cell progenitors predominate in about 25% of patients in blastic transformation (Champlin and Golde, 1985). T lymphocyte involvement has only rarely been reported, although recent findings would indicate that bilineal (T lymphoid/myeloid) *Ph*-positive progenitors may be implicated in some cases of blastic transformation (Akashi et al., 1993).

Tumor progression is a fundamental feature of cancer biology. Cancers do not arise *de novo* in their final form, but begin as small indolent growths, which gradually acquire characteristics associated with malignancy. Most leukemias, as is also the case with solid tumors, undergo clonal development and karyotypic evolution. As a result of the original leukemic clones acquiring additional genetic abnormalities, a proliferative advantage is conferred on subclones, which become increasingly malignant. About 10% of CML patients present at diagnosis with other chromosomal abnormalities in addition to the *Ph*-chromosome (Clarkson and Strife, 1991). At the time of blast crisis, approximately 8 out of 10 CML patients show additional chromosome abnormalities. Non-random translocations are most common, but additions, deletions, inversions, duplications and other structural chromosome abnormalities also occur frequently (Clarkson and Strife, 1991). In *Ph*-positive ALL, more than 90% of cases have identifiable chromosomal abnormalities at diagnosis, with structural changes occurring more frequently than numerical changes (75% vs. 60%). In addition, often other chromosome translocations are present besides the *Ph*-chromosome (Williams et al., 1990).

1.5. Molecular insight into the Philadelphia translocation

A cellular oncogene designated *ABL* is located on chromosome 9 in band 9q34. The possibility of *ABL* involvement in the *Ph*-translocation was substantiated, when translocation breakpoints were discovered within its 5' region (De Klein et al., 1982; Heisterkamp et al., 1983, 1983a; Groffen et al., 1987). In the *Ph*-translocation, part of it is moved to chromosome 22 (Heisterkamp and Groffen, 1991; Heisterkamp et al., 1993a). The majority of breakpoints in *ABL* occurs in the large intron between the two alternative first exons, 1B and 1A, and in that between 1A and 2 (Fig. 2). Some breakpoints occur in the main body of exons, sporadically upstream of exon 1B (Morris et al., 1990, and references therein). The location of the breakpoint is directly responsible for what happens on a molecular level in the *Ph*-translocation: a truncated *ABL* proto-oncogene, missing its regulatory promoter region, is joined to sequences on chromosome 22 (Fig. 3).

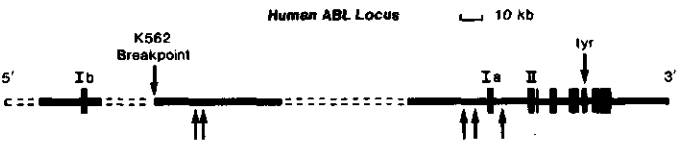

Figure 2. The human ABL locus. The genomic DNA of the ABL locus is represented by the horizontal lane, with the bracket and the solid lines indicating the regions not cloned and cloned to date. The alternative exons Ib and Ia and the common exon II are marked, as well as the exon containing the phosphotyrosine acceptor site (tyr; Groffen et al., 1983). Arrows below the graph point to the position of chromosomal breakpoints on chromosome 9 in 5 CML patient DNAs. The arrow above the map indicates the position of the breakpoint in the CML cell line K562, which has been used in numerous in vitro studies (figure taken from Heisterkamp and Groffen, 1991).

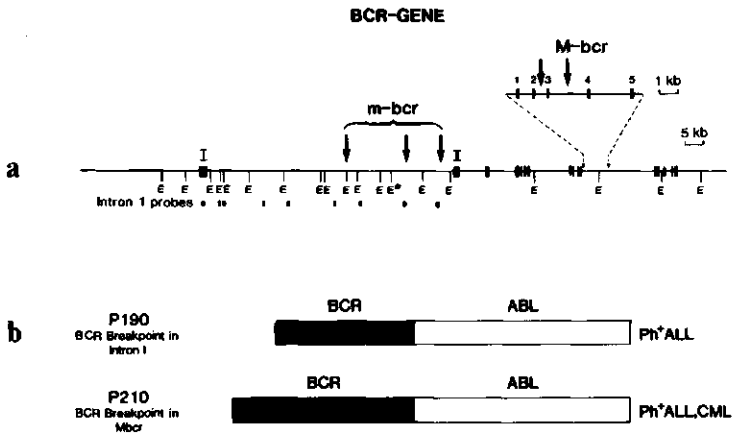

Figure 3. The human BCR locus and its chimaeric translational products. (A) More than 95% of breakpoints in CML are located within the M-bcr. In a subclass of Ph+ ALL, breakpoints are found within m-bcr. Exons are indicated with boxed areas in the restriction map. E = EcoR1, E denotes a polymorphic EcoRI site. (B) The chimaeric Bcr/Abl oncoproteins are shown schematically; the Abl moiety is the same in both types of patients, whereas the contribution of Bcr domains varies (see also Fig. 5; figure taken from Heisterkamp and Groffen, 1991).*

The breakpoints on chromosome 22 are restricted to relatively limited regions of DNA located within a gene designated *BCR* (*b*reakpoint *c*luster *r*egion; Fig. 3). The *BCR* gene is oriented with its 5' end toward the centromere of chromosome 22. Breakpoints occur within certain introns; as a consequence, what remains on chromosome 22 is part of a gene still containing its promoter and a varying number of 5' exons. The 3' exons are usually translocated to chromosome 9; their relevance for leukemogenesis is thought to be minor. The juxtaposition of the promoterless part of *ABL* to the 5' *BCR* segment seems to be the crucial event. More complex and variant translocations occur, in which the 5' *BCR*/3' *ABL* sequences are found on other chromosomes, or on an apparently normal chromosome 22. In all these cases, however, the unifying common event is the joining of *BCR* and *ABL* (reviewed in Heisterkamp and Groffen, 1991).

Because of their orientation with respect to each other, the two genes can be transcribed as one single chimaeric *BCR/ABL* gene (Shtivelman et al., 1985). The number of *ABL* exons found in the chimaeric transcript is relatively invariant (Fig.3). The contribution of the *BCR* gene to the chimaeric *BCR/ABL* oncogene differs. When the breakpoint lies within the first intron of *BCR* (within the minor breakpoint cluster region, or m-*bcr*), the hybrid transcript contains only *BCR* exon 1. In other instances, the break occurs within the major breakpoint cluster region (M-*bcr*) between exons 10 and 14 (or, by convention designated: M-*BCR* exons 1-5). Exon 1 of *BCR* as well as M-*BCR* exons 2 and 3 end after the first nucleotide of a triplet; since *ABL* exon 2 starts with the second nucleotide of a triplet, a correct open reading frame is maintained upon splicing of these *BCR* exons to *ABL* exon 2 (with the exception of the codon joining the two genes). The short, 7 kb *BCR/ABL* mRNA, that contains only *BCR* exon 1, encodes a protein 185,000-190,000 Dalton, and is designated P185 or P190 correspondingly. When the breakpoint is located in the M-*bcr* region, the messenger RNA's are around 8.5 kb in length. The corresponding proteins are 210.000 Dalton in size and referred to as P210. P190 is mainly found in juvenile and adult ALL, although it has occasionally been found in blastic phase of CML (Grosveld, 1990). The P210 is present in both CML and ALL.

- **The *ABL* proto-oncogene**

The *ABL* proto-oncogene was first identified in the mammalian genome (Goff et al., 1980) by its homology to the oncogene of Abelson murine leukemia virus (A-MuLV; Abelson and Rabstein, 1970). It has since been found to generate two more oncogenes, one of which is the v-*abl* of Hardy-Zuckerman feline sarcoma virus (HZ2) FeSV; Hardy-Zuckerman, 1970: Besmer et al., 1983). In the early 1980's ABL was implicated in human cancer for the first time, when it was shown that the proto-oncogene was translocated to the Philadelphia chromosome in CML (De Klein et al., 1982; Heisterkamp et al., 1983, 1983a; Groffen et al., 1987). This discovery, as was the case for *BCR*, heralded the beginning of intensive research into the molecular biology of the *ABL* gene.

The *ABL* proto-oncogene extends a region of at least 200 kb of DNA, its 3' end pointed toward the telomere of chromosome 9. The *ABL* gene has two alternative first exons, 1B and 1A, which are separated by a large 175 kb intron. The second intron is 17 kb. The main body of *ABL* exons spans only 32 kb.

The product of the c-*ABL* proto-oncogene is a non-receptor protein-tyrosine kinase that is ubiquitously expressed in mammalian cells. *ABL* homologs are found in different animal species and together constitute the *abl* family of tyrosine kinases (reviewed by Wang, 1993). The tyrosine specific protein kinase family includes transmembrane growth factor receptors like epidermal growth factor (EGF) receptor, insulin growth factor (IGF) receptor, platelet derived growth factor (PDGF) receptor and colony stimulating factor-1 (CSF-1, M-CSF, c-*fms*) receptor (Sefton et al., 1981; Groffen et al., 1983; Reddy et al., 1983). These receptors have the capacity to phosphorylate themselves (auto-phosphorylation). Abl is closely

related to a group of cytoplasmic or membrane associated tyrosine kinases that lack a transmembrane and/or extracellular domain, including proteins encoded by proto-oncogenes such as *SRC*, *ARG* and *FES/FPS*. However, the protein kinases are targeted to cellular membranes through N-myristoylation of an N-terminal sequence in the protein (reviewed by Resh, 1990)

The c-Abl protein contains at least four functional domains (Fig. 4): An N-terminal variable domain, encoded by two alternative exons (called Type I and IV in mouse c-*abl* (Meijer et al., 1987; Oppi et al., 1987), or Type 1a and 1b in human c-*abl* (Ben-Neriah et al., 1986; Shtivelman et al., 1986), a kinase regulatory domain which includes two *Src* homologous regions (SH3 and SH2), the tyrosine kinase or SH1 domain (this region harbors the enzymatic domain common to the oncogene family) and a large C-terminal segment that is unique to the Abl family of tyrosine kinases. Deletion of N-terminal domains within Abl activates the Abl-kinase (Franz et al., 1989; for further discussion see: '*Molecular mechanisms for BCR/ABL mediated leukemogenesis*'; this paragraph), as do a myriad of other mutations (Rees-Jones and Goff, 1988). SH2 domains recognize short peptide motifs bearing phosphotyrosine residues and function in protein-protein interactions (Koch et al. 1991; Pawson, 1995). Although SH2 domains are not required for catalytic activity, specific point mutations within these modules can inactivate the Abl-kinase (Mayer et al., 1992). SH3 domains are, like SH2 domains, also involved in signal transduction, through recognition of proline residues in peptide motifs, although their exact biological function is unclear (Koch et al., 1991; Pawson, 1995). In c-Abl(IV), deletion of the SH3 domain activates the kinase activity of Abl (Franz et al., 1989), as does insertional mutation in the region between the SH3 and the SH2 domain. This suggested that the spatial orientation of both domains is critical for regulation (Jackson and Baltimore, 1989) and that somehow the SH3 domain serves as a inhibitory regulator of the tyrosine kinase. Whether this inhibition is *trans* (through inhibitors) or *cis* (through intramolecular interaction) in nature, is currently not known (reviewed by Wang, 1993).

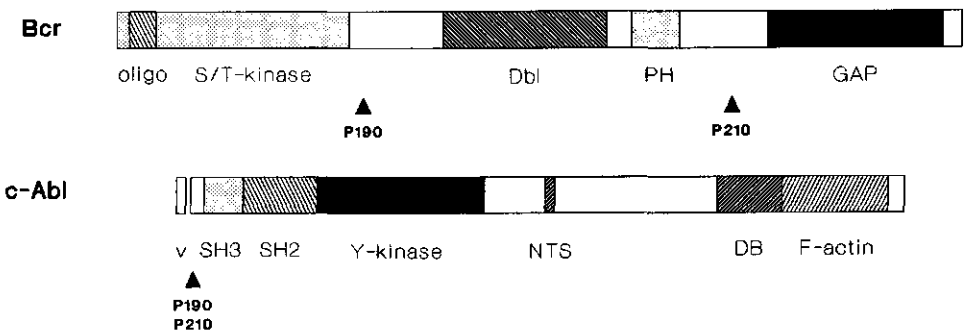

Figure 4. *Schematic depiction of structural domains within the Bcr protein (upper) and the Abl protein (lower). In p160^{Bcr} the N-terminal oligomerization domain (oligo), the approximate region responsible for the serine/threonine kinase activity in the protein (S/T-kinase), the Dbl-homology domain (Dbl), the pleckstrin homology domain (PH) and the C-terminal GAP domain (GAP) are indicated. In p145^{Abl}, the variable domain (v) is encoded by two alternatively spliced first exons (see text). Five additional domains include the Src homology domains SH3 (SH3), SH2 (SH2) and SH1, which is the tyrosine kinase domain (Y-kinase), a DNA binding domain (DB) and an F-actin binding domain (F-actinB). The putative nuclear translocation signal is marked with NTS. Arrows in the figure indicate the approximate positions in the Bcr protein and in the Abl protein, N-terminal and C-terminal of which, respectively, domains are included in the chimaeric Bcr/Abl P190 and P210 oncoproteins; the contribution of Bcr to Bcr/Abl in P190 and P210 differs, while the Abl moiety in both Bcr/Abl oncoproteins is identical (see also: Fig. 3).*

The c-Abl proto-oncoprotein is localized in both the cytoplasm, where it is weakly associated with F-actin, and the nucleus, where it co-localizes with chromatin. Nuclear translocation, DNA-binding, and F-actin binding are mediated by the large, non-catalytic C-terminal segment of the protein (Kipreos and Wang, 1992; McWirther and Wang, 1993a; Van Etten et al., 1989). The nuclear translocation of Abl is cell cycle dependent and corresponds to phosphorylation of serine/threonine residues in the protein. The DNA binding function of Abl is regulated by *cell cycle-dependent cyclin 2* (cdc2)-mediated phosphorylation (Kipreos and Wang, 1990; Kipreos and Wang, 1992).

The non-receptor tyrosine kinase $p145^{Abl}$ negatively regulates cell growth when overexpressed in fibroblasts (Sawyers et al., 1994), a function which contrasts with that of *abl* fusion oncogenes such as v-*abl* and *BCR/ABL*. *ABL* null-mutant mice are runted and die within 2 weeks after birth for unknown reasons. In addition, many show B and T cell lymphopenia, the cause of which also remains unknown (Tybulewic et al., 1991; Schwartzberg et al., 1991).

Although it seems clear that the Abl protein is involved in cell cycle regulation, at present, neither the mechanisms that regulate the normal c-Abl function nor the signal transduction pathways, that Abl is part of, are well understood.

- **The *BCR* gene**

The clustering of chromosomal breakpoints in a specific region of chromosome 22 (Groffen et al., 1984) in blood cells of leukemic patients, led to the discovery the *BCR* gene (Heisterkamp et al. 1985).

In human, the *BCR* gene is ubiquitously expressed. In the mouse, *bcr* expression is detectable as early as the zygote stage, continues throughout embryogenesis (unpublished observations) and is found in most adult tissues examined with a relatively high expression in brain (Heisterkamp et al., 1993).

The *BCR* gene encodes a 160 kDa cytoplasmic protein with several functional domains (Fig. 4). The N-terminal domain encoded by *BCR* exon 1 has an (associated) serine/threonine kinase activity *in vitro* (Maru and Witte, 1991), can oligomerize (McWirther and Wang, 1993), and is capable of binding other protein factors, among which $p145^{Abl}$ (Sawyers et al., 1991; McWirther et al., 1993). Bcr itself is phosphorylated on serine and threonine residues, the significance of which is currently not understood. The central part of Bcr has homology to the *Dbl* proto-oncogene, a guanine nucleotide-exchange factor (GEF) for human CDC42 (Eva and Aaronson, 1985; Hart et al., 1991), suggesting a similar catalytic activity for Bcr. The biological significance of the pleckstrin-homology domain in Bcr is as of yet unclear (Boguski and McCormick, 1994; Cohen et al., 1995).

In vitro, the C-terminal end of $p160^{Bcr}$ harbors GTPase activating protein (GAP) activity toward the small GTP-binding proteins Rac1 and Rac2 and CDC42 (Diekmann et al., 1991; Hart et al., 1992). Rac1 and Rac2 belong to the Rho family of small $p21^{Ras}$-like GTPases, members of which are involved in cyto-skeletal organization. Rac1 is required for growth-factor-induced membrane ruffling of Swiss 3T3 fibroblasts. Bcr can regulate this Rac1-mediated process *in vitro*, since microinjection of the purified Bcr GAP domain abolishes ruffling (Ridley et al., 1993). A number of other proteins are also GAPs towards Rac1 and Rac2 *in vitro*, including rhoGAP, ß-chimerin, n-chimerin, ABR and P190 (Diekmann et al., 1991; Leung et al., 1994; Ahmed et al., 1994; Heisterkamp et al., 1993b; Ridley et al., 1993).

Based on the information described above, Bcr is likely to play a role in signal transduction. To date, no naturally occurring mutants have been identified, other than the truncated Bcr in the Bcr/Abl fusion oncoprotein.

- **Molecular mechanisms for *BCR/ABL* mediated leukemogenesis**

Currently, at least three different c-abl derived oncogenes have been identified in oncogenic retroviruses known to cause cancer in mouse and cat (A-MuLV and (HZ2) FeSV respectively) and from human neoplasias (BCR/ABL in ALL and CML). In all of these oncogenic proteins, the Abl tyrosine kinase is constitutively activated. The mechanisms for oncogenic activation are different for the three oncogenic

forms. Activation of v-*abl* involves deletion of both the SH3 domain or the C-terminal part (A-MuLV or (HZ2) FeSV respectively) and fusion with viral protein (Gag) sequences. Activation of the transforming potential of Gag-v-Abl is primarily due to deletion of the SH3 domain (Fig. 5); additional mutations in the Abl moiety may contribute to the oncogenicity of Gag-v-Abl (Rees-Jones and Goff, 1988).

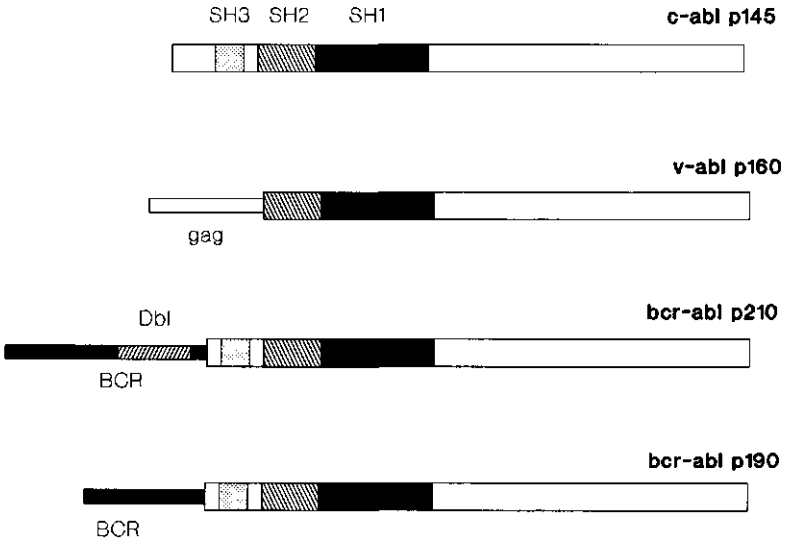

Figure 5. *Schematic representation of structural domains in the normal c-Abl protein and the chimaeric oncoproteins, Gag-v-Abl, P190 Bcr-ABl and P210 Bcr-Abl. In c-Abl, the SH3, SH2 and SH1 (kinase function) domains are marked. The presence of a viral (gag) and the variable Bcr amino acid sequences, in Gag-v-Abl and the chimaeric oncoproteins Bcr/Abl P210 and P190 respectively, are indicated in the lower three maps (N-terminal); note the presence of the Bcr DBL-homology domain in the Bcr/Abl P210 oncoprotein, but not in P190 (figure adapted from Grosveld, 1990).*

The c-*abl* gene is activated by a different mechanism in Philadelphia chromosome-positive leukemia. Fusion of *BCR* on chromosome 22 to c-*ABL* on chromosome 9, replaces N-terminal sequences of Abl with those of Bcr (Heisterkamp et al., 1983; Shtivelman et al., 1985; Hermans et al., 1987). The chimaeric Bcr/Abl oncoproteins differ in two ways from the Gag-v-Abl: their SH3 regions are still intact and they are not N-myristoylated (Fig. 5). No additional mutations are found in the Abl moiety of Bcr/Abl. Bcr/Abl oncoproteins are confined to the cytoplasm, in contrast to c-Abl or Gag-v-Abl. The chimaeric *BCR/ABL* oncoproteins have acquired a strong auto-phosphorylation activity, which is readily detectable *in vitro*. This is true for both *v-abl* and chimaeric *BCR/ABL* oncogenes. In Bcr/Abl, two regions in the first domain of Bcr are responsible for activating the Abl kinase in the chimaeric *BCR/ABL* gene product. One region, mapped to amino acids 28-68, deregulates the Abl tyrosine kinase activity and enhances the actin-binding function of Abl (McWhirter and Wang, 1991). The second region in the first Bcr domain, amino acids 176-242, may contribute to the deregulation of the catalytic activity through intrinsic binding to the Abl-SH2 domain (Konopka et al., 1984; Muller et al., 1991; McWhirter and Wang, 1991); it has been proposed that this deregulation involves interference with the putative negative-regulatory function

of the SH3 domain (Pendergast et al., 1991).

Although controversial (Groffen et al., unpublished results), there is some evidence that, unlike v-*abl*, *BCR/ABL* does not transform murine fibroblasts (NIH-3T3 cells; Daley et al., 1987). The weaker oncogenic activity of Bcr/Abl proteins is believed to relate at least in part to the subcellular localization of the different oncogenic forms of Abl (Wang, 1993). *BCR/ABL* can cooperate with the *myc* oncogene to transform Rat-1 fibroblasts, albeit with a much lower efficiency than v-*abl*. Similar *in vitro* assays have also indicated a difference in transforming ability between *BCR/ABL* P190 and *BCR/ABL* P210, which correlated with differences in auto-phosphorylation activity (Lugo and Witte, 1989; Lugo et al., 1990). The (auto-)phosphorylation activity of Abl *in vitro* is relatively weak; overexpression of c-*abl* is not transforming (Wang, 1993 + references therein).

1.6. *In vitro* studies of *BCR/ABL*

To decipher cellular mechanisms that underlie tumorigenic transformation of hematopoietic cells by *BCR/ABL* chimaeric oncoproteins, researchers have often resorted to the use of *in vitro* assay systems.

A classical method to examine the transforming (oncogenic) potential of a proto-oncogene is the NIH-3T3 transfection assay, in which the gene of interest is introduced into an immortalized cell line. Most NIH-3T3 transforming genes from human tumors appeared to be *ras* oncogenes. The assay is known to be relatively insensitive to morphological transformation by other oncogenes, such as *myc* and *myb*. The hybrid *BCR/ABL* oncoproteins do not, or at best poorly (Groffen et al., unpublished results), transform these mouse fibroblasts (Daley et al., 1987). However, in concert with another oncogene, *myc*, it they cause full transformation in Rat-1 fibroblasts (Lugo and Witte, 1990).

More important to the etiology of leukemia is the effect of *BCR/ABL* oncogenes on cells of hematopoietic origin. Lymphoid and myeloid cells can be transformed *in vitro* with retroviral constructs harboring *BCR/ABL* oncogenes (McLaughlin et al., 1987; Young and Witte, 1988; Daley et al., 1988). Both *BCR/ABL* P190 and P210 stimulate growth of immature lymphoid cells. These studies also indicated that *BCR/ABL* P190 is a more potent stimulator than *BCR/ABL* P210 (Lugo and Witte, 1990). BCR/ABL P210 expression abrogates the requirement for interleukin 3 (IL-3) of murine factor-dependent lymphoblastoid or myeloid cell lines (Daley et al., 1988). Similarly, leukemic B-cell progenitor cells, derived from *BCR/ABL* P190 transgenic mice, could be cultured independent of exogenous growths factors and stroma (Griffiths et al., 1992).

Recent studies, however, indicate that growth factor independence is probably not an early acquired trait of leukemic cells in human CML: neither human Philadelphia-positive hematopoietic progenitors nor chronic phase CML cells loose their growth factor requirement and normal proliferative responses to growth factors (Moore et al., 1973; Goldman et al., 1974; Metcalf et al., 1974; Lansdorp et al., 1985). It was suggested that *BCR/ABL* expression in chronic myelogenous leukemia alters the normal developmental controls in leukemic progenitor cells by suppression of apoptosis under conditions of limiting growth factor. This notion was corroborated by *in vitro* experimentation with hematopoietic cell cultures and temperature sensitive mutants of v-*abl* and *BCR/ABL* (Evans et al. 1993; Bedi et al., 1994a; Bedi et al., 1994b; Carlesso et al., 1994; Laneuville et al., 1994; McGahon et al., 1994; Kabarowski et al., 1994), suggesting that myeloid expansion is the result of prolonged cell survival rather than uncontrolled proliferation.

- **Cellular interactions of Bcr, Abl and Bcr/Abl**

Which molecular mechanisms and factors partake in cellular transformation, once *BCR/ABL* is expressed inside hematopoietic cells, has been the focus of research over recent years. A large number

of studies describes protein-protein interactions with Bcr, Bcr/Abl and/or Abl *in vitro* or in cultured cells. Proteins interacting with Abl include 3BP-1 (Cicchetti et al., 1992), Rb (Welch and Wang, 1993), Crk (Feller et al., 1994; Ren et al., 1994), Crkl (Ten Hoeve et al., 1994), Grb-2 (Ren et al., 1994; Pendergast et al., 1993; Puil et al., 1994), Nck (Ren et al., 1994), and actin (McWirther and Wang, 1993a). In addition, Abl has been shown to bind DNA (Kipreos and Wang, 1992). Bcr interacts with p21rac1/2 (Diekman et al., 1991; Ridley et al., 1993), CDC42Hs (Hart et al., 1992), Abl (Pendergast et al. 1993) and proteins of the 14-3-3 family (Reuther et al., 19940. Bcr/Abl binds and/or phosphorylates Bcr (Campbell et al., 1990; Lu et al., 1993), Grb-2 (Pendergast et al., 1994), ph-p53 (Campbell et al., 1990), Fes (Ernst et al., 1994), Shc (Puil et al., 1994; Matsuguchi et al., 1994), rasGAP, p190, p62 (Druker et al., 1992), the phosphatase Syp (Tauchi et al., 1994), c-cbl (Andoniou et al., 1994) and Crkl (Ten Hoeve et al., 1994b; Nichols et al, 1994; Oda et al., 1994). Some of these factors are specific to hematopoietic cells, others are not. Some factors engage in interactions with domains in the parental molecules, which are missing in the chimaeric Bcr/Abl oncoprotein. It is as of yet unclear whether such factors play a major role in transformation. Moreover, although many of these interactions are likely to be significant in an experimental setting, it is questionable that they all contribute equally *in vivo* to the process of Bcr/Abl mediated tumorigenesis in the hematopoietic cells of *Ph*-positive leukemia patients.

1.7. Animal studies in *Ph*-positive leukemia

Historically, a lot of information about gene function has been obtained through genetic analysis of mutant organisms (e.g. fruit fly, mouse). Whereas the discovery of mutant animals was merely governed by chance, recent revolutionary technical advances in mammalian genetics have made it possible to create animals with predetermined alterations in their genome. These techniques have already had a significant impact on our understanding of fundamental biology, in such areas as the developmental regulation of gene expression, cell lineage interaction and the effect of gene expression or ablation in the context of an intact organism. In the biomedical field, the opportunity has been created to generate animal models for human disease, to genetically engineer animals for production of proteins of therapeutic importance, and to develop human somatic gene therapy techniques. In agriculture, introduction of genetically manipulated species has been directed at optimizing quantitative yield and qualitative food product composition and enhancement of adaptation to adverse environments.

One of the first techniques aimed at germline alteration makes use of introduction of exogenous DNA sequences, *transgenes*, into the mouse genome (Fig. 6). This can either be achieved by microinjection of pronuclei of fertilized mouse eggs or retroviral infection of early embryos (Jaenisch, 1988). These embryos are transplanted into pseudopregnant female mice, and, with exception of when embryonically lethal transgene constructs are used, develop into transgenic animals.

A conceptually different strategy modifies endogenous DNA sequences in the mouse genome, rather than adding exogenous sequences. This technique is known as *gene targeting* or *targeted mutagenesis*. The advantage of this approach is that integration and integration site can be controlled. In transgenic technique, genomic integration is random rather than directed, and often in the form of tandemly integrated copies. Gene targeting relies on homologous recombination of cloned and altered (e.g., mutated or interrupted) DNA sequences with complementary sequences in the endogenous locus. In an initial step, one (or both) allele(s) of a particular gene is genetically altered in embryonal stem (ES) cells by means of a targeting vector (Capecchi, 1989). ES cells, although grown in culture, have retained their full developmental potential: ES cell contribution to all tissues is found, including the germline, upon injection of the cells in developing mouse blastulas (Evans and Kaufman, 1981; Martin, 1981;, Bradley et al., 1984). The injected embryos will be born as genetically mosaic (chimaeric) mice and transmit the targeted allele to their progeny (Fig. 7). Through interbreeding of fully heterozygous descendants, the gene of interest

can be fully inactivated or mutated. These animals are known as *null* mutants or, colloquially, *knock-out* mice.

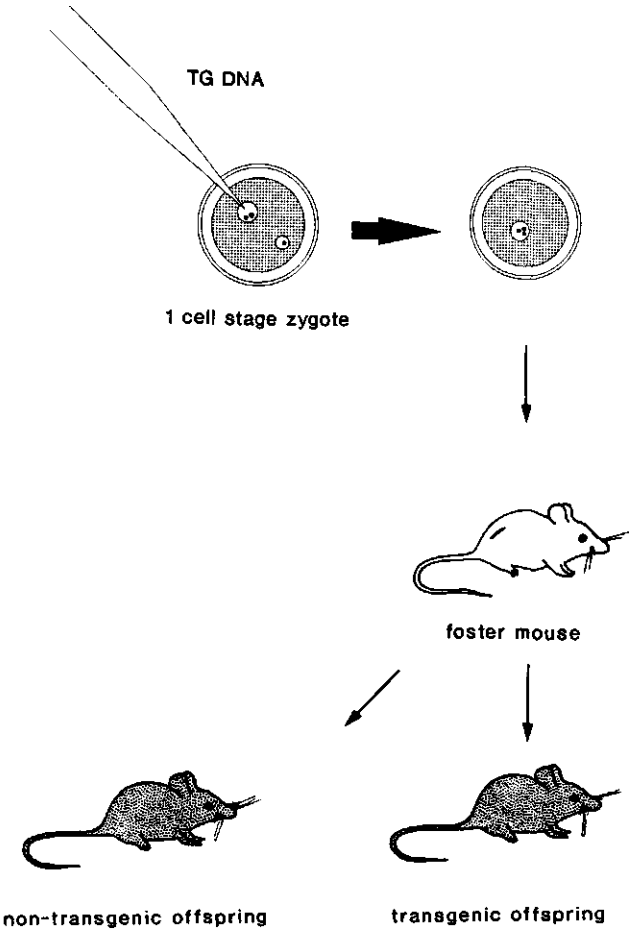

Figure 6. *Generation of transgenic animals through micro-injection of 1 cell stage embryos. The figure (above) depicts the micro-injection of the largest of two pronuclei in fertilized mouse eggs, with a DNA solution containing the transgene (TG) of interest. The transgene will randomly integrate into the mouse genome, and, from hereon, every newly formed cell in the dividing embryo will carry the transgene, including the germ line cells. Injected embryos are surgically transferred into the fallopian tube of pseudopregnant recipient female mice (foster mouse) and allowed to pass through gestation. Newborn mice are either transgenic or non-transgenic, depending on whether integration of the transgene occurred.*

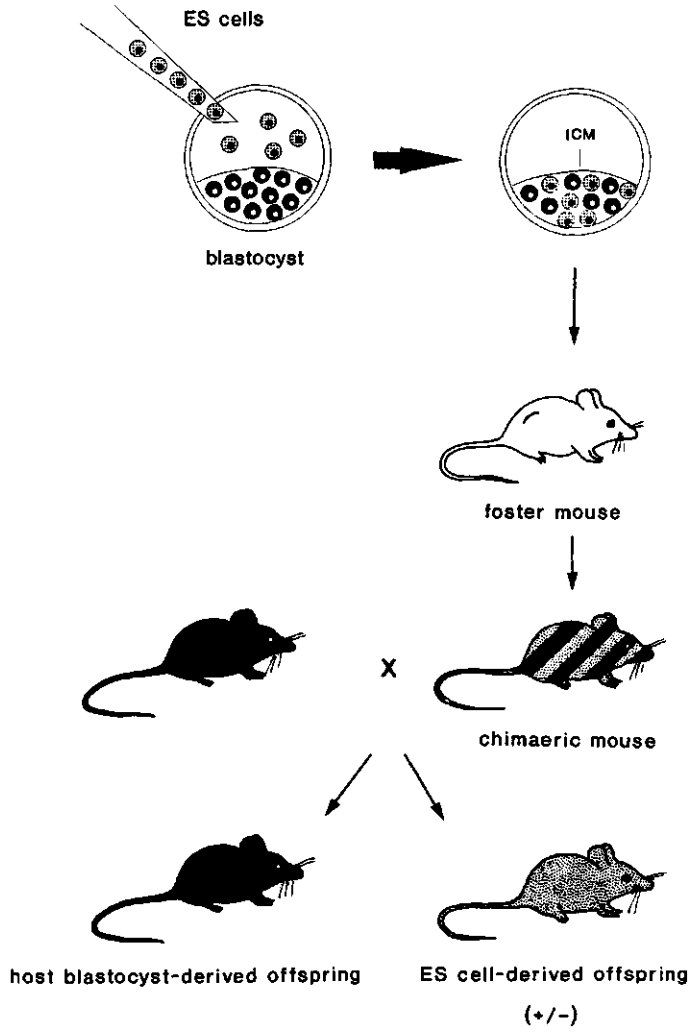

Figure 7. *Generation of mouse germ-line chimeras by introducing embryonal stem (ES) cells into mouse blastocysts. First, ES cells, in which one allele of a particular gene is genetically altered by the experimenter, are micro-injected into the blastocoel of a 4.5 day old host embryo (blastocyst; above). The injected ES cells (grey) combine with the existing embryonal stem cells (black) in the inner cell mass (ICM) to form a mosaic ICM, from which a chimaeric embryo will develop. The injected blastocysts are surgically transferred into the uterus of a pseudopregnant mouse and development is allowed to progress to birth. Above, the ES cells are derived from a mouse homozygous for a dominant agouti coat colour allele and the recipient blastocyst from a mouse homozygous for the recessive black allele. The chimaeric mice are composed of cells of both genotypes and therefore display coats with patches of each colour. When germ line transmission occurs, the dominant agouti coat colour gene, which was ES cell derived, and the mutated allele will be passed on from the chimaeric mice to offspring, thereby generating animals uniformly heterozygous (+/-) for the introduced mutation. Offspring from matings between heterozygous animals will consist of wildtype (+/+; not mutated), heterozygous (+/-; one allele mutated in every cell) and null-mutant animals (-/-), the latter of which no longer carry a normal functional gene (i.e. both alleles of the gene of interest are mutated).*

The importance of *in vivo* models to study Philadelphia-positive leukemia is manifold. A well defined mouse model provides a means by which tumorigenesis can be studied from its earliest stages onward and factors and mechanisms that eventually contribute to malignant progression of the leukemic cells can be uncovered. Besides an 'unlimited' provision of tumor material for analysis, more importantly, the availability of a mouse model provides a means by which cancer treatment regimes can be tested. In addition, identification of cellular components and/or pathways that contribute to the onset or progression of leukemia may eventually lead to the discovery and development of new drugs.

Two conceptionally different approaches have made use of transplantation of *BCR/ABL*-positive murine bone marrow cells into syngeneic recipients or microinjection of *BCR/ABL* transgenes into fertilized oocytes respectively.

- **Chronic myeloid leukemia; retroviral approach**

Over the most recent years, attempts to generate a reliable animal model for the study of CML have been thwarted by lack of reliability and reproducibility (Daley, 1993). Several investigators have tried to create a mouse model for CML through reconstitution of irradiated recipient mice with *BCR/ABL*-positive bone marrow (Elefanty et al., 1990, Kelliher et al., 1990, Daley et al., 1990, Daley et al., 1991; Gishizky et al. 1993). Mouse bone marrow cells were infected with retroviral vectors bearing *BCR/ABL* coding regions. Although some mice were obtained that developed leukemia, the kinetics and the nature of the induced disease were, shown to depend largely on infection conditions, retroviral and internal regulatory sequences used, and also on genetic background of the irradiated recipient mice. In essence, the outcome of the disease depended on which hematopoietic progenitor cell was targeted by retroviral vectors (Elefanty et al. 1992; Kelliher et al., 1993). Myelo-proliferative disorders could be obtained by manipulating these experimental conditions. However, myeloid disease develops in only a small number of transplanted mice (Daley, 1993); the myeloproliferative disorder in these mice was referred to as CML-like rather than CML (Elefanty and Cory, 1990; Gishizky et al., 1993). *BCR/ABL* P190 and P210 were found to cause similar disease in a retroviral setting (Kelliher et al., 1991). Even with v-*abl*, which under natural conditions exclusively induces pre-B cell leukemia in mice, myeloproliferative illness could be produced (Kelliher et al., 1990). All in all, the disease pattern resulting from retroviral infections appears very difficult to reproduce (Daley, 1993 and personal communication). A major obstacle in retroviral technology is to target the infection to stem cells. This is essential, since CML is thought to be a clonal disease originating in the hematopoietic stem cell (Fialkow et al.'77).

- **Transgenic mouse models for CML and ALL**

Transgenic mouse technology was first introduced in 1980 (Palmiter and Brinster, 1985). Essentially, a transgene is micro-injected into a one-cell stage zygote, and will consequently be integrated into the genome of the developing embryo. The advantage of a transgenic model as an alternative to retrovirally infected bone marrow transplantation becomes immediately apparent: once integrated into the genome of a 1-cell stage zygote, a transgene is per definition present in all derived cells, including the hematopoietic stem cell. Transgenic technology is a powerful experimental tool in the study of gene function in development and disease.

In *BCR/ABL* transgenic animals, every cell including hematopoietic stem cells will contain a *BCR/ABL* transgene. Development of disease is independent of influences of retroviral sequences or other experimental factors, such as infection and bone marrow culture conditions. Hence, a transgenic mouse model solely reflects the tumorigenic properties of *BCR/ABL* oncoproteins. An initial study was carried out with a hybrid *BCR*-v-*abl* transgene (Hariharan et al., 1989) in mice. Depending on the choice of regulatory sequences, mainly T lymphomas and few pre-B lymphomas (promoter sequence: immunoglobulin heavy chain enhancer, Eμ) or exclusively T lymphomas (promoter sequence: myeloproliferative sarcoma virus long terminal repeat; MPSV-LTR) were found. However, the v-*abl* oncogene in BCR-v-*abl* has

accumulated a multitude of mutations in the structural part of the *abl* oncogene, that do not occur in either the human *ABL* or the *BCR/ABL* oncogene (Rees-Jones and Goff, 1988; Groffen et al., 1992). These mutations contribute to the transforming ability of v-*abl*. Besides the fact that v-*abl* causes a hematopoietic disease distinct from that caused by *BCR/ABL* (Scott et al., 1991), the hybrid *BCR/v-abl* protein does not exist outside the laboratory. These observations complicated interpretation and comparison of results with those of others who did use *BCR/ABL* in for example retrovirus-infected bone marrow studies.

In an attempt to generate a true transgenic mouse model for CML, a 'minigene' had been designed (Heisterkamp et al., 1993c), in which expression of a P210 or P190 transgene was controlled by the human *BCR* promoter, as is the case in the Philadelphia translocation. With these constructs, however, no live transgenic progeny were ever obtained: although a small number of 'expressing' transgenic embryos apparently passed through development normally up to term, embryonic malformation and resorption were found significantly more frequent than in control pregnancies (Heisterkamp et al., 1993c). The observed effects presumably directly related to *BCR* promoter activity during development, as well as to yet unknown pleiotropic lethal effects of the P210 fusion protein on embryogenesis.

To circumvent the problem of embryonic lethality a different promoter system was chosen: metallothionein (MT) promoters are inducible and transcriptionally active in a non-tissue specific manner (Mayo and Palmiter, 1981; Durnam and Palmiter, 1981; Stuart et al., 1984). The first construct made was one that encoded a Bcr/Abl P190 oncoprotein. Although transgene expression was detectable without induction, it did not interfere with embryogenesis: transgenic founder animals were born in relatively large numbers. Within two months after birth the animals quite rapidly developed a pathological condition: 80% of the founder animals died of acute leukemia. Predominant disease was clinically classified as pre-B cell ALL and was indistinguishable from human ALL (Heisterkamp et al., 1990). Hence, this animal model unambiguously demonstrated the specific relation between *BCR/ABL* P190 oncogene expression and the development of pre-B cell leukemia.

1.8. Scope of this thesis

The availability of primary human tumor material for study is often restricted. Moreover, such tissues represent terminally advanced stages of tumorigenesis, and do not provide any insight into early events in tumorigenesis. To decipher cellular mechanisms that underlie tumorigenic transformation of hematopoietic cells by *BCR/ABL* chimaeric oncoproteins, researchers have often resorted to the use of *in vitro* assay systems. Unfortunately, the chosen assay conditions are not always relevant to human leukemia. Therefore, the availability of *in vivo* models to study Philadelphia-positive leukemia is paramount: a well defined mouse model provides a means by which tumorigenesis can be studied, from its earliest stages onward, and factors and mechanisms, that eventually contribute to malignant progression of the leukemic cells, can be uncovered. Besides an 'unlimited' provision of tumor material for analysis, more importantly, the availability of a mouse model provides a means by which cancer treatment regimes can be tested. In addition, identification of cellular components and/or pathways that contribute to the onset or progression of leukemia may eventually lead to the discovery and development of new drugs.

In 1990, the group of Groffen and Heisterkamp reported on a transgenic mouse model for Philadelphia-positive acute lymphoblastic leukemia (ALL). The disease that developed in the *BCR/ABL* P190 transgenic mice resembled the human disease closely. With the establishment of a model for leukemia

in an *in vivo* setting, the possibility was created to investigate the development of a 'human' leukemia from its earliest stages onward, and to study factors and mechanisms that eventually contribute to malignant progression of the disease. Part of this graduate study focussed on initiating a detailed biological analysis of this and other mouse models for Ph^+ leukemia: tumor evolution, a common feature of cancer biology, was studied by cytogenetic analysis of leukemia, and oncogenic specificity of *BCR/ABL* P190 and differences in oncogenicity between the *BCR/ABL* P190 and P210 oncogenes, were investigated using *de novo* generated transgenic animals (Chapters 2, 3, 4 and 5). A start was made with the evaluation of relatively simple anti-cancer treatment protocols by us (Chapter 6) and by other groups.

The biochemical reason why cellular transformation occurs in Philadelphia-positive leukemia has been known for quite some time now: the Abl tyrosine kinase, which is under normal circumstances implicated in cell cycle regulation, becomes constitutively activated through fusion to Bcr sequences as a result of the t(9:22)(q34;q11) translocation. The reason why, on a (molecular) biological level, cells lose growth control, remains elusive. The role of Bcr in the disease, other than that the fused sequences somehow activate the Abl kinase, is unclear. At the beginning of this graduate study, nothing was known about the normal cellular function(s) of the $p160^{Bcr}$ protein. In 1991, it was discovered that Bcr retains a GTPase activating protein (GAP) function toward certain small GTP-binding proteins of the $p21^{Ras}$ subfamily $p21^{Rac/Rho}$. Subsequently it was found that the Rac1 protein plays a role in mitogen stimulated membrane ruffling in fibroblasts, indicating a possible role for Bcr in cell growth regulation. At approximately the same time, Rac1 and Rac2 were connected *in vitro* with the respiratory burst (NADPH) oxidase, a catalytic system that is responsible for bactericidal oxygen radical production in neutrophils and macrophages, and in B-cells. The Bcr protein was meanwhile unfolding as a complex multidomain protein, harboring sequence homology with the Dbl oncogene, a guanidine-nucleotide exchange factor (GEF) for the small GTP-binding protein CDC42, pleckstrin, the major substrate for protein kinase C in platelets, with ß-chimerin, n-chimerin, ABR, rhoGAP and p190, all proteins with an overlapping *in vitro* GAP activity toward Rac1 and Rac2. Besides, Bcr harbors a unique serine/threonine kinase activity, is capable of oligomerization and was shown to interact with several cellular factors *in vitro*. Although *in vitro* the BcrGAP activity showed overlap with a whole myriad of biochemically related proteins, the biological relevance of this regulatory domain was not clear. Recently it was speculated that the *BCR* gene plays a role in normal hematopoiesis and is also implicated *Ph*-positive leukemia. On the basis of the data described above, and more importantly, because of its connection to *Ph*-positive leukemia, we set off to generate *bcr* null-mutant mice to examine the function of $p160^{Bcr}$ in normal and leukemic cell in the context of the whole organism (Chapter 7). To this end, the innovative gene targeting technology was used.

The *bcr* gene is highly expressed in mammalian brain. The expression pattern of a biochemically related protein, n-chimerin, overlaps with that of *bcr*. N-chimerin was shown to be involved in certain cognitive processes in birds and possibly in mice as well. Notwithstanding the function of Bcr in cells of hematopoietic origin, these observations suggest a possible different function for this protein in brain. In an attempt to further delineate a putative function of Bcr in the brain we have studied the regional expression and developmental expression kinetics of *bcr* in rodent brain (Chapter 8).

'Two "mouses"'. By Mia (3)

CHAPTER 2

Clonal Development and Karyotype Evolution during Leukemogenesis of *BCR/ABL* Transgenic Mice

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Clonal Development and Karyotype Evolution During Leukemogenesis of BCR/ABL Transgenic Mice

By Jan Willem Voncken, Christine Morris, Paul Pattengale, Gunther Dennert, Christie Kikly, John Groffen, and Nora Heisterkamp

The Philadelphia (Ph) translocation is responsible for the generation of the chimeric BCR/ABL oncogene. The Ph chromosome constitutes the earliest detectable chromosome abnormality in chronic myelogenous leukemia and is also found in acute lymphoblastic leukemia. Mice transgenic for a P190 BCR/ABL-producing DNA construct develop lymphoblastic leukemia/lymphoma and provide an opportunity to study early stages of the disease as well as progression. In this study, we have karyotyped the bone marrow of 10 19-day-old BCR/ABL P190 transgenic mice from a line that reproducibly develops leukemia/lymphoma. Leukemic cells from 17 terminally ill transgenic founders and progeny were also karyotyped as well as bone marrow transplant recipients of leukemic donor marrow. Karyotypically visible aberrations were absent from the early stages of BCR/ABL P190-generated leukemia and normal metaphases could be found even in the terminal stages of the disease. A high frequency of aneuploidy was found in advanced leukemia, with a marked preference for the gain of mouse chromosomes 12, 14, or 17. These results point to a primary role for BCR/ABL in leukemogenesis and suggest a destabilizing effect of the BCR/ABL gene on the regulation of cell division.

THE PHILADELPHIA (Ph) chromosome, specifically found in chronic myelogenous leukemia (CML) and Ph-positive acute lymphoblastic leukemia (ALL), is the result of a reciprocal translocation between chromosomes 9 and 22.[1-3] The translocation causes the ABL oncogene from chromosome 9 to fuse with part of the BCR gene located on chromosome 22. In approximately half of cases of Ph-positive ALL this chimeric gene produces a P190 BCR/ABL fusion protein, containing only those amino acids encoded by the first exon of the BCR gene; in the remaining cases of Ph-positive ALL and in CML a fusion protein with a larger BCR gene contribution, P210, is found.[4-6] The oncogenic role of these fusion proteins is suggested by the fact that the Ph chromosome is a consistent abnormality present at the time of diagnosis in the leukemic cells of CML and ALL patients, and also by the oncogenic outcome of transgenic and other in vivo experiments using the BCR/ABL fusion gene.[7-10]

Cancer is thought to be a multistep process that passes through different stages, each accompanied by gene mutations.[11,12] This process is exemplified by CML. Debate continues as to whether a predisposed leukemic cell, which lacks the Ph chromosome, exists in CML,[13,14] but the Ph chromosome is always found at diagnosis, usually as the sole karyotypic abnormality.[15] With progression of the disease, additional chromosome aberrations are observed in as many as 80% of patients after the transition from chronic to acute stage.[16,17] ALL is often accompanied by nonrandom chromosome abnormalities at presentation, among which the Ph chromosome is one of the most frequently seen.[18]

To investigate the role of BCR/ABL in leukemia, we have generated mice transgenic for a DNA construct that produces the P190 protein. Eight founder mice rapidly succumbed to leukemia (both myeloid and lymphoid) within a short period (58 days) after birth, demonstrating the leukemogenic activity of the activated ABL oncogene.[10] In the present study, we have asked whether the onset and progression of BCR/ABL P190-generated leukemia in transgenic mice is accompanied by the appearance of chromosome abnormalities such as those found in humans. Our results show a normal karyotype in initial stages and support the assumption that the activated BCR/ABL gene alone plays a key role in the development of leukemia. Later stages of the disease are characterized by nonrandom numerical changes of mouse chromosomes that have a genetic analogy to human chromosomes involved in leukemia.

MATERIALS AND METHODS

Transgenic mice. Transgenic mice were generated as previously described[10] using a BCR/ABL construct encoding the P190 fusion protein. In different lines the transgenic DNA construct is expected to have been integrated independently on different chromosomes. In cases in which the copy number of the transgene was higher than one, integration most often occurs in a tandem, head-to-tail fashion in one particular locus. There is no evidence that integration of transgene DNA into the mouse genome exhibits site-specificity.[19]

Founder animals were the offspring of matings between C57Bl/CBA F_1 animals. Transgenic progeny were the result of matings between founders and C57Bl × CBA F_1 mice. Successive generations were the result of similar matings. Mice identification numbers have three, six, nine, or 12 digits and indicate founders, F1, F2, and F3 animals. Pathologic analysis was performed on autopsy material obtained at killing from mice that were generally terminally ill. Mice were diagnosed with lymphoblastic lymphoma/leukemia (LL) or ALL alone. Of all founder and progeny transgenic mice (n = 100), 50% had died of leukemia/lymphoma before

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70 days of age. Of the line established from founder no. 623, animals died of or were killed with terminal disease on an average of 68 days after birth (range, 41 to 147 days). A more detailed characterization of these mice will be presented elsewhere (manuscript in preparation).

Bone marrow (BM) transplants. BM from founder no. 713 and from progeny animal no. 623-844-1037 (both transgenic BCR/ABL, P190) was collected from femurs and transplanted into C57Bl × CBA F_1 animals that had received a lethal radiation dosis of 1,000 rad (recipient S.2-3) or a sublethal dosis of 500 rad (all other recipients). Animal no. 713 was diagnosed with ALL and killed at 64 days. Its terminal white blood cell count (WBC) was 28×10^9/L. Donor no. 623-844-1037 was diagnosed with LL. Transplants were performed as indicated in Fig 1.

Cytogenetics. Karyotypic analysis was performed directly on material collected at autopsy. A clone was defined as two cells having trisomy or a structural change and three cells having monosomy of a particular chromosome. Heparinized peripheral blood (PB) or BM was collected in RPMI 1640 medium supplemented with 15% fetal bovine serum. PB cells were used only when WBCs were higher than 50×10^9/L; nonleukemic blood and blood containing less lymphoblasts did not yield sufficient metaphases under these culture conditions. Cells were washed once, then incubated with 100 ng/mL colcemid (GIBCO, Grand Island, NY) for 1 hour at 37°C, given a hypotonic shock in 0.075 mol/L KCl for 20 minutes at 37°C, and fixed and washed with cold 1:3 (vol/vol) acetic acid:methanol. Chromosomes were trypsin G-banded according to Seabright.[20] Chromosome banding terminology was as described by Nesbitt and Franke.[21]

Southern blot analysis. DNAs were isolated from spleen, lymph nodes, muscle, blood, and kidney as described.[10] DNAs digested with *Eco*RI were run on 0.7% gels, blotted to nitrocellulose, and hybridized to a 1.2-kb *Msp* I/*Eco*RI J_H probe. Posthybridization washings were at 0.1X SSC at 65°C.

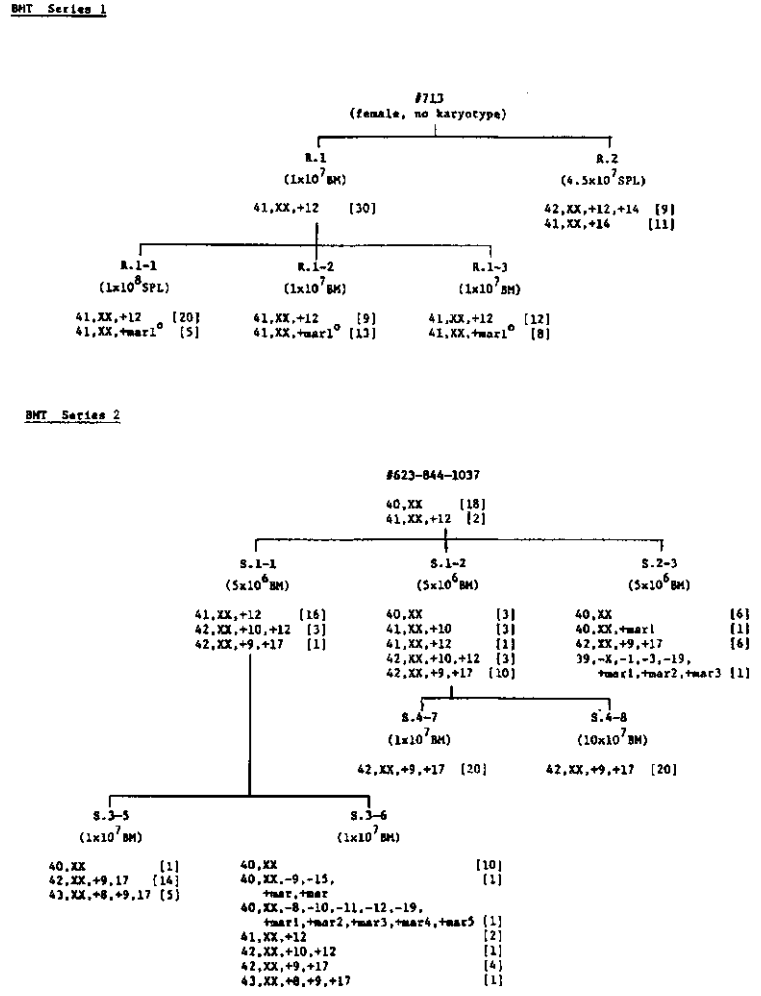

Fig 1. Transplant strategy and karyotype findings for recipients of BM or splenocyte (SPL) cells from leukemic mouse donors no. 713 (series 1) and 623-844-1037 (series 2). The number of cells that had a particular karyotype is in brackets.

RESULTS

The transgenic mice expressing P190 BCR/ABL described previously[10] all succumbed to leukemia before progeny was obtained. New transgenic founders were made and progeny was obtained from some of these. Animals developed LL and were evaluated karyotypically at different stages of disease.

Karyotypes of transgenic BCR/ABL P190 founder mice and their progeny. A total of 17 mice were analyzed cytogenetically. Karyotypic findings in leukemic cells isolated in the terminal stages of disease are detailed in Table 1 and summarized in Table 2 for the three founder animals (nos. 529, 538, and 632) and 14 progeny animals derived from founders no. 632, 629, 626, and 623.

Abnormal karyotypes were found in 15 of the 17 animals analyzed and ranged from 10% to 100% of metaphase cells scored. Trisomy predominated and was nonrandom with a preference for chromosomes 10, 12, 14, and 17 (Table 1, Fig 2A through E). Founder animal no. 538 showed a t(12;16). Two of the 17 animals analyzed had normal karyotypes. The first animal (no. 626-729) had a leukemic BM, but lymph nodes and PB were normal. The second animal (no. 623-844-1035) was diagnosed with lymphoblastic lymphoma.

Karyotypically normal cells were found in eight of the animals, even in those mice with a high WBC. For example, mice no. 529, 632-767, and 623-844-1037, all with a WBC above 100×10^9, had at least 80% normal metaphases (Table 1). These high WBCs suggest that the karyotypically normal cells were leukemic.

Nine of the 17 animals showed evidence of clonal evolution. For example, animals no. 623-881 and 623-844 showed normal metaphases as well as two or three abnormal clones, which seem to have been derived through the sequential gain of chromosomes (Table 1). There was also evidence of independent clone formation. For example, mouse no. 626-730 had five apparently unrelated abnormal karyotypes, in addition to normal metaphases (Table 1). Animal no. 623-844-1051 showed evolution within clones as well as independent clone formation (Table 1).

Karyotypes of leukemic BM transplant recipients. BM transplant procedures were used to investigate cytogenetic changes during tumor progression. The transplant strategy is outlined in Fig 1. Survival times were from 16 to 48 days and WBCs were elevated at least fourfold (not shown). Karyotype analysis was performed on material collected at autopsy.

Sublethally irradiated C57Bl × CBA hosts were transplanted with leukemic BM or spleen cells from two different BCR/ABL P190 transgenic mice. BM or splenocytes from founder mouse no. 713 that had ALL were transplanted into four recipients. Two of these recipients, R.1 and R.2, developed ALL after 48 and 69 days, respectively. Transplantation of the leukemic cells of R.1 to three secondary recipients resulted in the rapid development of disease with death at 15 or 16 days. Serial transfers to tertiary recipients resulted in an increasingly aggressive type of leukemia with a short latency period (9 days; results not shown).

Founder mouse no. 713 was not karyotyped. The primary recipient R.1 had an extra chromosome 12 in all leukemic cells, and R.2 had an extra chromosome 14 in one clone and an extra chromosome 12 and 14 in a second clone (Fig 1). The three secondary recipients of R.1 all had the trisomy 12 clone of R.1 and a second clone with a small marker chromosome, the origin of which could not be identified, but which was identical in all three animals (Fig 1).

We also used Southern blot hybridization of different transgenic tissue DNAs to an Ig J_H probe to follow tumor progression in the transplant series. The spleen of the original donor (no. 713) showed a germline *Eco*RI fragment, a less intense rearranged band of approximately 5.5 kb, and two intense rearranged bands of approximately 4.3 and 3 kb (Fig 3). This finding suggested the presence of more than one major dominant clone in this animal. DNA from spleen and an affected lymph node of recipient R.1 showed both 4.3- and 3-kb rearranged bands, with lymph node DNA containing only a faint germline band. Splenic DNA of R.2 lacked the 4.3- and 3-kb rearranged bands but showed two bands of approximately 6.5 and 4 kb. Splenic DNA from animals R.1-1, 1-2, and 1-3 exhibited the same pattern as seen in the donor R.1 (Fig 3). Recipients of R.1-1 BM (animals R.1-1-1 and 1-1-2) had a prominent 4.3-kb band and a weak 3-kb band (Fig 3). The lack of novel rearranged bands suggests that the clone containing the marker chromosome originated from the trisomy 12 clone.

The second transplant donor (no. 623-844-1037) had only one abnormal clone, containing an extra chromosome 12 in 10% of its leukemic cells (Table 1). Spleen DNA, however, exhibited at least seven rearranged bands with a J_H probe (data not shown). Consistent with this finding, transplanted animals S.1-1, 1-2, and 2-3 (all primary recipients) showed six different cytogenetic clones, including the trisomy 12 clone (Fig 1). Clones characterized by trisomies 10 and 12 and by trisomies 9 and 17 were found in more than one of the recipients and were presumably present as a minority, undetected clone in the original donor mouse. The trisomy 9 and 17 clone appeared to have a selective advantage in transplanted cells because it was present in all the transplanted animals of this series, ie, in both recipients of S.1-1 BM (animals 3-5 and 3-6), and as the dominant clone in both recipients of S.1-2 BM (animals S.4-7 and 4-8; Figs 1 and 2F).

Thus, Southern blot analysis of the BM transplant series suggested distinct clonal patterns, and supported the presumed presence of unrelated clones, as seen cytogenetically.

Early stages of P190 leukemia. The cytogenetics studies we have described above were based on leukemic samples taken from animals in late to very advanced stages of the disease. It was possible that the karyotype changes detected were present early in the disease and, together with the BCR/ABL gene product, were jointly responsible for the development of overt leukemia. Alternatively, their appearance could have been associated predominantly with disease progression. To investigate these possibilities, we examined the karyotype of mice early in the development of leukemia. For this purpose, a breeding line of animals

Table 1. Karyotype of Transgenic P190 BCR/ABL Leukemic Founders and Progeny

Mouse No.*	Src†	No. of Cells	Karyotype	Age (d)‡	WBC (10^3/L)
626-729§	BM	50	40,XX	70	2
623-844-1035‖	BM	20	40,XY	143	23
529	PB	4	41,XY,+5	43	347
		16	40,XY		
632-767	PB	1	41,XX,+10	68	301
		2	42,XX,+10,+17		
		3	41,XX,+?19		
		24	40,XX		
623-881	BM	3	40,XX	45	127
		5	42,XX,+10,+14		
		12	43,XX,+10,+12,+14		
623-844	BM	2	43,XX,+12,+14,+mar	52	22
		4	41,XX,+14		
		10	42,XX,+12,+14		
		14	40,XX		
623-844-1037	PB	2	41,XX,+12	41	120
		18	40,XX		
623-844-1051	PB	1	40,XX	66	127
		1	43,XX,+10,+12,+18		
		2	42,XX,+12,+12		
		2	42,XX,+10,+12		
		5	41,XX,+12		
		9	42,XX,+10,+14		
538	PB	20	40,XY,t(12;16)(F1;B5)	38	196
632	PB	1	42,XY,+12,+12	79	153
		1	42,XY,+12,+mar_1		
		2	42,XY,+10,+12		
		4	42,XY,+12,+mar_2		
		22	41,XY,+12		
632-749	PB	7	42,XY,+?13,+17	43	55
		23	43,XY,+10,+?13,+17,		
632-753	PB	25	41,XX,+14	60	376
629-719	BM	1	41,XX,+17	99	7
		1	43,XX,+5,+17,+17		
		1	42,X,−X,−19,+14,+17,+mar,+mar_3		
		8	40,XX		
		8	42,X,−X,−19,+14,+17,+mar,+mar_3,+mar_3		
		10	42,XX,+17,+17		
629-723	BM	20	42,XX,+5,+17,del(6)(B1;B3)	39	4
626-730	PB	1	41,XY,+9	55	60
		1	41,XY,+mar		
		2	42,XY,+mar,+mar		
		2	40,XY,t(5;12)(B;F1)		
		5	40,XY		
		5	41,XY,+12		
623-884	BM	1	44,XY,+1,+10,+14,+17	147	47
		1	45,XY,+1,+10,+12,+14,+17		
		18	43,XY,+1,+14,+17		
623-844-1035-1087	PB	1	42,XY,+12,+12	45	187
		1	42,XY,+14,+17		
		9	41,XY,+12		

*The first three-digit numbers indicate distinct transgenic mouse lines; subsequent three-digit and four-digit numbers indicate F1 progeny and F2 progeny, and so on.

†Source of lymphoblasts.

‡Age at which animal was killed with terminal disease.

§Animal had evolving lymphoblastic leukemia at death, with no lymph node but some marrow involvement.

‖Animal was diagnosed with lymphoblastic lymphoma, with apparent soft tissue involvement.

founded by mouse no. 623 was used. These animals showed an average age of death due to overt leukemia similar to that found in the other P190 BCR/ABL transgenic mice.

The appearance of BCR/ABL-expressing cells in PB was taken as a measure for the development of leukemia, and trial samples were taken from a test group to determine the earliest appearance of such cells in the circulation (data not shown). PB samples of 3 μL were drawn at regular intervals

Table 2. Summary of BCR/ABL Mice With Terminal Disease

Founders	3
Progeny	14
Total no. of animals analyzed	17
No. of animals with normal karyotype	2
No. of animals with karyotypically normal and abnormal cells	8
No. of animals with only karyotypically abnormal cells	7
Total no. of animals analyzed	17

and assayed for BCR/ABL expression using reverse transcriptase and the polymerase chain reaction.[22] Based on these data, 10 transgenic animals, all progeny of mouse no. 623-844-1035, were killed at 19 days of age and their BM evaluated. The course of the disease in additional progeny of animal no. 623-844-1035 was identical to that in other transgenic lines. At 19 days, the peripheral WBC was normal, there was no evidence for the development of lymphomas, and the BM showed normal development. In these particular animals BCR/ABL expression was detected in the BM of all and in the PB of four. Of these 10 animals, only one, no. 1118 (Table 3), had chromosome abnormalities; all other animals had a normal karyotype. There was a low level of apparently random aneuploidy in no. 1118, with four different cells showing a gain of a different chromosome. These data show that clones characterized by chromosome change develop in later stages of the leukemia.

DISCUSSION

Previous experiments suggested that the activated BCR/ABL oncogene was the primary factor causing LL in our transgenic mice.[10] We can assume that the presence of the activated oncogene in many lymphoid cells would lead to multiple leukemias in single mice. Our present results support those concepts. The prime role of BCR/ABL in causing leukemia was supported by the early leukemia studies (Table 3). Only one of 10 animals had chromosomal abnormalities and none showed clonal growth. Later-stage leukemias, including those with large numbers of leukemic cells, showed many normal metaphases (39% of the total scored) and 8 of the 15 animals with chromosome abnormalities showed some normal metaphases (Tables 2 and 4). These findings are a strong argument for the expansion of a pool of early leukemic cells that have BCR/ABL as a sole mutation. However, we can not exclude the possibility that cooperation between BCR/ABL and other oncogenes is necessary to generate overt leukemia. Such oncogenes could have been activated by mutations, not visible cytogenetically, of a similar form to structural alterations of the p53 gene present in more advanced stages of CML.[23,24]

The idea of a multiple origin of the leukemia is supported by the transplantation of the BM of donor no. 623-844-1037, which gave rise, in different recipients, to independent clones characterized by IgH gene change or abnormal chromosome number. Other animals also showed evidence for independently derived leukemic clones. These multiple clones probably reflected the development of separate leukemias from different BCR/ABL-transformed lymphoid cells in single mice. Further cytogenetic/genetic-based progression of these clones, and their competition with each other, could be expected. There was no evidence of a common, specific cytogenetic change that was essential for the development of leukemias.

A high frequency of chromosome aneuploidy was noted in the advanced leukemias. The four cells showing gain of a different chromosome in the early leukemic mouse no. 1118 (Table 3) may represent a pool of cytogenetic variation from which cells with proliferative advantage become dominant and form clones. It is of interest that one of the abnormal cells in that animal had an extra chromosome 12,

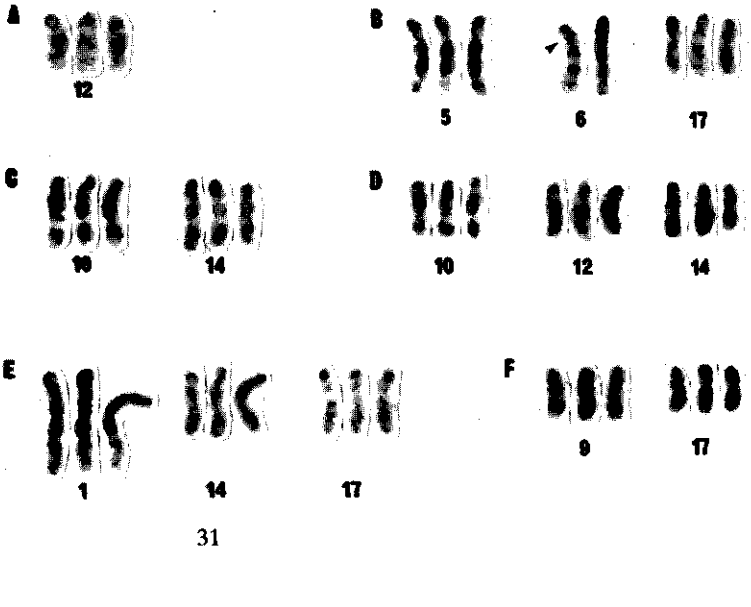

Fig 2. Partial karyotypes of abnormal clones detected in leukemic cells of (A) mouse no. 632 [41,XY,+12]; (B) mouse no. 629-723 [42,XX,+5,+17,del(6)(B1B3)]; (C) mouse no. 623-844-1051 [42,XX,+10,+14]; (D) mouse no. 623-881 [43,XX,+10,+12,+14]; (E) mouse no. 623-884 [43,XY,+1,+14,+17]; and (F) BM transplant recipient no. S.3-5 [42,XX,+9,+17]. The arrowhead marks the deleted chromosome 6 in mouse no. 629-723.

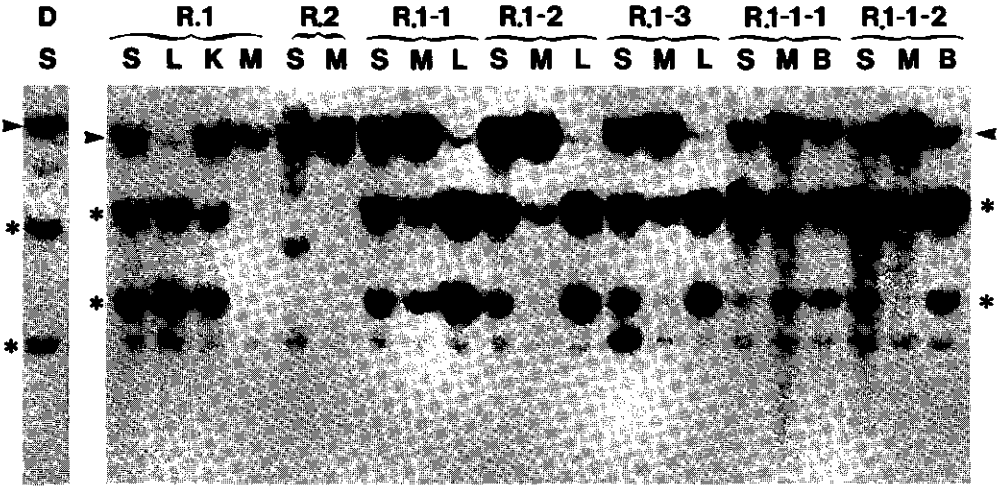

Fig 3. Ig J_H rearrangements in leukemic BM transplant recipients. Individual mice (D, donor no. 713; see text for other mice) are indicated above the lanes. Arrows and the two asterisks point to the germline and two rearranged 4.3- and 3-kb *Eco*RI fragments, respectively. The source of DNA is indicated above the figure: S, spleen; L, lymph node; K, kidney; M, muscle; B, blood.

one of the common trisomies found in advanced leukemia in other P190 BCR/ABL mice.

The chromosome changes tended to be nonrandom in occurrence (Table 4). Most prominent was the acquisition of extra copies 10, 12, 14, and 17, either alone or in combination. The gain of chromosomes 10, 12, and 17 was also found in the second BM transplant series (Fig 1), although the original donor showed only trisomy 12 in 10% of its cells. The first BM transplant series showed gain of chromosomes 12 and 14. The chromosomes involved in these nonrandom gains carry a number of loci known to be associated with cancer, cell growth, and development. Chromosome 10 carries the *Steel* locus, the *bcr* gene, the *myb* proto-oncogene, *Ahi*-1 (a site of MoMuLV integration in Abelson murine leukemia virus-induced pre–B-cell lymphomas), *fyn*, *ros*-I, and *Braf*.[25] Mouse chromosome 10 exhibits four main regions of synteny with human chromosomes: 6q, 10, 12q, and 21q.[25] Mouse chromosome 12 contains several MoMuLV integration sites, as well as the *fos* proto-oncogene, the transforming growth factor-β3 (TGF-β3) locus, N-*myc*, L-*myc*1, and the Ig heavy chain locus. This chromosome has main regions homologous to human chromosomes 2p (N-MYC) and 14q (TGF-β3, FOS, IgH). Mouse chromosome 14 also contains MoMuLV integration sites, the T-cell receptor-α (TCR-α) and -δ loci and the *Rb*-1 gene, and has synteny with human chromosomes 10, 13q (RB-1), and 14q (TCR-α and -δ). Finally, mouse chromosome 17 has MoMuLV integration sites, the *Pim*-1 gene, tumor necrosis factor-α (TNF-α) and -β, the H-2 complex, and shows significant homology with human chromosome 6p (PIM, TNF-α, TNF-β, HLA).[26,27]

How do these data compare with chromosome abnormalities found in human ALL? The leukemic cells of patients

Table 3. Karyotype of BCR/ABL P190 Mice Killed at 19 Days

Mouse No.*	Age (d)	WBC (10^9/L)	Source	No. of Cells	Karyotype
1116	19	0.8	BM	20	40,XX
1117	19	2.1	BM	20	40,XX
1118	19	0.6	BM	1	41,XX,+?6
				1	41,XX,+12
				1	41,XX,+16
				1	41,XX,+mar
				16	40,XX
1119	19	1.8	BM	21	40,XX
1120	19	1.8	BM	21	40,XX
1121	19	1.6	BM	20	40,XX
1122	19	1.3	BM	20	40,XY
1123	19	1.5	BM	14	40,XY
1124	19	1.0	BM	20	40,XY
1125	19	0.6	BM	20	40,XY

*Full designation of these mice is 623-844-1035-(number shown); all are G3.

Table 4. Nonrandom Chromosome Duplications in BCR/ABL P190 Leukemic Mice

Chromosome Abnormality	No. of Cells*	%	No. of Different Animals†	%
+6	25	6	3 (2)	17
+10	59	14	6 (2)	28
+12	81	20	7 (3)	44
+14	97	24	7 (3)	39
+17	94	23	6 (3)	33
None	159	39	10 (5)	61

*Total number of metaphases analyzed is 411 and includes all animals in Table 1.

†Total number of different animals is 17; the numbers in parentheses reflect the number of different transgenic lines examined (ie, breeding lines derived from different founders; of a total of 6) in which this abnormality was seen.

with ALL show a high incidence of clonal chromosome abnormalities, often complex, and including both structural and numerical changes.[16] Hyperdiploidy is frequently seen, and chromosome numbers usually range from 50 to 60. Identical karyotypes are rare between cases, but the addition of chromosomes 4, 6, 10, 14, 17, 18, 21, or X is commonly seen.[16,28] Interestingly, the homologs of some of these chromosomes were involved in the transgenic mice: human chromosome 6 (mouse chromosome 10 and 17) and human chromosome 14 (mouse chromosome 12 and 14) were the most obvious of these. This correlation between findings in the two species could indicate the presence of genes on these chromosomes, such as those described above, and others yet unidentified that are associated with an increase in proliferative potential. Structural changes are found in approximately one-third of human ALL patients. Specific translocations include the t(9;22), which is found in 2% to 6% of childhood and 19% to 30% of adult cases when leukemia is diagnosed. Chromosome abnormalities, in addition to the Ph chromosome, tend to be variable.[16] In this respect, the nonrandom chromosome changes and clonal evolution of our BCR/ABL P190 mice is more comparable with the nonrandom chromosome changes of human blast-phase CML.[16]

It has been argued that the initial development of Ph-positive CML in humans is unrelated to the presence of the Ph chromosome,[13,14] and that a primary Ph-negative leukemic clone(s) has an increased susceptibility, of an unknown nature, to acquire chromosomal abnormalities. This instability leads to the t(9;22) translocation and CML develops. The data described in this study provide no support for such model. In our mice, it is clear that expression of BCR/ABL precedes the development of chromosome abnormalities.

Cytogenetic variation is a marked feature of most forms of cancer, particularly their advanced stages, and is interpreted as a consequence of neoplasia. It has been postulated that the basic defect in Ph-positive CML lies in discordant maturation of cytoplasm and nucleus that results directly or indirectly from action of the BCR/ABL protein.[29] Discordant maturation of cytoplasm and nucleus is likely to disrupt regulation of the cell cycle leading to nondisjunction and aneuploidy. Evidence that BCR/ABL disrupts cell cycle regulation also comes from experiments with the (murine) 32D cell line, which, when infected with an active BCR/ABL retroviral construct, shows an increase in cell size and a striking increase in the percentage of cells in the G2/M phase of the cell cycle.[30]

Our results suggest that BCR/ABL expression is the prime event initiating Ph-positive leukemia and that cytogenetic/genetic instability, which sets the stage for additional mutations and progression of the leukemia, is either an indirect effect of this prime event or a direct effect of continued BCR/ABL expression.

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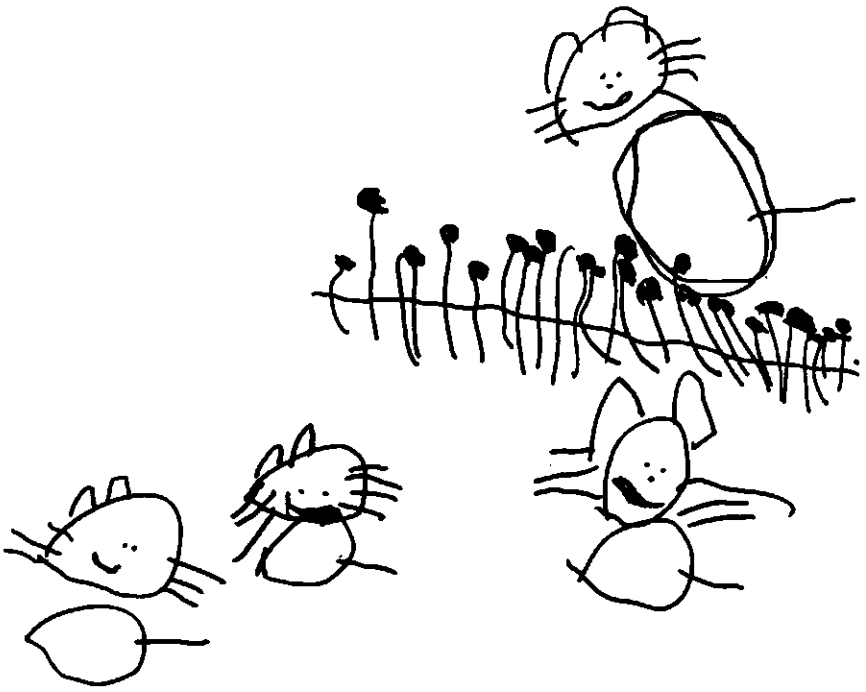

'Happy mice with flowers'. By Paula (4)

CHAPTER 3

Transgene Methylation and Leukemogenesis in *BCR/ABL* Transgenic Mice

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Transgene Methylation and Leukemogenesis in *BCR/ABL* Transgenic Mice

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Abstract

DNA methylation is recognized as a mechanism by which gene expression can be regulated, directly or indirectly. In transgenic organisms, transgene inactivation through methylation has been projected as a host defense mechanism against transcription of foreign genes. This results in transgene inactivation, thereby rendering eukaryotic transgenic technology and gene therapy ineffective. We have created transgenic mouse lines for a human *BCR/ABL* oncogene. While most lines consistently produced pre-B lymphoid leukemia, animals in some lines failed to develop disease. In four of these lines, 626, 629, 787 and 793, a variable penetrance of disease was noted. Southern analysis of the 5-prime part of the transgene by isoschizomeric restriction analysis with HpaII/MspI showed a close correlation between transgene methylation in this region, and lack of transgene expression and absence of malignant disease in these animal lines. Attempts to induce leukemia in vivo, among other methods through combined use of mitogens and the demethylating agent 5-azacytidine, were not successful, suggesting that transgene silencing was not the direct result of de novo methylation.

Introduction

Methylation of deoxycytidine in the eukaryotic dinucleotide combination 5'-CG-3' (CpG) can stably repress genetic activity of genes and is part of normal post-replication regulatory mechanisms governing differentiation and development (Bird, 1992; Tate and Bird, 1993; Eden and Cedar, 1994 + references therein). The methylation status of CpG islands on genes is also implicated in imprinting: differential methylation patterns distinguishes alleles of different parental origin (Allen et al., 1990; Barlow, 1993; Li et al., 1993; Rainier and Feinberg, 1994). Genetical inactivation of one of two X chromosomes in somatic cells in female mammals results in dosage compensation of X-linked genes between XX females and XY males (Kay et al., 1993). It is generally accepted that methylation of the inactive X chromosome helps to maintain its inactive state (Allen et al., 1990). In cancer, abnormal methylation patterns have been recognized in specific genes and in certain instances linked to malignant progression of the disease (Feinberg and Vogelstein, 1983a, 1983b; Jones, 1986; Achten et al., 1991; Felgner et al., 1991; Kochanek et al., 1991; Malinen et al., 1991; Nelkin et al., 1991; Wahlforss et al., 1992; Razin and Cedar, 1994). In prokaryotes DNA methylation is part of a defense mechanism against foreign, in particular viral, DNA and protects host DNA from auto-degradation by its own restriction endonucleases (reviewed by Doerfler, 1992). *De novo* methylation of foreign DNA has also been postulated to function as a eukaryotic defense against transcription of potentially harmful genes (Doerfler, 1992). Gene inactivation in virus-transformed mammalian cells correlates with methylation of specific viral sequences, and can be reversed by treatment with 5-azacytidine, a demethylating agent (Groudine et al., 1981; Groffen et al., 1982; Hoeben et al., 1992). Transgenic technology in plants (Weber et al., 1990; Kilby et al., 1992; Meyer and Heidmann, 1994) and animals (Engler et al., 1991; Lettmann et al., 1991) has revealed that transgenes are also subject to inactivation; transgene methylation patterns often

reflect a species-specific branding (Chaillet, 1994). Although currently the mechanisms by which (trans)gene inactivation is brought about are poorly understood, it is of considerable importance for practical reasons: gene technologies such as gene therapy depend on stable integration and expression of what may be considered, at least in part, foreign sequences (Richards and Huber, 1993; Challita and Kohn, 1994).

In Philadelphia (Ph) positive leukemia (Ph+ acute lymphoblastic leukemia (ALL) and chronic myelogenous leukemia (CML)), the *BCR* gene on chromosome 22 undergoes a reciprocal translocation with the ABL proto-oncogene on chromosome 9. The resulting fusion gene is transcribed in leukemic cells and is responsible for the development of leukemia (reviewed by Van Etten, 1993; and Heisterkamp et al., 1993a). Depending on the exact position of the breakpoint in the *BCR* locus, chimeric *BCR/ABL* proteins will be 190 kDa (ALL) or 210 kDa (ALL and CML) in size (reviewed by Heisterkamp and Groffen, 1991). We have previously generated mice transgenic for a *BCR/ABL* transgene encoding the P190 oncoprotein: transgenic *BCR/ABL* P190 animals develop pre-B cell leukemia/lymphoma (Heisterkamp et al., 1990; Voncken et al. 1992a; Voncken et al., 1992b). Of the in total 14 lines, 1 line did not develop leukemia at all. Surprisingly, four lines displayed a variable penetrance of the disease: within these lines some animals did, others did not develop disease. The purpose of this study was to further characterize the transgenic mice carrying the human *BCR/ABL* P190 transgene and to investigate possible mechanisms that influence expression of the transgene in the latter lines.

Materials and methods

Transgenic construct

The P190 producing *BCR/ABL* construct has been studied extensively in our laboratory (Heisterkamp et al., 1990; Voncken et al. 1992a; Voncken et al, 1992b). Briefly, human *BCR* exon 1 and human ABL exon 2 are separated by intervening sequences derived from respectively *BCR* intron 1 and ABL intron 1, which provide a splice donor and acceptor site. ABL intron 2 is also retained in the construct, while ABL exons 3 to 11 and 3'-untranslated sequences are incorporated as cDNA. Intron sequences have been reported to enhance transgene expression (Brinster et al., 1988; Palmiter et al., 1991). The transgene was placed under transcriptional control of a segment of the mouse metallothionein-1 (mMT) promoter. The MT-*BCR/ABL* P190 (Fig. 1) construct contains only part of the mouse MT promoter, identical to that used in earlier studies (Heisterkamp et al. 1990; Voncken et al., 1992a, Voncken et al., 1992b).

Transgenic technology

Mice transgenic for *BCR/ABL* P190 were generated essentially as described (Heisterkamp et al., 1990). Transgenic founders were identified by Southern analysis. Progeny was obtained through breeding to C57BL/CBA F1, C57BL/6, DBA/2 (Jackson Laboratories, MA). F1 progeny are identified by numbers, of which the first three digits identify the founder animal. The transgenic animal lines used in these studies have been described in detail elsewhere (Voncken et al., 1992a; Voncken et al., 1992b).

Autopsy

Autopsies on terminally ill or apparently healthy animals for methylation studies were carried out as described previously (Pattengale et al., 1989). Histological examination concentrated on hematopoietic tissues (bone marrow, spleen, lymph nodes) and served to confirm the health status of the animals, whether leukemic or not.

Transgene methylation

Genomic DNA was isolated from spleens, involved lymph nodes and bone marrow as described (Voncken et al., 1992a). DNAs digested with MspI or HpaII were run on 1.2% agarose gels, blotted to Nytran (Micron Separations Inc., MA, USA) and hybridized to an 0.6 XhoI-BglII probe (Fig. 2). Post hybridization washings were at 0.03x standard saline-citrate, 65°C.

Analysis of BCR/ABL expression

At sacrifice, a sample of bone marrow, spleen, lymph node or blood was used for RNA isolation. Expression in blood and in other tissues was evaluated by reverse transcription (RT) followed by the polymerase chain reaction (PCR). RNA isolation was carried out as described (Voncken et al., 1992b).

In vivo induction trials

Transgene activation *in vivo* was attempted with heavy metals, a specific B-cell mitogen and demethylating agents. 5-Azacytidine (5-Aza) and 5-Fluorouracil (5–FU) treatment protocols were adapted from other studies (Lee et al. 1983; Harrison et al. 1990; Naya et al., 1991 and Constaninoulakis et al., 1992). In total, 6 different induction regimes were tested: I, Animals were subjected to 2 intraperitoneal (ip) bolus injections with lipopolysaccharide (LPS) (day 0: 10 μg LPS/animal (ip), day 33: 20 μg LPS/animal (ip). II, Animals were exposed for 60 days to cadmium (100 μM $CdSO_4$) in drinking water, which was provided ad libitum; the experiment was terminated after 180 days. III: Animals were given two bolus injections (ip) with 5-Aza, 4 mg/kg body weight (bw) and 12 mg/kg bw respectively, 12 hours apart. IV: Animals received an intravenous (iv) bolus injection of 150 mg 5–FU/kg bw on day 0, followed by two 5-Aza injections (ip) on day 4 (as in III). V: An (iv) 5–FU injection on day 0 (as in IV) was followed by 3 successive (ip) injections with 2.5 mg 5-Aza/kg bw on days 3, 6 and 9 respectively. VI: Animals were injected (iv) with 150 mg 5–FU/kg bw on day 0, followed by 4 injections with 4 mg/kg bw 5-Aza (ip) at weekly intervals, starting at day 6. Non-transgenic control animals in experiential groups III-VI were injected with 5-Aza, control transgenic animals were injected with diluent only (5–FU: alkaline PBS (pH 8.0), 5-Aza: 0.9% saline). WBCs were monitored for up to 2-3 months after initiation of the trials; WBC measurements were done manually with a hemocytometer. Blood samples for RT/PCR analysis were taken at regular intervals. When monitoring for transgene expression, blood samples from leukemic mice of the 623 (Voncken et al. 1992b) and non-transgenic controls were taken along as respectively positive and negative controls . Animals were monitored for development of leukemia, and examined for lymphomas or other tumors at sacrifice.

In vitro induction trials

Healthy transgenic animals were treated with 150 mg 5–FU/kg bw. One week later bone marrow cells or splenocytes were isolated. Bone marrow cells were expelled from femur and tibia with a syringe/25 G needle, using cold PBS. Single cell splenocyte suspensions were obtained by mincing the spleen with sterile surgical blades and mashing the tissue bits through a 40 micron sterile wire grid. The suspensions were allowed to settle for 5 minutes in a 15 ml conical tube, after which the supernatant (i.e. single cell suspension) was transferred to a fresh tube. Erythrocytes were lysed in hypotonic ammonium chloride/ammonium carbonate, after which cells were sedimented and washed once in PBS, divided and plated out in 6 well plates in IMDM medium (Gibco/BRL) supplemented with 30% FBS, penicillium, streptomycin, L-glutamine and 100 μM ß-mercaptoethanol. Cells were cultured in I: 0, 0.03, 0.3 or 3.0 μM 5-Aza for 2x 72 hrs; II, for 7 days in medium after which they were exposed to 0.1 μM or 1.0 μM 5-Aza for 24 hours, in the presence or absence of a heavy metal cocktail (100 μM $ZnCl_2$/1 μM $CdCl_2$), after which the cells were washed and cultured for an additional 3 days; III: for 9 days in medium after which they were exposed to heavy metals (100 μM $ZnCl_2$/1 μM $CdCl_2$) in the presence or absence of 1.0 μM 5-Aza for 72 hours, after which the cells were washed and cultured for an additional 2 days. Cultures were then terminated, harvested and analyzed for transgene expression using RT/PCR; expression analysis was also done at the time tissue isolation, prior to culturing (zero-point controls).

Results

Transgenic founders and progeny

Table I gives an overview of transgenic animal lines generated, their offspring and the development of disease in these lines. In 50% of the founders

Table I. *BCR/ABL* related disease in transgenic founder animals and offspring

Founder	disease[a]	Progeny	disease[a]
618	LLY	none	-
620	LLY	none	-
628	ALL	none	-
631	LLY	none	-
713	ALL	none	-
790	LLY[b]	none	-
796	LLY[b]	none	-
623	LLY	F1-F8	ALL/LLY
632	ALL/LLY	F1	ALL/LLY
626	no disease	F1	variable penetrance[c]
629	no disease	F1	variable penetrance[c]
787	no disease	F1	variable penetrance[c]
793	no disease	F1	variable penetrance[c]
791	no disease	F1-F3	no disease

a: Abbreviations: ALL: acute lymphoblastic leukemia, LLY: lymphoblastic lymphoma.
b: Animals were found dead at 31 days (790) and 55 days (796). Although no histological analysis was carried out, gross pathological findings were indicative of a leukemic condition.
c: Among siblings in these animal lines, a variable penetrance of disease was noted: some animals developed BCR/ABL related disease, others remained disease-free.

disease developed before offspring was sired. Of one founder, 623, an transgenic animal line was established successfully; this transgenic mouse line has been studied in detail (Voncken et al., 1992a and 1992b). Bone marrow of founder animal 713 was used for bone marrow transplantation experiments (Voncken et al., 1992a). Some founders did not develop disease, although some of their progeny did (626, 629, 787, 793). While only the 791-line did not develop acute lymphoblastic leukemia/lymphoma, four lines, 626, 629, 787 and 793 showed what appeared as a variable penetrance of disease: some progeny did, other did not develop leukemia. The estimated transgene copy number of the transgenic founder animals in the current study varied between 1 and 6, with the exception of 787 (copy number: >25). A detailed description of founder characteristics has been presented elsewhere (Voncken et al., 1992b).

Transgene methylation

We looked for altered transgenic methylation patterns in the 791 and the 626, 629, 787 and 793 lines. Human *BCR* exon 1 contains multiple MspI/HpaII restriction sites as indicated in figure 2. Because of the human origin of the transgene sequences (Fig. 1; Heisterkamp et al., 1990), it was possible to discriminate transgene from the endogenous mouse *bcr* gene and thus evaluate the methylation status of *BCR* exon 1. The degree of methylation of a DNA sequence can be assessed by restriction analysis with the isoschizomers MspI and HpaII: MspI will digest DNA independent of its methylation status, while HpaII will only digest DNA if the restriction sites in unmethylated. As is

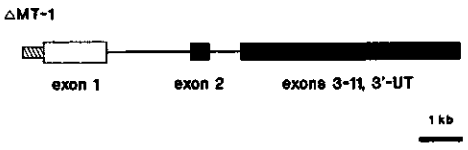

Figure 1. The MT-BCR/ABL P190 transgene. The transgene was constructed from human (BCR/ABL) and from mouse sequences (metallothoinein promoter: MT-1). A more detailed description of the transgene is presented elsewhere (Heisterkamp et al., 1990; Voncken et al., 1995b). The human BCR exon 1 is indicated by the open box, human ABL exons 2 and 3-11 by dark boxes. The hatched box represents the MT-1 promoter.

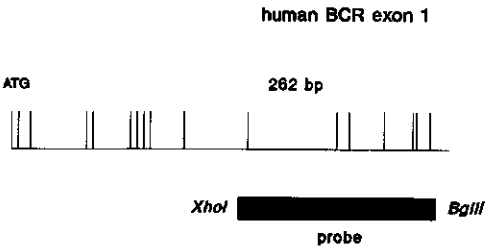

Figure 2. MspI/HpaII restriction sites in human BCR exon 1; the MspI/HpaII restriction sites are indicated by vertical lines. The dark bar underneath the map represents the 0.6 kb XhoI-BglII probe used for methylation studies. The hybridizing human BCR restriction fragment of 262 base pairs is indicated on top; the flanking 3'-restriction fragments were too small to detect by Southern analysis.

indicated in figure 3: hypomethylation of the *BCR* exon 1 region produced indistinguishable restriction/hybridization patterns for both isoschizomers HpaII and MspI, while methylation allowed for MspI digestion only. Table II presents an overview of the methylation and transcriptional status of the transgene in a number of transgenic lines and founders. All animals that had an unmethylated *BCR* exon 1, expressed the transgene (not shown) and had developed disease at the time of sacrifice. Within the 623 line, animals without symptoms of leukemia also carried an unmethylated transgene in their bone marrow. Three founder animals, that revealed a methylated transgene and did not develop disease themselves, sired offspring that did develop leukemia (Table II). Analysis of the methylation status of founder 787 and progeny was not possible, due to its high transgene copy number.

Influence of genetic background on transgene activity

Recently, it was proposed that a strain specific modifier controls methylation of foreign DNA sequences in mice of different genetic background (Allan et al., 1990; Engler et al., 1991). We wanted to determine whether in-crossing of some of the original B6CBA/F1 lines into a 'low' methylating mouse strain (DBA/2) or a 'high' methylating background (C57BL/6) had an influence on transgene activation in the lines with variable penetrance of disease (626 and 629). The 623 mouse line was also bred to both C57BL/6 and DBA/2 mice, while line 791, the only line that never developed *BCR/ABL* related disease, was bred into DBA background alone. Breeding was maintained for at least two generations. Neither the C57BL/6 nor the DBA/2 background had little if any effect on development of leukemia in the 623 line (not shown). None of the other lines, 626, 629 and 791 developed leukemia when bred into the DBA/2 background. Transgene activity has remained constant for over more than 10 generations in the 623 line (in a B6CBA/F1 background).

Induction of transgene activity in vivo

In transgenic mice, exposure to zinc or cadmium causes upregulation of MT promoter activity and transgene expression in liver (Dyer and Messing, 1989; Morahan et al., 1989; Rafferty et al., 1993). We do not know whether hepatic *BCR/ABL* transgene expression levels increase after exposure to zinc or cadmium, since development of leukemic disease was taken as the initial criterium for further analysis of an animal. However, no hepatic pathology was noted upon sacrifice.

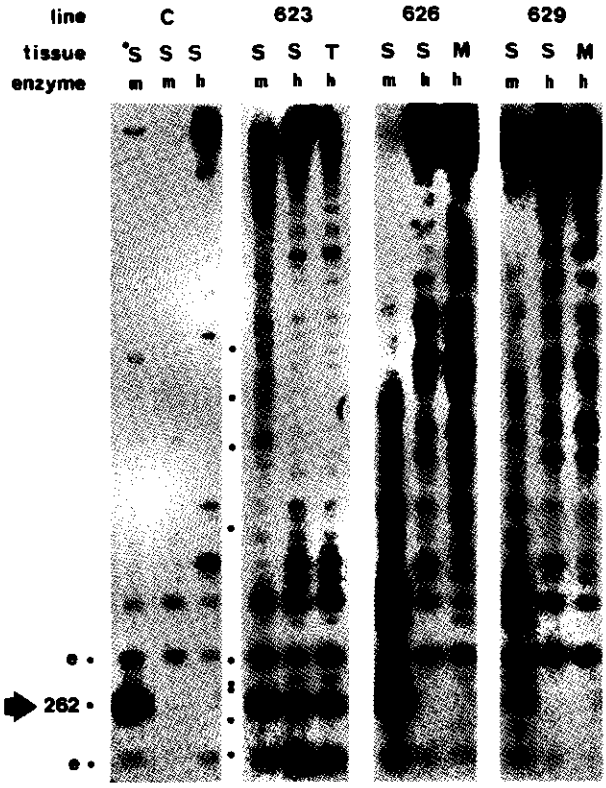

Figure 3. Methylation analysis of BCR exon 1 sequences in the human BCR/ABL P190 transgene. Panel 623 gives an example of an unmethylated transgene, the panels 626 and 629 of methylated transgenes. The expected human BCR fragment size (of 262 bp) under non-methylated conditions (see also: Fig. 2) is marked with an arrow; e indicates endogenous mouse bcr sequences. line: representative animals from different mouse lines are indicated by the founder number of a line (623, 626 and 629 respectively); to the far left, non-transgenic (C) mouse DNA spiked (•) with recombinant transgene DNA and non-transgenic mouse DNA are depicted. tissue: S: spleen, T: tumor, M: bone marrow. enzyme: m: MspI digestion, h: HpaII digestion. The dots at the left of panel 623 represent PhiX(HaeIII) marker DNA fragment sizes.

In an attempt to evoke *BCR/ABL* expression through de-methylation, we set out to induce transcriptional activity using combinations of mitogenic and demethylating agents. Mouse lines 626 and 629 were considered the best candidates for experimental induction. Since leukemia can evolve in a clonal fashion, Southern analysis for altered methylation patterns in treated animals was not considered realistic unless the animals developed overt leukemia. Therefore, development of leukemic disease was taken as an initial criterium for further analysis. The effect of the induction protocols on appearance of *BCR/ABL* expression in circulatory nucleated cells was however closely monitored by means of RT/PCR on peripheral blood samples. Experimental groups IV, V and VI (see 'Materials and Methods') comprised animals of both the 626 and 629 lines; Experiments I, II and III were done with the 629 line only. Although the expected biological responses to the various treatments were noted (such as leukopenia [LPS; 5-FU], loss of coat color [TBI]), none of the

Table II. Transgene methylation and disease in founders and offspring.

founder	disease[a]; (CpG status[b])	progeny	disease[a]; (CpG status[b])
713	ALL; na[c]	BMT[d]	ALL; *U*
623[e]	LLY; nd	progeny[e]	ALL/LLY; *U*
626	–; *M*	626-730	LLY; *U*
		626-728	–; *M*
		626-729	–; *M*
		626-1354	–; *M*
629	–; *M*	629-719	LLY; *U*
		629-723	LLY; na[c]
		629-741	ALL; na[c]
		629-742	-; na[c]
		629-1505	–; *M*
		629-1514	–; *M*
		629-1520	–; *M*
793	–; *M*	793-908	LLY; *U*
		793-1031	LLY; *U*
		793-1030	–; *M*
791[f]	–; *M*	791-894	–; *M*
		791-902	–; *M*

a: Abbreviations: ALL: acute lymphoblastic leukemia, LLY: lymphoblastic lymphoma
b: Methylation status of CpG dinucleotides in BCR exon 1: U, unmethylated; M, methylated.
c: No material was available for Southern analysis from these animals.
d: Bone marrow of 713 was used for serial transplantation experiments (Voncken et al., 1992a), hematopoietic tissues of recipients were analyzed for methylation status and were consistently found unmethylated.
e: Animals of the 623 line developed ALL/LLY with a 100% incidence; this line has been studied in detail (Voncken et al., 1992a; Voncken et al., 1992b); the transgene is consistently unmethylated in the first BCR exon.
f: None of the animals of the 791 line developed BCR/ABL-related disease: the transgene is consistently methylated in its BCR sequence.

treatment regimes employing 5–FU and/or 5-Aza delineated lead to leukemogenesis in transgenic mouse lines 626 and 629, although similar protocols were successfully applied in other models (Thomas and Williams, 1992). In one instance a brief *BCR/ABL* P190 transgene activation was detectable in peripheral blood in two animals when subjected to protocol III: *BCR/ABL* mRNA was detected in

one animal between 3 and 5 weeks after the last 5-Aza induction, while the other animal was positive for *BCR/ABL* expression at only one time point (5 weeks). The same animals, when treated more than 6 months later with protocol VI, failed to show transgene activation. All animals were kept up to 6-12 month following treatment, after which they were sacrificed, examined for gross pathology and some of the animals for transgene expression. None of the animals had leukemic symptoms or expressed the transgene. All animals were kept up to 6-12 month following treatment, after which they were sacrificed, examined for gross pathology and some of the animals for transgene expression. None of the animals had leukemic symptoms or expressed the transgene. None of the treatment protocols led to any other disease in transgenic or control non-transgenic animals.

Induction of transgene activity in vitro

Attempts to induce transgene induction through de-methylation *in vitro* (see Materials and Methods) were also to no avail: none of the cultures revealed transgene activation under any condition, as monitored by RT/PCR. All zero-point controls were negative for transgene expression. Since some of the animals used for these *in vitro* studies had been pre-treated with 5-FU, we have assumed that absence of detectable transgene expression in short term bone marrow cultures most likely reflects the events that take place in the bone marrow with *in vivo* treatment protocols as well. We tentatively concluded from these combined experiments that attempts to evoke transgene expression through demethylation by administration of 5-azacytidine was ineffective in our *BCR/ABL* P190 transgenic animals.

Discussion

The finding that transgene methylation patterns in the *BCR/ABL* mouse lines presented here correlate with transcription of the *BCR/ABL* transgene and development of pre-B cell leukemia or lymphoma and clearly illustrates that P190 Bcr/Abl is, directly or indirectly, responsible for malignant transformation of immature B-cells in this animal model (Heisterkamp et al., 1990; Voncken et al., 1992a; Voncken et al., 1992b).

The *BCR/ABL* P190 transgene is fairly simple in its composition: the promoter is not particularly strong and no specific elements, like locus control regions (Talbot et al. 1989,; Collis et al., 1990), have been included, that would confer position independent and copy number dependent expression characteristics upon the transgene. Based on random integration in the mouse genome, one expects to find inactive transgenes more frequently (i.e. through integration into heterochromatin). However, effectively 1 out of 14 lines was non-productive. Although incorporation of transgenes into transcriptionally active regions in the genome might be occur more readily, the findings suggest that *BCR/ABL* P190 transgene expression is relatively insensitive to chromosomal positioning. Alternatively, this might indicate that the transgene harbors regulatory elements that we are currently unaware of. We have previously observed that *BCR/ABL* expression patterns in a variety of mouse tissues parallels endogenous *bcr* gene expression (Voncken et al., 1992b; Heisterkamp et al., 1993b). Expression of Bcr in rodent brain is high and found in specific structures (Fioretos et al., 1995). Interestingly, in the 626, 629 and 787 lines, transgene expression was detected by RT/PCR in brain and kidney (not shown), while absent in hematopoietic tissues (bone marrow and spleen). Although to date no cis-activating elements, analogous to those described for other genes (Stenzel-Poore et al., 1992; Aronow et al., 1992), have been described for *BCR* coding or intervening sequences, the above observations would not exclude this possibility.

Expression characteristics of (trans)genes are principally directed by upstream regulatory sequences. Metallothoinein (MT) genes (Glanville et al., 1981) are ubiquitously expressed in animals tissues and cell lines (Stuart et al., 1984; and references therein). The mouse MT-1 promoter has been widely used for control of (trans)gene

expression in cells and animals. MT-directed transgene expression in animals is generally too low for detection by Western analysis, except for example in tumor tissue (Iwamoto et al., 1993). We have noted similar transgene behavior in several MT-*BCR/ABL* models (Voncken et al., 1995b). The detectability of transgene encoded protein in terminal cancers is probably the result of progressively malignant tumor evolution rather than a prerequisite for (clonal) tumorigenesis. *BCR/ABL* P190 transgene expression is detectable by RT/PCR in fertilized oocytes (unpublished observations) and throughout a variety of tissues during mouse development (Voncken et al., 1992b). Transgenic animals develop leukemia without prior experimental exposure to heavy metals.

From a mechanistic point of view, the variable penetrance of disease in four individual mouse lines was of considerable interest to us: controlled induction of transgene expression may help unravel early events in *BCR/ABL* mediated neoplastic transformation. We therefore attempted to induce transgene expression in individual animals lacking transgene expression, in lines that displayed a variable penetrance of the disease. Unsuccessful attempts to induce transgene expression in methylated lines included bolus injection with LPS, exposure to cadmium and zinc in the drinking water or a single dose sublethal (650 rad) total body irradiation (TBI). LPS is a known B-cell mitogen and an activator of metallothoinein expression (De et al., 1990; Leibbrandt and Koropatnick, 1994). Repeated LPS administration to transgenic 623-animals did not change the course of disease, as compared to a non-treated transgenic control group, either (not shown), indicating that onset and development of the disease is relatively insensitive to *BCR/ABL* P190 expression levels, or alternatively that the transgene is, because of the truncated mMT-I promoter, non-responsive to LPS-induction. The choice of alternative regimes was based on the premise that in order for 5-Aza to exert its demethylating effect, active cellular replication was necessary. In concordance with this idea, high doses of 5-Aza and/or 5-Aza2dC were only thyro-carcinogenic in mice in combination with a non-genotoxic goitrogen (Thomas and Williams, 1992). Similar studies in liver have shown that a only following a non-genotoxic hepatotrophic stimulus and partial hepatectomy, 5-Aza administration is capable of initiating tumorigenesis (Denda et al., 1985). These results suggested that the effect of 5-Aza is best observed when administered during active cell growth (division). In good agreement with the need for proliferative activity, *in vitro* effects of 5-Aza on cell cultures are usually very rapid (Harrison et al., 1983; Lee et al., 1992). To this end, we first treated our mice with 5–FU. The cytological and histo-pathological effects of 5–FU have been described in detail (Radley and Scurfield, 1979; Hoyer and Nielsen, 1992): by treating animals with 5–FU, the bone marrow is enriched for actively dividing hematopoietic progenitors (Shih et al., 1992). Following 5–FU, the animals received one or more injections with 5-Aza (VI, V, VI). 5-Aza had also been administered alone (III). However, none of the animals in any protocol developed leukemia, indicating that transgene activation through de-methylation is not effective in our *BCR/ABL* transgenic line. 5–FU is a known myelo-suppressant, the cytotoxicity of 5-Aza is not well understood. The possibility exists that either one or both agents are selectively harmful to cells of the B-lineage. The transient transgene expression (protocol III) was not sufficient to precipitate leukemogenesis. We and others have deduced from earlier studies that *BCR/ABL* expression needs to be sustained in order for tumor evolution toward a more malignant cancer to occur (Voncken et al. 1992a; Voncken et al., 1995b).

Recently, it was shown that transgene expression and imprinting are epigenetically controlled by genotype-specific modifier genes: *trans*acting modifier gene products were found responsible for differences in the ability of various mouse strains to methylate foreign DNA, C57BL/6 mice showing the highest degree of methylation, DBA/2 the lowest (Allen et al., 1990; Engler et al., 1991). We tested whether the *BCR/ABL* transgene activity could be altered by back-crossing the founders to either C57BL/6 or DBA/2 mice. However, breeding over at least two generations into a C57BL/6 or DBA/2

background mice, did neither activate transgene expression in the inactive 626, 629 and 791 lines nor affect mortality rate in the active 623 line (not shown). The 623 line has been studied for over 10 generations in our laboratory and shows no signs of diminished transgene activity. Variable penetrance in third generation animals (F2) has never been observed. This raised the possibility that transgene copies separated through germ line transmission. However, transgene segregation as a result of multiple chromosomal integration was rejected on the ground of Southern analysis (i.e. confirmation of single integration site and low transgene copy number). An alternative, the existence of a 'silencer gene' that segregates in the F1 generation, has not been explored.

Although the variable penetrance of disease among sibling animals appears to point to a rather direct transgene silencing mechanism, the failure to induce any expression both *in vitro* and *in vivo* seems to refute this deduction. Taken together, the findings seem to indicate that, although a clear marker, transgene methylation is not the sole factor determining transgene silencing; indeed epigenetic changes to DNA, such as CpG methylation, often function to maintain and propagate the newly established transcriptional state of a responding locus (Bird, 1992; Tate and Bird, 1993 + references therein). Mechanisms that regulate *de novo* methylation of foreign DNA sequences *in vivo* are not well understood (Doerfler, 1992). Adenoviral sequences, retroviral sequences and transgenes that include prokaryotic and viral sequences are subject to methylation in transgenic mice and cell lines (Lettman et al., 1992; Engler et al., 1992). Recognition of species specific sequences (MT-1 promoter) in our transgene may prevent direct inactivation through methylation. Both *BCR* alleles are actively transcribed, both in human (Fioretos et al., 1994) and in mouse (Voncken et al., 1995a). It would therefore seem unlikely that *BCR* sequences in our transgene would be 'branded' for allelic inactivation. Conversely, functional 'counter'-mechanisms, such as forementioned *cis* activating elements could help explain sustained activity of transgenes throughout many generations, despite clear harm to the organism.

In summary, we show that there is a close correlation between transgene activity, development of disease and transgene methylation in our mouse model for Philadelphia positive acute lymphoblastic leukemia, as revealed by combined isoschizomeric restriction analysis of *BCR* exon 1 sequences and expression studies. Attempts to induce transgene expression in mice, which showed *BCR/ABL* transgene methylation, with several protocols employing both tissue directed (LPS, 5-FU plus 5-Aza) and general induction protocols (heavy metals, TBI) were unsuccessful: no *BCR/ABL* related hematopoietic disorders were induced. *In vivo* observations also seem to confirm that sustained expression of *BCR/ABL* P190 is required for leukemia to develop. Based on failure to detect any transgene expression *in vitro* by means of the very sensitive RT/PCR method, we speculate that methylation is not the cause for transgene silencing, but that CpG methylation in the 5-prime region of the transgene possibly serves to preserve an inactive transgene and as such provides a marker for transcriptional activity. The scientific and clinical application of sophisticated genetic technologies, such as transgenic technique and gene therapy will benefit form further elucidation of mechanisms that cause transcriptional silencing of exogenous DNA sequences in mammalian cells.

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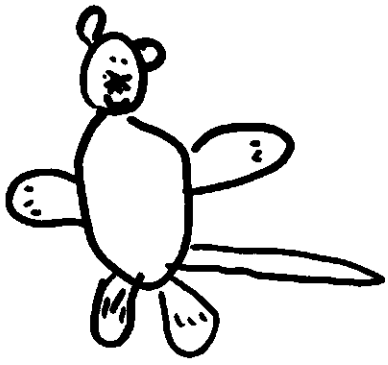

'A big mouse'. By Aminah (5)

CHAPTER 4

Restricted Oncogenicity of *BCR/ABL* P190 in Transgenic Mice

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Restricted Oncogenicity of BCR/ABL p190 in Transgenic Mice[1]

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ABSTRACT

A chimeric *BCR/ABL* oncogene encoding the p190 protein has been introduced into the mouse germline using microinjection of one-cell fertilized eggs. Founder and progeny transgenic animals, when becoming ill, were found to develop lymphoblastic leukemia/lymphoma which was transplantable to compatible recipients. Lymphoblasts were arrested at the pre-B stage of development. Expression of BCR/ABL was not detected in peripheral blood during the early stages of leukemia but became evident as the disease progressed. However, the transgene was expressed early in development in bone marrow and was also transcribed in nonhematopoietic tissues although this did not result in tumorigenesis. These results strongly suggest that the oncogenicity of BCR/ABL is limited to hematopoietic cells, including pre-B cells or their progenitors.

INTRODUCTION

The Ph chromosome, the result of a reciprocal translocation between chromosomes 22 and 9 (1–3), is specifically associated with CML[3] and is also found in a percentage of adult and pediatric patients with ALL (4, 5).

Due to the translocation, the *ABL* protooncogene from chromosome 9 is fused to the *BCR* gene on chromosome 22 and together they form a chimeric gene. The *BCR* gene contributes promoter and 5′ exons, and the *ABL* gene contributes its 3′ exons. The activity of the ABL gene product, normally a tyrosine kinase with a low level of autophosphorylation activity, is substantially increased in the BCR/ABL protein. Moreover, the fusion of parts of the *BCR* gene with the *ABL* combines two kinase activities within one protein (6, 7). The two major forms of BCR/ABL proteins, p210 and p190, differ in the amount of BCR amino acid residues included. On a genomic level, p190 contains only *BCR* gene exon 1, whereas p210 contains exon 1 as well as several other BCR exons. p190 appears to be associated mainly with Ph-positive ALL, whereas p210 is found both in Ph-positive ALL and in CML (for reviews see Refs. 8–10).

To investigate the role of the chimeric gene in leukemia, BCR/ABL p210 retroviral constructs have been introduced into 5-fluorouracil-treated mouse bone marrow and transplanted into lethally irradiated recipients. Different types of hematopoietic disease were found. In one study 13 of 30 recipients (43%) developed either a CML-like myeloproliferative disease, lymphoblastic leukemia, or macrophage cell type tumors within 5 months (11). In a different study recipients eventually died of hematopoietic disease involving macrophage, lymphoid, erythroid, and mast cell lineages with the incidence (69–100%) of the exact type of disease being strain dependent (12). Myelomonocytic leukemias, granulocytic leukemias, and lymphocytic leukemia/lymphomas were found with p210 constructs (13).

These types of studies have tested only the oncogenic potential of the *BCR/ABL* gene in cells types limited to the hematopoietic system and even there the outcome could have been determined by selection of hematopoietic cells capable of being infected by these viruses. A different model system is created through the use of transgenic mice because the gene of interest will be present in every cell. In a previous study, we have generated mice transgenic for a BCR/ABL p190 construct. Of ten founder mice, eight died rapidly, within 58 days of birth, of acute leukemia (the majority lymphoid and possibly some myeloid) (14). The two remaining mice were found to be chimeric and did not express the construct in their bone marrow; the unexpected early onset of the disease precluded further studies or breeding with these mice.

To examine p190-associated malignancies in more detail and over a prolonged period of time, we have now generated new founders and have established a transgenic line from one, in which the progeny reproducible develop cancer. We find that BCR/ABL expression is not restricted to bone marrow cells. Surprisingly, however, the types of malignancies found were limited to pre-B leukemias and lymphomas even in animals with prolonged survival time and over 4 generations.

MATERIALS AND METHODS

Transgenic Mice. Mice transgenic for the BCR/ABL p190 construct were generated as previously described (14). Founder animals were the offspring of matings between C57BL × CBA F_1 animals. Transgenic progeny was the result of matings between transgenics and C57BL × CBA F_1 mice. F_1 progeny are identified by six-digit numbers. The first three digits are those of the founder of that specific line.

Autopsies on terminally ill mice were performed as described previously (15). Routine histology examinations included brain, heart, lung, liver, kidney, spleen, and thymus. Tissue sections were fixed in 10% formalin, 90% B5 (90% B5 = 66 g $HgCl_2$ and 14 g NaAc/liter). Peripheral blood and bone marrow smears were stained with Wright-Giemsa and evaluated histologically. WBC were performed manually. Mice were diagnosed with lymphoblastic lymphoma/leukemia or ALL alone. Of all founder and progeny transgenic mice included in this study (n = 100), 50% had died within 70 days of birth and 72% had died (range, 31–199 days) of leukemia/lymphoma at an arbitrarily chosen experiment end point. Of the line established from founder 623, animals died of or were sacrificed with terminal disease on average 68 days after birth (range, 41–147 days).

Southern Blot Analysis. DNAs were isolated from spleens and involved lymph nodes as described (14). DNAs digested with *Eco*RI were run on 0.7% agarose gels, blotted to nitrocellulose, and hybridized to a 1.2-kilobase *Mspl/Eco*RI J_H probe located immediately 3′ to J1-J4. Posthybridization washings were at 0.3× standard saline-citrate, 65°C.

Analysis of BCR/ABL Expression in Peripheral Blood. WBC and BCR/ABL expression assays were performed regularly on peripheral blood from transgenic and control mice. Briefly, approximately 10 μl of blood were withdrawn from the tail artery using a 25-gauge needle, and

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[3] The abbreviations used are: CML, chronic myeloid leukemia; ALL, acute lymphoblastic leukemia; RT/PCR, reverse transcriptase/polymerase chain reactions.

3 μl heparinized blood were used for RNA isolation. RBC were lysed after addition of 50 volumes 0.144 M NH_4Cl-5 volumes 0.01 M NH_4HCO_3. WBC were pelleted and lysed in 500 μl "solution D" (16). RNA was isolated essentially as described (16). The RNA pellet was suspended in 10 μl d H_2O and RT/PCR were performed on 2 μl essentially as described (14) using "ALL E" and "ALL F" oligonucleotides as amplimers and "ALL G" as hybridization probe (17). To test the sensitivity of detection under our experimental conditions, a series of dilutions of blood from a leukemic mouse (terminal WBC, 85×10^9/liter) with normal blood was performed. These samples were subjected to RT/PCR and hybridized to the ALL G probe. A detection level of one expressing cell among 10,000 nucleated cells was found.

To verify that a certain RNA sample was suitable for RT/PCR reactions, it was tested for mouse actin expression using as 3′ amplimer (5′- CGGTTGGCCTTAGGGTTCAGGG-3′) and as 5′ amplimer (5′-GTGGGCCGCTCTAGGCACCAA-3′) (18). In some experiments, an oligonucleotide probe (5′-ACTCCTATGTGGGTGACGAGG-3′) contained within the amplified region was used for hybridization probe.

RNA Isolation and Expression in Other Tissues. RNAs were isolated as described (16) using guanidine isothiocyanate. To test for early expression, timed matings between transgenic males and nontransgenic C57BL × CBA F_1 females were set up and embryos were isolated between day 14 and day 16 of gestation. Tail DNAs were Southern blotted to determine which animals were transgenic. Tissues isolated from the embryos were homogenized immediately in solution D and stored at −80°C. Preterm and 1 day postpartum bone marrow RNA was isolated by homogenizing an entire hind limb. From 2 to 3 μg of total RNA were used per RT/PCR reaction. RT/PCR products were separated on 1.5% agarose gels, blotted to Nytran (Schleicher and Schuell), and hybridized as indicated above.

RNase Protection Assays. RNase protection assays were performed essentially as described (18). To construct a probe for BCR/ABL detection, a 1.4-kilobase segment of the BCR/ABL p190 mRNA was amplified using RT/PCR with amplimers ALL E and CML H. CML H is located near the kinase domain of ABL and consists of: 5′—CCAAAGCAATACTCCAAATGC—3′. A 450-base pair 5′ *Taq*I— 3′ *Kpn*I BCR/ABL complementary DNA fragment from this 1.4-kilobase amplified product was inserted into pSK digested with *Cla*I/*Kpn*I. A segment of 3′ ABL sequences was removed by *Ava*II digestion. The *Ava*II site was blunted by a filling-in reaction; the DNA was digested with *Hin*dIII and the insert was ligated into pSK digested with *Hin*dIII/*Hin*cII. The segment contains 147 base pairs of BCR exon 1 sequences and 93 base pairs of ABL exon 2. The construct was linearized with *Bam*HI. A 306-base pair probe was synthesized containing the 240 bp BCR/ABL fragment plus 66 base pairs of pSK sequence using T7 RNA polymerase and [α-^{32}P]UTP. Probes were gel purified prior to hybridization. Twenty μg of total RNA were used for BCR/ABL hybridizations. An approximately 20-fold excess of probe was used. As control, mouse actin complementary RNA probes were used and 1 μg of total RNA. To quantitate individual signals, cpm were determined using a Betascope 603 blot analyzer (Betagen). Relative amounts of actin and BCR/ABL mRNA were calculated, after correction for background and the actual length of the mRNAs.

RESULTS

Pathology of BCR/ABL p190 Mice. A total of 14 new founder mice were generated that were transgenic for the BCR/ABL p190 construct (14) controlled by the metallothionein promoter. Estimated transgene copy numbers varied from less than 1 to approximately 26 (Table 1). Two animals died of undetermined causes. Four animals were still alive and apparently disease free after 20 months. However, three of these have produced progeny which developed malignancies. Eight (57%) founders have died with a diagnosable disease. Transgenic progeny was obtained from a number of animals (#632, 623, 626, 629, 787, 791, and 793). The type of disease in the progeny was similar to that of the founders, with an average incidence of 67%. Founder animal 623 was used to establish a permanent transgenic breeding line.

Table 1 *Transgenic BCR/ABL P190 founders*

Animal	Transgene copy no.	Age at death (days)	WBC at death ($\times 10^9$/liter)	Diagnosis	IGH[a]
618	8	28	20	LL	G
620	2	31	ND	LL	R2
631	5	37	4	LL	R2
628	2	67	10	ALL[b]	R3
632	6	79	153	LL	R2
713	2	76	28	ALL	R3
623	2	198	12	LL	R2
626	4	Alive	—	—	—
629[c]	6	600	ND	ND	ND
787	26	Alive	—	—	—
790	11	31	ND	ND	ND
791	<1	Alive	—	—	—
793	1	Alive	—	—	—
796	16	55	8	LL	R2

[a] IGH, immunoglobulin J_H rearrangement in splenic DNA; LL, lymphoblastic leukemia/lymphoblastic lymphoma; ALL, acute lymphoblastic leukemia; ND, not determined; G, germline; R1, one rearranged band; R2, two rearranged bands.
[b] Animal died during routine sedation. Autopsy showed bone marrow packed with neoplastic lymphoblasts.
[c] Animal was found dead with no external signs of tumors.

Of the eight founders that died, six developed lymphoblastic leukemia/lymphoma and two developed lymphoblastic leukemia (Table 1). Fixed and stained tissue sections showed a varying degree of involvement of lymph nodes with neoplastic lymphoblasts in all sick founders and offspring analyzed in detail with the exception of animals 713 and 628. None of the animals showed involvement of cell types other than lymphoblasts. Many animals exhibited pronounced splenomegaly. Gross morphological examination and histological examination of fixed and stained tissue sections of samples of brain, liver, kidney, lungs, heart, and thymus showed no abnormalities other than those associated with the development of lymphomas.

Cell Type Affected. Southern blot analysis was used to examine immunoglobulin heavy chain rearrangements in the neoplastic lymphoblasts using a mouse J_H probe. Splenic DNAs from animals such as 713 and 632 showed 3 or 2 rearranged bands (Fig. 1, *Lanes 7* and *6*; Table 1). Other splenic DNAs lacked visible IGH rearrangements (618, Table 1; 629–723 and 632–752, Fig. 1, *Lanes 9* and *8*). Comparable results were also obtained with other DNA samples in that some appeared to have an IGH germline configuration, whereas others showed few (2–4) prominent rearranged bands indicative of a mono- or biclonal population of cells (Fig. 1; Table 1). The immunoglobulin κ light chain constant region (C_κ) was germline in all samples tested (results not shown).

Peripheral blood from 5 randomly chosen progeny animals (626–730, 632–749, 632–753, 632–767, 623–881) representing three different lines and from transplant recipients of mouse 713 bone marrow (also see below) were phenotyped in detail and were found to uniformly exhibit a pre-B immunophenotype: the samples were all positive for the B220/Ly5 (pan-B), BP-3 (pan B), and BP-1 (B-cell precursor) markers and were negative for the Thy-1 and M1/70 markers which are found on cells of T-cell and myeloid lineages, respectively. Surface immunoglobulin was absent (results not shown and Ref. 19). The genotypic analysis on the other samples using Southern blots of immunoglobulin gene segment rearrangements is in agreement with this conclusion.

The leukemia was found to be transplantable. Bone marrow from two randomly chosen leukemic mice, 713 and 623–844–1037 (an F2 descendant of 623) was transferred to sublethally

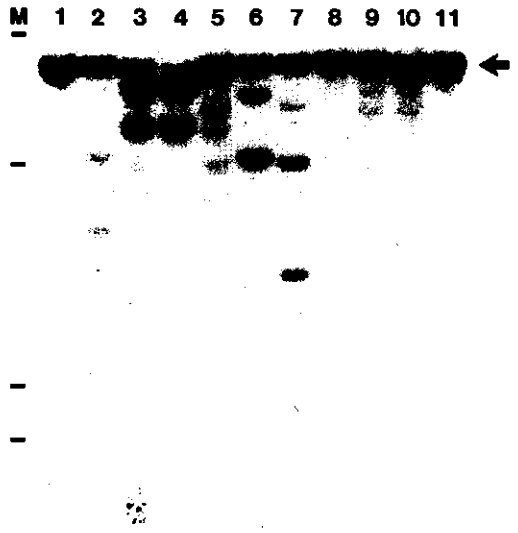

Fig. 1. Southern blot analysis of IGH rearrangements in involved splenic DNAs. DNAs digested with *Eco*RI include those of founders 618 (*Lane 1*), 620 (*Lane 2*), 631 (*Lane 3*), 631 involved lymph node (*Lane 4*), 628 (*Lane 5*), 632 (*Lane 6*), 713 (*Lane 7*). F1 animals include 632–752 (*Lane 8*) and 629–723 (*Lane 9*). Control splenic and tail DNA is shown in *Lanes 10* and *11*. The position of the 6.4-, 4.4-, 2.3-, and 2.0-kilobase markers of a λ/*Hin*dIII digest is shown to the *left* in *Lane M*. *Arrow*, germ line fragment.

(500 rads) irradiated syngeneic recipients. After a latency period (27–69 days) leukemia developed in some (713) or all (623 line) of the recipients. Upon secondary and tertiary passage, cells from the 713 line became increasingly malignant, with 1×10^6 cells causing overt leukemia in nonirradiated hosts in as little as 14 and 9 days. Analysis of peripheral blood cells from three of the secondary transplant recipients showed the same phenotype as the leukemic cells of the transgenic mice described above (not shown).

Expression of p190 in the Development of Leukemia/Lymphoma. These experiments demonstrated the association between the presence of the BCR/ABL oncogene and the development of a transplantable pre-B leukemia/lymphoma in the founder transgenic mice. However, not all animals became leukemic and it is possible that in some the transgene was integrated into a transcriptionally silent region.

A number of founders were followed prospectively over an extended period of time for the possible appearance of BCR/ABL expression in their peripheral blood using RT/PCR. No expression could be detected in 626 and 629, although both were evaluated up to 3 months of age (not shown). Both animals were still alive at 20 months but had sired progeny which expressed BCR/ABL and developed leukemia/lymphoma. Similarly, 787 and 793 sired expressing progeny.

Subsequent expression studies were performed with progeny of founder 623 (Table 1). The 623 transgenic line has been bred for more than 4 generations, and within each generation animals developed lymphoblastic leukemia/lymphoma. Eight of such animals from two randomly chosen nests with transgenic mice were assayed at a relatively early age. The first appearance of BCR/ABL expression in peripheral blood was at day 20 in two animals. At 30 days, 7 of 8 mice were positive and all mice of this set were dead by 193 days (Fig. 2). In a second experiment, ten 19-day-old mice were sacrificed and assayed for BCR/ABL expression both in bone marrow and in blood. Morphologically, bone marrows of these animals appeared normal. Control reactions using actin amplimers showed that all samples were suitable for RT/PCR (Fig. 3). Six of 10 mice lacked clear expression in peripheral blood at this detection level (also see "Materials and Methods"), but all had a positive bone marrow (Fig. 3). Taken together, these results showed that the *BCR/ABL* transgene was not detectably expressed in the peripheral WBC population of apparently healthy founders, or of progeny of 623. However, expression was clearly present in bone marrow cells at 19 days after birth.

To investigate the earliest occurrence of expression in bone marrow, samples from 5–6 days of age, day 1, and preterm at

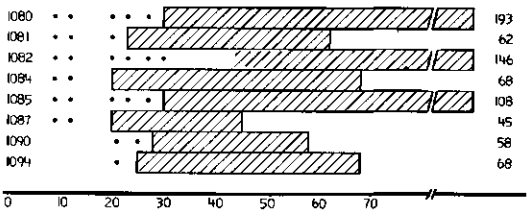

Fig. 2. Expression of BCR/ABL in peripheral blood. *Left ordinate*, animals; all animals are F2 and F3 progeny of 623. ▨, from which age on, in days after birth, expression could be detected; ●, individual sampling points. Additional sampling points (not shown) were taken for most mice subsequent to the detection of expression. Blood of mouse 1082 was still negative for BCR/ABL expression at the last sample taken (*i.e.*, 30 days). *Right ordinate*, age of the animals at death or sacrifice due to overt disease.

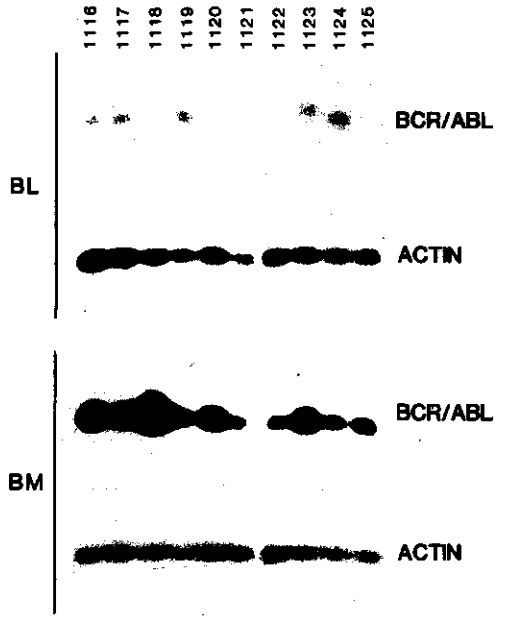

Fig. 3. BCR/ABL expression in the bone marrow and blood of 19-day-old mice. *Top abscissa*, identifying numbers of the individual mice. Animals 1116–1125 are all F3 descendants of 623. Samples of RNA from blood (*BL*) or bone marrow (*BM*) were subjected to RT/PCR with the appropriate primers and hybridized to a BCR/ABL or an actin oligonucleotide probe. Exposure time was 21 h for BCR/ABL and 1 h for actin.

between 14 and 16 days of gestation were analyzed. All transgenic samples showed clear expression whereas nontransgenic siblings were uniformly negative (Table 2). These results suggest a prolonged expression of the BCR/ABL p190 construct in bone marrow.

Expression of p190 mRNA in Other Transgenic Tissues. RNA isolated from transgenic brains and livers of the 14–16-day-old fetuses, 1 day and 5–6-day-old mice also showed BCR/ABL expression (Table 2). An examination of different RNAs from somewhat older (13–22 days) transgenics from the 623 line using PCR showed continued expression in brain and liver, and also BCR/ABL mRNA in kidney, spleen, and muscle (Table 2).

The RT/PCR method is very sensitive and able to detect small amounts of expression. However, since expression of BCR/ABL could not be detected in peripheral blood samples (not shown) taken simultaneously with those for other RNA isolations, it did not seem likely that the signals seen in other tissues were from BCR/ABL expressing bone marrow cells which had escaped into the circulation. To provide a more quantitative measure of the different levels of expression seen and to confirm the endogenous tissue-specific BCR/ABL expression an RNase protection assay (20) was used.

A protection probe was constructed which spanned the BCR/ABL junction of the p190 mRNA and contained both BCR and ABL sequences (Fig. 4*A*). Different lymphoblastic lymphoma RNA samples from one founder and progeny of four different transgenic lines were examined for BCR/ABL expression (Fig. 4*B*). With 20 μg of total lymphoma RNA, BCR/ABL signals were generated comparable to those obtained with an actin probe in 1 μg total RNA (Fig. 3*B*). RNAs from brain, liver, kidney, muscle, and spleen of the 13–16-day-old mice (also see Table 2) were then examined (Fig. 5). Actin showed a differential level of expression in different organs. BCR/ABL expression was relatively low but a consistent level of protection was

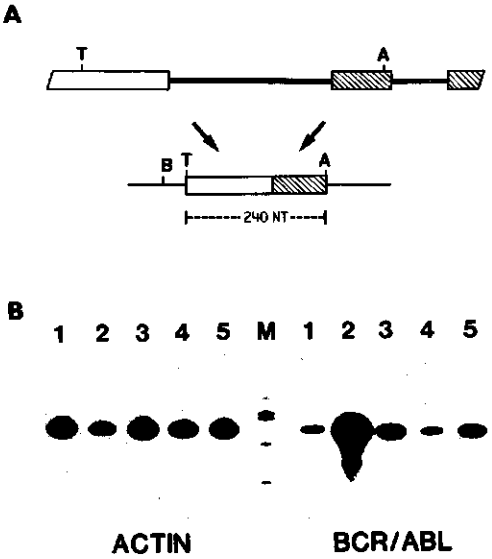

Fig. 4. RNase protection analysis of BCR/ABL expression. *A*, RNase protection probe. *Upper line*, part of the BCR/ABL DNA construct used (14). □, BCR coding sequences; ▨, ABL coding sequences. *Lower line*, full-length mRNA protection probe including plasmid sequences. *A*, *Ava*II; *B*, *Bam*HI; *T*, *Taq*I. *B*, expression of BCR/ABL in lymphoid tumors. RNAs (1 μg for actin, 20 μg for BCR/ABL) include those isolated from lymphoblastic lymphomas of founder 796 (*Lane 1*); 629–719 (*Lane 2*); 632–753 (*Lane 3*); 787–1018 (*Lane 4*); 623–880–1051 (*Lane 5*). *M*, ΦX/*Hae*III marker. Exposure time was 10 h with intensifying screen.

Table 2 *Expression of BCR/ABL in different tissues using RT/PCR*

Age of animal[a]	Expression in: Bone marrow	Brain	Liver	Kidney	Muscle	Spleen	Animal no.[b]
14/16 pc.	+	+	+				1132i
14/16 pc.	+	+	+				1132l
14/16 pc.	+	+	+				1132m
Control	−	−	−				1132k
1 d.	+	+	+				1211a
1 d.	+	+	+				1211b
1 d.	+	+	+				1211c
1 d.	+	+	+				1211e
Control		−					1211d
5–6 d.	+	+					1132b
5–6 d.	+	+					1132c
5–6 d.	+	+					1132d
5–6 d.	+	+					1132e
Control	−						1132a
13 d.	+	+	+	+	±	−	1262
13 d.	+	+	+	+	±	±	1263
16 d.	+	+	+	+	±	+	1255
16 d.	+	+	+	+	±	±	1260
19 d.	+	+	+	+	±	+	1254
19 d.	+	+	+	+	±	+	1259
22 d.	+	+	±	+	±	±	1256
22 d.	+	+	+	+	±	±	1257

[a] Expressed in days after birth (d) or after conception (pc).
[b] Progeny of 1132 and 1211 are F4 and F5 animals. All other mice are F3 generation of the 623 line. Hybridization signals are designated as readily detectable (+), absent at this detection level (−), or very weak (±) as compared to the other samples. Indicated control animals are nontransgenic mice.

seen in the tissues examined. The highest levels were found in brain, kidney, and spleen (Fig. 5). Compared to actin, BCR/ABL expression was approximately 200-fold lower in brain; 400–600-fold lower in kidney, muscle, and liver; and 1400-fold lower in spleen. In brain, protection of the chimeric mRNA was detected as early as 14–16 days *in utero* (not shown). In comparison with the (average) expression levels in the lymphomas, the (average) relative levels of BCR/ABL expression were calculated as lymphoma:brain:kidney/spleen:liver/muscle as 100:2:1:0.3. These experiments conclusively demonstrate that the *BCR/ABL* transgene is expressed not only in bone marrow but also in nonhematopoietic tissues such as brain, kidney and muscle.

DISCUSSION

We have examined the development of malignancy in 14 founder mice and progeny transgenic for a p190 BCR/ABL construct over a prolonged period of time. When any of the total of 100 animals developed cancer, it was a lymphoblastic leukemia/lymphoma, between 31 and 199 days after birth. In a previous study (14) transgenic animals had a somewhat more virulent development of overt disease than these animals and more significantly, the cause of death in 2 of the 8 previous mice was diagnosed as myeloid leukemia. The constructs used in both studies were identical, as was the mouse genetic background (C57BL × CBA F_1) used to generate the transgenic founders. The observed differences could be attributed to the low numbers of transgenic mice (8 genuinely *BCR/ABL* transgenic mice) analyzed in the first study, environmental factors and/or less than optimal pathological diagnosis in the previous

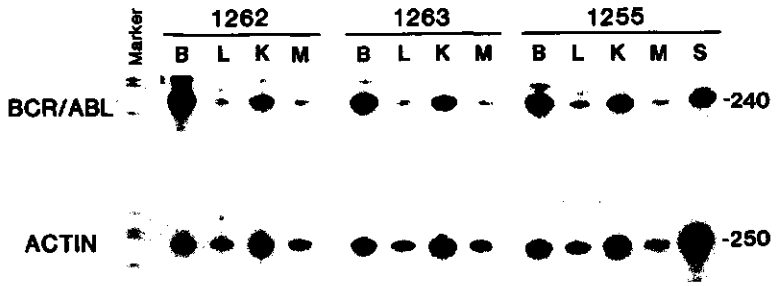

Fig. 5. BCR/ABL expression in different tissues using an RNase protection assay. Mouse identification numbers are shown above the panels. *B*, brain; *L*, liver; *K*, kidney; *M*, muscle; *S*, spleen. Marker is ΦX/*Hae*III. The size (in nucleotides) of the protected BCR/ABL or actin fragments is as shown. Exposure times were 15 h at room temperature for actin and 216 h at −80°C with intensifying screen for BCR/ABL.

study since the two mice diagnosed with myeloid leukemia were found dead (14). An advantage of this relative lower initial morbidity was that we were able to establish breeding lines and characterize the affected cell population in more detail. Phenotypic analysis of cell-surface markers on leukemic peripheral blood cells of different samples consistently indicated a pre-B phenotype (this study and Ref. 19). Thus, a pre-B cell type was affected both in these animals as well as was suggested in the animals of the previous study (14) and parallels the dominant cell type observed in Ph-positive lymphoblastic leukemia in humans. These data differ considerably from those published by others (21) who, among other differences, found that the majority of mice developed T-cell lymphomas. However, considerable variation in the constructs used are obvious. Most importantly, in the study of Hariharan *et al.*, a *bcr/v-abl* construct was used for injection. In comparison with mouse *abl*, *v-abl* has undergone multiple deletions and amino acid substitutions which are thought to enhance tumorigenicity (22). Our experiments to examine possible BCR/ABL expression in peripheral blood of founders yielded a somewhat surprising result in that expression was not found in the circulating WBC of early leukemic mice. One of many possible explanations for this restricted expression is that the BCR/ABL construct is turned on only in immature (precursor) cells and is silenced when the cells mature. However, the metallothionein promoter used to control expression of the construct is active in many tissues and is not known to be regulated in such a way in hematopoietic cells. It would therefore seem more likely that some sequence belonging to the *BCR* or *ABL* part of the transgene is responsible for this regulation.

A different explanation is that the continued BCR/ABL expression in bone marrow directly or indirectly causes maturation arrest or causes the cells to die. Because maturation/viability and BCR/ABL expression would be mutually exclusive, only cells not expressing the transgene would be able to mature and be subsequently found in peripheral blood. The *BCR/ABL*-expressing cells, because of their immaturity, would remain within the bone marrow until either crowding and/or mutations leading to termination of stromal dependence cause migration into the peripheral blood. Such mutations for cells of the B-cell lineage which render the cells, *e.g.*, interleukin 7 and/or stromal cell-independent have been demonstrated in *in vitro* lines derived from the blood of these transgenic mice (19).

Previously, it has been shown that BCR/ABL (p210) can transform rat fibroblasts (23) but not NIH 3T3 cells (24) suggesting that BCR/ABL might also cause the development of solid tumors. We have observed morphological changes in both NIH 3T3 and Rat-2 cells upon transfection of BCR/ABL p210 and/or p190.[4] These changes were quite subtle in comparison with those caused by other transforming oncogenes and we were unable to unambiguously determine if BCR/ABL causes transformation of cultured fibroblasts. However, *in vivo*, in a mouse model, the consequences of BCR/ABL expression with respect to tumorigenesis in other cell types should be more significant than in immortal and *in vitro* cultured fibroblasts.

Since in our mice the transgene is transcribed in tissues other than bone marrow, why do no malignancies develop there? There are at least two possible explanations for these findings. Since the absolute level of BCR/ABL expression found in brain is on the average approximately 50-fold lower (range, 25–500-fold) than that found in lymphomas it is possible that a certain threshold level is required for the generation of tumors. However, the lymphomas consist predominantly of a single type of cell, represent terminal stage disease tissues, and may contain malignant cells which were selected for high BCR/ABL expression after leaving the bone marrow. The brain sample will contain a heterogenous collection of cell types with a possible very high level of expression in some cell types and none in others. It is therefore difficult to prove that an overall 50-fold difference in expression levels is realistic from the viewpoint of a single cell.

A second possibility is that only the bone marrow precursor cells expressing BCR/ABL contain those effector molecules that are necessary for mediating the oncogenic effect of the BCR/ABL protein. This view is consistent with the demonstrated critical role of the normal *ABL* protooncogene in a restricted population of cells of the immune system (25, 26). The normal *BCR* gene product has sequence similarity with a GTPase activator protein for $p21^{rho}$, and was shown to have GTPase activator protein activity for $p21^{rac1}$ (27). $p21^{rac}$ is a member of the GTP-binding protein superfamily of signal-transducing proteins, is ubiquitously expressed, and is involved in secretory processes in myeloid cells (28, 29).

The restricted oncogenicity of BCR/ABL for a certain type of hematopoietic progenitor cells may thus be a reflection of cellular functions of the *BCR* and/or *ABL* genes in signal transduction pathways found only in those types of cells. This would further explain why the Ph chromosome, which one would expect to arise by chance in many proliferating tissues, is found only in blood cell cancers.

[4] Unpublished data.

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'A jumping mouse'. By Aarti (8)

CHAPTER 5

BCR/ABL P210 and P190 Cause Distinct Leukemia in Transgenic Mice

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BCR/ABL P210 and P190 Cause Distinct Leukemia in Transgenic Mice

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Abstract

DNA constructs encoding *BCR/ABL* P210 have been introduced into the mouse germ line using microinjection of one-cell fertilized eggs. Kinetics of *BCR/ABL* P210 expression in transgenic mice were very similar to those of *BCR/ABL* P190 constructs in transgenic mice: mRNA transcripts were detectable early in embryonic development and also in hematopoietic tissue of adult animals. Expression of *BCR/ABL* in peripheral blood preceded development of overt disease. P210 founder and progeny transgenic animals, when becoming ill, developed leukemia of both B and T cell origin after a relatively long latency period. In contrast, P190 transgenic mice exclusively developed leukemia of B-cell origin with a relatively short period of latency. The delayed progression of *BCR/ABL* P210 associated disease in the transgenic mice is consistent with the apparent indolence of human CML during the chronic phase. We conclude that in transgenic models, comparable expression of *BCR/ABL* P210 and *BCR/ABL* P190 results in clinically distinct conditions. The observed dissimilarities are most likely due to intrinsically different properties of the P190 and P210 oncoproteins and may also involve sequences that control transgene expression.

Introduction

A reciprocal translocation between chromosomes 9 and 22 results in the formation of the Philadelphia (*Ph*) chromosome (Nowell and Hungerford, 1960; de Klein et al., 1982; Rowley, 1973). Although invariably associated with chronic myeloid leukemia (CML), the *Ph*-chromosome also occurs in a percentage of adult and childhood acute lymphoblastic leukemias (ALL) (Sandberg et al., 1980; Priest et al., 1980) and in acute non-lymphocytic leukemia (ANLL). The [t(9;22)(q34;q11)] translocation fuses *BCR* to the *ABL* proto-oncogene. Activation of the Abl tyrosine kinase in hybrid Bcr/Abl oncoproteins is considered essential for the development of leukemia (reviewed in Van Etten, 1993). The two major forms of Bcr/Abl proteins, P190 and P210, differ only in the amount of Bcr amino acid residues included. P190 contains only residues encoded by *BCR* gene exon 1, whereas P210 contains residues encoded by *BCR* exons 1 to 13 or 14, depending on the exact location of the breakpoint on chromosome 22. P190 appears to be associated mainly with Ph-positive ALL, whereas P210 is found both in Ph-positive ALL and in CML (for reviews see Kurzrock, Gutterman and Talpaz, 1988; Sawyers, Denny and Witte, 1991; Heisterkamp and Groffen, 1991).

The 160 kDa *BCR* gene product contains several distinct domains. The segment encoded by exon 1 harbors a serine/threonine kinase activity *in vitro* and is held responsible for activating *ABL* in the chimeric *BCR/ABL* gene product (Maru et al., 1991; McWhirter and Wang, 1991; Muller et al., 1991). *BCR* exons 3-10, which are only present in *BCR/ABL* P210 but not in *BCR/ABL* P190, share sequence homology with a guanine nucleotide-exchange factor for human *CDC42*: the *DBL* proto-oncogene (Eva and Aaronson, 1985; Hart et al., 1991). The C-terminal end of $p160^{Bcr}$ has GTPase activating protein (GAP) activity toward the small GTP-binding proteins Rac1/2 and CDC42 *in vitro* (Diekmann et al., 1991; Hart et al., 1992). Examination of *bcr* null mutant mice recently

connected $p160^{Bcr}$ GAP *in vivo* to regulation of superoxide production by the NADPH-oxidase system in myeloid and B lymphoid cells (Voncken et al., 1995).

The non-receptor tyrosine kinase $p145^{Abl}$ negatively regulates cell growth when overexpressed in fibroblasts (Sawyers et al., 1994), a function which contrasts with that of *abl* fusion oncogenes such as v-*abl* and *BCR/ABL*. *ABL* null mutant mice are runted and die within 2 weeks after birth. In addition, many show B and T cell lymphopenia, the cause of which remains unknown (Tybulewic et al., 1991; Schwartzberg et al., 1991).

To investigate the role of the chimeric *BCR/ABL* gene in leukemia, *BCR/ABL* P210 encoding retroviral constructs have been introduced into mouse bone marrow and transplanted into lethally irradiated recipients. Different types of hematopoietic disease were found. In one study, 43% of the recipients developed either a CML-like myeloproliferative disease, lymphoblastic leukemia, or macrophage cell type tumors within 5 months (Daley et al., 1990). Other investigators reported *BCR/ABL* mediated hematopoietic disease involving macrophage, lymphoid, erythroid and mast cell lineages. The incidence (69-100%) of the exact type of disease was strain-dependent (Elefanty et al., 1990). Myelomonocytic leukemias, granulocytic leukemias and lymphocytic leukemia/lymphomas were found both with P210 and P190 (Kelliher et al., 1990; Kelliher et al., 1991). It appears that the kinetics and the nature of the induced disease in this experimental setting, besides on choice of internal promoter, depend on culturing conditions, infection procedure and genetic background of the grafted mice (Elefanty et al., 1992; Kelliher et al., 1993). It was further concluded that retroviral regulatory sequences may have influenced the outcome of the experiments. A major obstacle in retroviral technology is targeting the infection to stem cells. This is essential since CML is thought to be a clonal disease originating in the hematopoietic stem cell (Fialkow et al., 1977).

A different model system is created through the use of transgenic mice as the gene of interest will be present in every cell. In a previous study, we have generated mice transgenic for a *BCR/ABL* P190 construct under the transcriptional regulation of a mouse metallothionein (MT-1) promoter; most transgenic founder animals and progeny died rapidly of an acute leukemia, which was clinically classified as ALL (Heisterkamp et al., 1990; Voncken et al., 1992a, Voncken et al., 1992b). Initial attempts to generate a transgenic mouse model for human CML with a *BCR/ABL* P210 transgene controlled by the human *BCR* promoter failed, due to deleterious effects of the construct on embryogenesis (Heisterkamp et al. 1993). Here we present the generation of mouse lines transgenic for MT-*BCR/ABL* P210 constructs, and describe the development of hematopoietic neoplasia caused by the P210 transgenes.

Materials and Methods

Transgenes and mice

The δMT-*BCR/ABL* P210 (see Fig. 1) construct contains only part of the mouse MT promoter, identical to that used before to generate δMT-*BCR/ABL* P190 transgenic animals (Heisterkamp et al., 1990). The δMT-*BCR/ABL* construct was generated by ligating a 5' ClaI-SalI fragment containing the truncated MT-1 promoter (a 200 bp SstI-BglII fragment; Stuart et al., 1984) and *BCR* gene exon 1 till the SalI site to a large SalI-ClaI fragment encompassing the remainder of *BCR/ABL*. The latter fragment was from the previously described cosmid cl(8)der, a *BCR/ABL* DNA construct containing the human *BCR* gene promoter (Heisterkamp et al., 1993). The SalI-ClaI fragments were ligated into vector pHEP and packaged *in vitro*. The 32.5 kb cosmid insert was separated from the vector by pulsed field gel electrophoresis. In this construct the entire *BCR* coding region up to *Mbcr* exon 3 (*BCR* exon 14) and *ABL* exons 2-8 are contained within one large cDNA fragment. The rest of *ABL* includes introns and is a genomic sequence. The MT-*BCR/ABL* P210 was constructed from three segments: (a) the complete murine MT-1 promoter fused to *BCR* exon 1 plus a segment of 3' intron (b) the intron

immediately preceding *BCR* gene exon 2, additional *BCR* gene exons up to and including Mbcr exon 2 and Mbcr exon 3 with flanking introns (c) *ABL* exon 2 with 5' and 3' introns, and *ABL* exon 3-11 as cDNA.

Transgenic δMT-*BCR/ABL* P190 animals have been described in detail elsewhere (Heisterkamp et al., 1990; Voncken et al. 1992a; Voncken et al., 1992b). Mice transgenic for the MT-*BCR/ABL* P210 constructs were generated as described (Heisterkamp et al., 1990). Founder animals were the offspring of matings between C57BL/CBA F_1 animals. Transgenic progeny was the result of matings between transgenics and C57BLxCBA F_1 mice, *bcr* null mutant (Voncken et al., 1995) or P53 null mutant mice (Donehower et al., 1992). Progeny are identified by eight-digit numbers; the first four digits are those of the founder of that specific line.

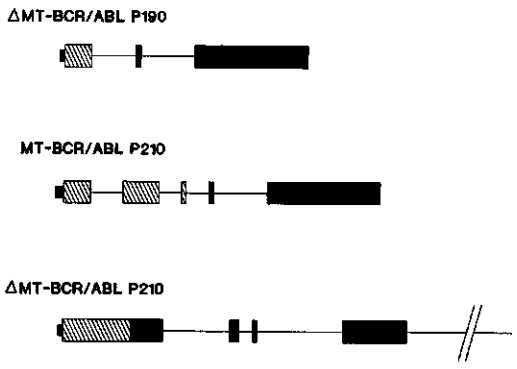

Figure 1. *Human BCR/ABL transgenic constructs. Expression of the MT-BCR/ABL P210 transgene (middle line) is regulated by the complete mouse metallothionein (MT-1) gene promoter, whereas the δMT-BCR/ABL P210 transgene (lower line) is controlled by a part of the MT promoter. Promoter sequences in the δMT-BCR/ABL P190 construct (upper line) are identical to those of the δMT-BCR/ABL P210 construct. Thin lines indicate intron sequences. Hatched boxes represent BCR gene exons, and black boxes ABL gene exons; the small black boxes 5' to BCR exon 1 represent murine MT-1 promoter sequences.*

Histology

Pathologic analysis was performed as described previously (Pattengale et al., 1989), on autopsy material obtained at sacrifice from mice which were generally terminally ill. Routine histology examinations included bone marrow, lymph nodes, liver, kidney, spleen and thymus. Tissue sections were fixed in 10% formalin, 90% B5. All MT-*BCR/ABL* P210 males displayed fertility problems: severely reduced testis size and aspermia were frequently encountered. For this reason only a limited amount of offspring was obtained from MT-*BCR/ABL* founders. The δMT-*BCR/ABL* P210 and P190 animals apparently had normal fertility.

Periodic monitoring of peripheral blood for signs of disease

WBC counts and *BCR/ABL* expression assays were performed regularly on peripheral blood from transgenic and control mice. Briefly, approximately 70 μl of blood was obtained via retro-orbital sinus bleeding. White blood cell (WBC) counts were performed manually, as were differential white blood cell counts. Peripheral blood films were stained with Wright-Giemsa and evaluated histologically. Evidence of myeloid involvement (differential counts) in the period that preceded the development of overt leukemia in *BCR/ABL* P210 transgenic animals was not found. 50 μl heparinized blood was used for RNA isolation. Red cells were lysed in two successive treatments with 20 volumes 0.144 M NH_4Cl, 5 volumes 0.01 M NH_4HCO_3, interchanged with washes in normal PBS. White cells were pelleted and lysed in 500 μl "solution D"; RNA was isolated essentially as described (Chomcyznski and Sacchi, 1987). The RNA pellet was suspended in 40 μl dH_2O and reverse transcriptase/polymerase chain reactions (RT/PCR) were performed on 8 μl essentially as described (Voncken et al., 1992b) using "CML A" and "CML H" oligonucleotides as amplimers and "CML B" as hybridization probe (Kawasaki et al., 1988); in every experiment RNA from the human CML cell line K562 was used as a positive methodological control.

In previous experiments, the level of sensitivity of

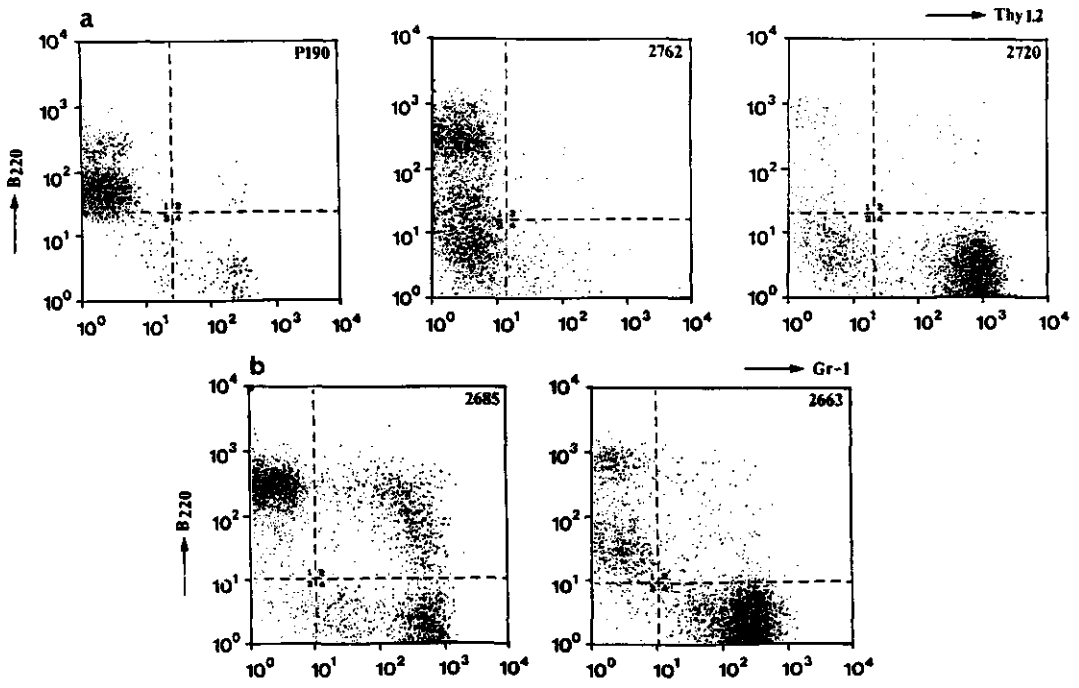

Figure 2. Flow cytometric analysis of BCR/ABL positive tumors: Panel a shows a pre-B cell leukemia lymphoma of a sMT-BCR/ABL P190 mouse, a B lymphocytic leukemia/lymphoma of a sMT-BCR/ABL P210 mouse (2762) and a small T lymphocytic leukemia of a sMT-BCR/ABL P210 animal (2720). Panel b shows two examples of granulocytic involvement in P210 related leukemia (see 'Results'): animal 2685 B-lymphocytic leukemia with cells staining double positive for B220 and GR-1; mouse 2663 revealed acute myeloblastic leukemia. All flowcytometric analyses were performed on peripheral blood samples. The animal identification numbers are printed in the top right corner of the respective graphs.

the RT/PCR method for *BCR/ABL* expression was established at one in 100,000 nucleated cells. To verify, that a certain RNA sample was suitable forRT/PCR reactions, it was tested for mouse actin expression using as 3' amplimer (5'-CGGTTGGC-CTTAGGGTTCAGGG-3') and as 5' amplimer (5'-GTGGGCCGCTCTAGGCACCAA-3') (Alonso et al., 1986). In some experiments, an oligonucleotide probe (5'-ACTCCTATGTGGGTGACGAGG-3') contained within the amplified region was used for hybridization probe.

Composition of hematopoietic tumors and peripheral blood samples

Composition of hematopoietic tissues was determined by double-color flow cytometry on a FACScan (Beckton-Dickinson, NJ). Briefly, tissues were disaggregated and washed twice in cold PBS. Approximately $1x10^6$ white cells were stained with phycoerythrin (PE) and/or fluorescein-isothiocyanate (FITC)-conjugated antibodies for 20 to 30 minutes in 50-100 μl RPMI containing 3% fetal bovine serum and 0.1% sodium azide on ice in the dark. Cells were then washed in PBS fixed in 1% paraformaldehyde and analyzed. Monoclonal antibodies used include: anti-CD45R (RA3-6B2), anti-CD45 (30F11.1), anti-CD3$_\epsilon$ (145-2C11), anti Thy1.2 (53-2.1), anti-GR-1 (RB6-8C5) (PharMingen, CA) and anti-CD11b (M1/70.15) (Caltag, California).

RNA isolation and expression in other tissues

RNAs were isolated as described (Chomcyznski and Sacchi, 1987) using guanidine-isothiocyanate. To test for early expression, timed matings between transgenic males and non-transgenic C57BlxCBA F1 females were set up and embryos isolated between day 13 and 15 of gestation. Three fetuses were pooled and homogenized immediately in solution D and stored at -80 °C. Bone marrow, spleen or tumor

RNA was isolated after homogenizing part of the tissue in slnD. 1-3 μg of total RNA was used per RT/PCR reaction. RT/PCR products were separated on 1% agarose gels, blotted to Nytran (Schleicher and Schuell) and hybridized as indicated above.

Immunoblotting

Tissues were minced and homogenized in a Potter homogenizer in 2x SDS sample buffer and analyzed by Western blotting as described (Harlow and Lane, 1988). The chimeric Bcr/Abl P190 and P210 oncoproteins were detected with the mouse monoclonal antibody Ab-3 (anti-c-Abl; Oncogene Science, Inc., Uniondale N.Y.)

Results

Transgenes and transgenic animals

To circumvent the problem of embryonic lethality observed with the human *BCR* promoter (Heisterkamp et al., 1993b), the mouse metallothionein-1 promoter was chosen as an alternative regulatory sequence for *BCR/ABL* P210 transgenes. Analysis of a mouse model for human ALL in which this MT promoter was utilized to control P190 expression (the δMT-*BCR/ABL* P190 construct (also see Fig. 1); Heisterkamp et al. 1990; Voncken et al. 1992a) revealed that transgenic animals developed pre-B cell leukemia without prior exposure to heavy metals. *BCR/ABL* mRNA could be detected, by RT/PCR, as early as the 1 cell stage embryo and was found in all tissues examined at later stages (Voncken et al. 1992b). This low level of transgene expression is apparently enough to predispose for cancer development.

Subsequently, two *BCR/ABL* P210 DNA constructs were made (Fig. 1), both controlled by the murine MT-1 promoter. They differ in the length of the MT promoter segment included and in their intron/exon structure (see Fig. 1). The δMT-*BCR/ABL* P210 and δMT-*BCR/ABL* P190 constructs share identical MT promoter sequences; MT-*BCR/ABL* P210 and δMT-*BCR/ABL* P190 harbour identical *BCR* exon 1/intron 1 and *ABL* sequences (Fig. 1).

Transgenic founder and progeny animals were generated with both *BCR/ABL* P210 constructs (Table I) and hematological tumors were obtained. Animals containing a "double" δMT-*BCR/ABL* P210 transgene were also generated by breeding (i.e. P210/P210). However, the transgene copy number had no influence on the kinetics and/or type of disease that developed (not shown). In addition, the δMT-*BCR/ABL* transgene was introduced into a *bcr*(+/–) and a *P53*(+/–) genetic background. The lack of one copy of the endogenous *bcr* or *P53* gene had no apparent influence on the type of disease which developed or its latency period (Table I).

Disease

Unlike mice transgenic for *BCR/ABL* P190, which developed pre-B cell acute lymphoblastic leukemia, *BCR/ABL* P210 animals developed B as well as T cell leukemias with approximately the same frequency; no difference was found in type of disease between the two *BCR/ABL* P210 constructs (Table I). Tumor phenotypes were confirmed by flowcytometric analysis (Fig. 2). Incidence of hematologic disease in δMT-*BCR/ABL* P210 mice was 58% and in MT-*BCR/ABL* P210 53%; by comparison, δMT-*BCR/ABL* P190 had a disease incidence of nearly 100% (Fig. 3; also see Groffen et al., 1993).

In contrast to studies by others which reported the development of T cell neoplasias upon injection of either *BCR/ABL* P190 or P210 retroviral vectors directly into the thymus (Clark et al., 1993), we found no evidence that T lymphoid cells are targets for *BCR/ABL* P190: to date, among more than 20

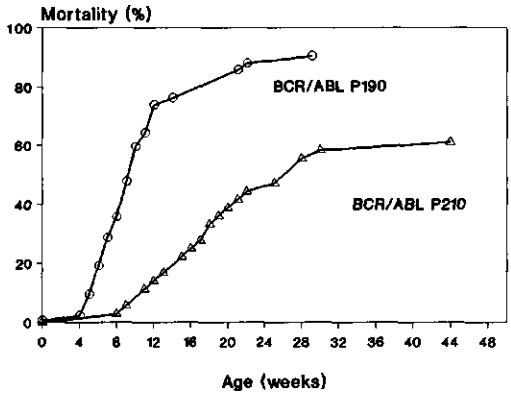

Figure 3. *Mortality curve of BCR/ABL P190 versus BCR/ABL P210 transgenic mice. Both δMT-BCR/ABL P210 and MT-BCR/ABL P210 groups included F_0 (founder) and F_1 and F_2 progeny animals (open triangles; n=36). A comparable selection of animals (including F_0, F_1 and F_2 animals of both sexes) is represented in the δMT-BCR/ABL P190 graph (open circles; n=42).*

different founders and their transgenic offspring, none have shown any leukemia other than of the B-lineage.

Neoplasias in *BCR/ABL* P210 animals were of both B and T cell type. B lymphoid leukemias or lymphomas were invariably accompanied by splenomegaly, involvement of lymph nodes, but never of the thymus. Most T-cell lymphoid leukemia involved the thymus, frequently lymph nodes and spleen; white blood cell counts were often extremely high. Mature lymphocytic leukemia (B or T) was further characterized by absence of mitotic figures in the peripheral blood. In both B and T-lymphoid tumors, a coexisting but separate and presumably non-neoplastic prominent hematopoiesis (erythropoiesis and megakaryocytosis) was frequently observed in the spleen (not shown). Since clonality studies (immunoglobulin heavy or light chain and/or T-cell receptor rearrangements) and/or Bcr/Abl protein levels were not evaluated in isolated megakaryocyte population, we can not exclude the possibility that these phenomena related to direct oncogenic action of *BCR/ABL*. Macrophage tumors were not found in either *BCR/ABL* P190 or *BCR/ABL* P210 animals and primary granulocytic disease was rarely found. All leukemias were rapid in onset; no evidence for chronic disease was found in the peripheral blood.

At some terminal stages of disease, myeloid involvement was found: circulating mature neutrophil counts were elevated in one case (1994/2663), and a relatively high percentage of cells in the bone marrow compartment of another terminally sick animal (1994/2685) stained positive for both B220 and GR-1 (Fig. 2b). Myeloblastic leukemia with a sarcomatous component in animal 2663 involved the red pulp in the spleen, lymph nodes, liver and kidney (Table 1). Since mouse 2685 revealed a lymphoid proliferative disorder at an earlier stage, this myeloid involvement could be secondary to lymphoid tumorigenesis (i.e. a deregulated cytokine production) as has been suggested before (Elefanty et al. 1990; Elefanty et al. 1992). Alternatively, *BCR/ABL* P210 expression could disturb differentiation or proliferation of relatively rare myeloid-lymphoid precursors. Such rare stem cell origins in Ph-positive leukemia are occasionally encountered (Akashi et al., 1993a and 1993b); also switching within myeloid and pre-B lymphoid tumor lineages has been reported before (Martin et al., 1993), demonstrating a close developmental link between these hematopoietic lineages.

Taken together, we conclude from our data that P190 and P210 cause clinically distinct disorders in a transgenic setting.

Kinetics of transgene expression

To investigate whether differences in type of disease could be associated with different levels of *BCR/ABL* expression, total protein was isolated from different end-stage tumors. However, tumors of all three transgenic animal lines expressed comparably high levels of the chimeric Bcr/Abl oncoproteins (Fig. 4).

We also examined transgene expression on an RNA level using RT/PCR early in development and in bone marrow. Both P210 transgenes were actively expressed early in utero (not shown) and in bone marrow of different transgenic animal lines (see:

Table I. Tumorigenesis in *BCR/ABL* P210 transgenic animals.

Animal	Age[1] (wks)	Tissue involvement[2] BM	SPL	THY	LN	WBC[3] ($x10^6$/ml)	Diagnosis[4]
MT-*BCR/ABL* P210							
1063	11	+	+	–	+	110	pre-B lymphoblastic leukemia
1063-1164	13	+	+	–	+	53	small B-lymphocytic leukemia
1102-1684	17	+	+	+	+	376	small T lymphocytic leukemia
1102-1691	15	+	+	+	+	219	small T lymphocytic leukemia
2160-2302	20	+	+	–	+	nd	B lymphoblastic leukemia/lymphoma
2179	15	+	+	+	+	nd	small T lymphocytic leukemia
δMT-*BCR/ABL* P210							
1994-2725	11	+	+	+	+	84	T lymphoblastic leukemia
1994-2762	12	+	+	–	+	29	small B lymphocytic leukemia/lymphoma
1994-2777	9	+	+	+	+	56	T lymphocytic leukemia
1994-2781	28	+	+	–	+	196	pre-B lymphoblastic leukemia
1994-2784	28	+	–	+	+	nd	T lymphoblastic lymphoma
bcr(+/–)•							
1994/2683	22	+	+	–	+	1	pre-B lymphoblastic lymphoma
1994/2685	21	+	+	–	+	1	B lymphocytic leukemia/lymphoma[§]
1994/2720	19	+	+	+	+	128	small T lymphoblastic leukemia
1994/2744	18	+	+	+	+	nd	T lymphocytic leukemia
1994/2748	25	+	+	–	+	19	T lymphoblastic leukemia
P53(+/–)•							
1994/2619	18	+	+	+	+	nd	T lymphoblastic leukemia
1994/2661	44	+	+	+	+	126	T cell lymphoma leukemia
1994/2663	54	+	+	–	+	98	myeloblastic leukemia[§*]
1994/2710	14	+	+	–	+	2	lymphoblastic lymphoma (B or T)

1: Age at death; autopsies were performed on terminally ill animals or animals were followed until they perished.
2: Tissue involvement was established by gross pathological and histological examination.
3: White blood cell count was either determined at or a few days before death.
*4: Phenotype of lymphoid tumors was assessed by flowcytometry (see: 'Materials and Methods'). §: These animals displayed granulocytic involvement in the peripheral blood at terminal stages of disease. *: diagnosis of myeloblastic leukemia in animal 2663 was based on pattern, general morphology and anatomic sites involved (see also: 'Results'). •: (+/–) indicates that animals were heterozygous for either the bcr or the P53 gene.*

Table II. Transgene expression in bone marrow of different *BCR/ABL* P210 lines

δMT-*BCR/ABL* P210			MT-*BCR/ABL* P210		
animal of line[1]	TG[2] xpr	disease in mouse line: founder or offspring	animal of line[1]	TG[2] xpr	disease in mouse line: founder or offspring
168	*nd*	*no*			
170	+	+	1063	+	+
171	*nd*	*no*	1102	+	+
1989	+	*nd*	1105	*nd*	*no*
1994	+	+	2148	+	–
2039	–	–	2160	+	+
2043	+	–	2179	+	+

1: Animal lines are represented by founder identification numbers. 2: Transgene expression in bone marrow was evaluated in at least one transgenic animal per line by RT/PCR. nd: not determined, no: no offspring sired by this founder.

Table II), without prior exposure to zinc or cadmium. As was the case in the δMT-*BCR/ABL* P190 animal model, appearance of *BCR/ABL* P210 expressing cells in the peripheral blood preceded the development of overt leukemia (Voncken et al., 1992b). However, not all transgenic animal lines that expressed the transgene in the bone marrow developed peripheral expression (Table II). Moreover, appearance of transgene expression in circulating blood cells did not necessarily herald subsequent development of disease: some animals, of which transgene expression in circulating nucleated cells had been confirmed between 10 and 16 weeks of age, remained disease free, some past 16 months of age. These observations were valid for both *BCR/ABL* P210 constructs. In P190 animals, a close correlation exists between transgene expression and development of disease (Voncken et al., unpublished observations). The differences between P190 and P210 transgenic lines is also quite apparent in the mortality curves (Fig. 3). The large differences between transgenic animals as far as latency time is concerned indicates that *BCR/ABL* P210 expression is not the sole cause of leukemia, but rather predisposes for the cancer. In agreement with current concepts, additional (epi)genetic mutations, directly or indirectly resulting from *BCR/ABL* expression, most likely contribute to malignant tumor evolution.

Discussion

Transgenic mouse models allow direct comparison of P210 and P190 associated disease

In recent years, attempts to generate a reliable animal model for the study of CML have been thwarted by lack of reliabilty and reproducibility (Daley, 1993). The outcome of bone marrow transplantation experiments employing retroviral *BCR/ABL* constructs was shown to depend largely on infection conditions, retroviral and internal regulatory sequences used and also on genetic

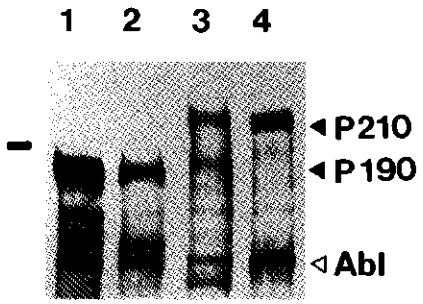

Figure 4. *Bcr/Abl expression in primary tumors of transgenic animals. A high molecular weight protein marker of 200 kDa is indicated to the left (square). Lane 1 and 2: P190 expression in lymphomas of two different sMT-BCR/ABL P190 animals. Lane 3: P210 expression in a T cell lymphoma (thymoma) in a sMT-BCR/ABL P210 mouse of the 1994-line and Lane 4: transgene expression in a B-cell lymphoma of an MT-BCR/ABL P210 animal.*

background of the irradiated recipient mice; in essence, the outcome of the disease depends on which hematopoietic progenitor cell was targeted by retroviral vectors (Elefanty et al. 1992; Kelliher et al., 1993). Myelo-proliferative disorders could be obtained by manipulating these experimental conditions. In this fashion, *BCR/ABL* P190 and P210 were shown to cause similar disease (Kelliher et al., 1991). Even with v-*abl*, which under natural conditions exclusively induces pre-B cell leukemia in mice, myeloproliferative illness could be produced (Kelliher et al., 1990).

In transgenic animals, every cell including hematopoietic stem cells will contain a *BCR/ABL* transgene. The disease which develops is independent of influences of retroviral sequences or other experimental factors, such as infection and bone marrow culture conditions. Hence, a transgenic mouse model solely reflects the tumorigenetic properties of *BCR/ABL* oncoproteins. The present study shows that, although the transgenic constructs are very similar in composition, *BCR/ABL* P190 and P210 tumor biology clearly differ. The longer latency time of P210 associated tumorigenesis, independent of whether a B or T lineage tumor develops, would seem to indicate intrinsic variation in biochemical properties between the two distinct oncoproteins.

Influence of Bcr protein moiety and Bcr gene promoter on leukemogenesis

It has been reported that P190 Bcr/Abl has a higher auto-phosphorylation activity than P210 *in vitro*, a property which correlated with its higher transforming ability in cultured fibroblast cell lines (Lugo et al., 1990). Such differences in tyrosine kinase activity might for example have a differential effect on the differentiation of certain hematopoietic lineages and may help explain the altered disease-spectrum associated with P210 as compared to P190.

The combined findings in our transgenic mice demonstrate that, as in human leukemia, the additional Bcr domains included in the P210 oncoprotein modulate the outcome of the disease. The mechanisms which govern these distinct neoplastic processes are however not understood. As indicated above, the Bcr domains modify the tyrosine kinase activity of the Abl moiety (reviewed in Van Etten, 1993) and also F-actin binding activities of c-Abl (McWhirter and Wang, 1991). In addition, the Dbl-homology (Eva and Aaronson, 1985) and pleckstrin-homology domains (Mayer et al., 1993) only present in P210 may allow protein-protein interactions with other cellular factors, which can not occur with P190.

Apart from its structural role in the Bcr/Abl oncoprotein, there appears to be a second discrete mechanism through which *BCR* may contribute to leukemogenesis. We have recently shown that the effect of *bcr* gene ablation specifically affects hematopoietic cells in mice affected by *BCR/ABL* in man and mouse (Voncken et al., 1995). It seems

reasonable to conclude from our data that a correlation exists between the cell type affected in Ph-positive leukemia and Bcr function. The *BCR* promoter controls expression of both the remaining non-rearranged *BCR* allele and the *BCR/ABL* oncogene in human leukemia. It is very well possible that regulatory sequences that normally govern *BCR* expression have substantial influence in directing *BCR/ABL* mediated tumorigenesis. The prominent myeloid development in *BCR/ABL* P210 mediated human CML may be related to the presence of factors in myeloid lineages that regulate *BCR* expression through specific elements in the *BCR* promoter. These factors may be less prevalent in human T and B cell lineages and preferentially direct *BCR* promoted tumorigenesis in granulocytes.

All experimentally derived *BCR/ABL* lymphoid tumors were generated with regulatory sequences other than the *BCR* promoter. To date, all animals models for human Ph-positive leukemia, whether generated through retroviral (Elefanty et al., 1990; Daley et al. 1990; Kelliher et al., 1991; Gishizky et al., 1993) or transgenic technologies (Hariharan et al., 1989; Heisterkamp et al., 1990; this study), develop predominantly lymphoid malignancy of B and T type. Although hematologic tumors in Ph-positive ALL are principally of the pre-B phenotype, B lymphoid involvement occurs only in about 25% of cases of human CML in blast crisis and T cell involvement is rarely seen in human. We suggest that regulatory sequences within the *BCR* gene are important for the type of disease found in human and mouse. This concept contrasts with the postulate that neither *BCR* sequences nor myeloid cell promoter elements are essential for myeloid tumorigenic transformation (Kelliher et al., 1990) and that the *BCR* sequences that distinguish *BCR/ABL* P190 and *BCR/ABL* P210 do not control the selection of particular types of hematopoietic cells (Kelliher et al., 1991).

Myeloid expansion in BCR/ABL P210 transgenic mice does not result in transformation

BCR/ABL expression in CML results in myeloid expansion. It was suggested, that this is the consequence of a reduced programmed cell death (Carlesso et al., 1994; Laneuville et al., 1994; Kabarowski et al., 1994 and references therein), which plays an important part in normal hematopoietic homeostasis. Preliminary experiments performed in our laboratory, with isolated bone marrow cells in semi-solid medium from P210 and P190 animals seem to indicate an absolute increase in primitive myeloid progenitor cells (CFU-GM) and total number of myeloid colonies in our P210 mouse model at the 50% mortality time-point, while a decrease in myeloid colony formation is observed in the P190 bone marrow at a comparable time-point. These two opposing effects of P190 and P210 again seem to reflect a biological difference between the two oncoproteins.

The expansion of the myeloid cell pool in human CML is believed to effectively increase their susceptibility to additional mutagenic events, which ultimately accompany malignant transition from chronic phase to fatal blast crisis. The idea that *BCR/ABL* expression causes genomic instability was corroborated by the appearance of karyotypic abnormalities at relatively early stages of disease and their nearly invariable presence in malignant terminal tumors in our *BCR/ABL* P190 transgenic mouse model (Voncken et al., 1992a). The absence of a chronic phase in the present study (e.g. by elevated peripheral granulocyte count), despite an elevated primitive myeloid progenitor pool in the bone marrow, could be a direct result of the restricted size of the cell population subject to secondary mutational events in a small animal like the mouse. A similar scenario was suggested to explain the absence of

retinoblastoma in heterozygote retino-blastoma susceptibility gene (Rb)-mutant animals (Jacks et al., 1992).

In human, about 50% of cases of Ph-positive ALL carry the *BCR/ABL* P190 translocation, the other 50% the P210. Consequently, the issue whether Ph+ ALL is actually CML *in* blastic phase has been controversial for some time. Recently, however, clinical and molecular studies have established that indeed Ph+ ALL and acute phase CML are two distinct diseases (reviewed by Berger, 1993). All results of the present study support this concept. In transgenic mice, *BCR/ABL* P210 clearly causes a distinct disease from *BCR/ABL* P190. This is caused by inherently distinct properties of P190 and P210, possibly including the intrinsically different kinase activities of the *ABL* moiety in the hybrid oncoproteins and/or cellular interactions of the Dbl and pleckstrin homology domains or as of yet to be identified domains in Bcr. In addition, regulatory sequences upstream from or within the *BCR* gene are likely to contribute to the occurrence of myeloid leukemia in human and presumably in the mouse. These ideas will be the subject of future investigations in our laboratory.

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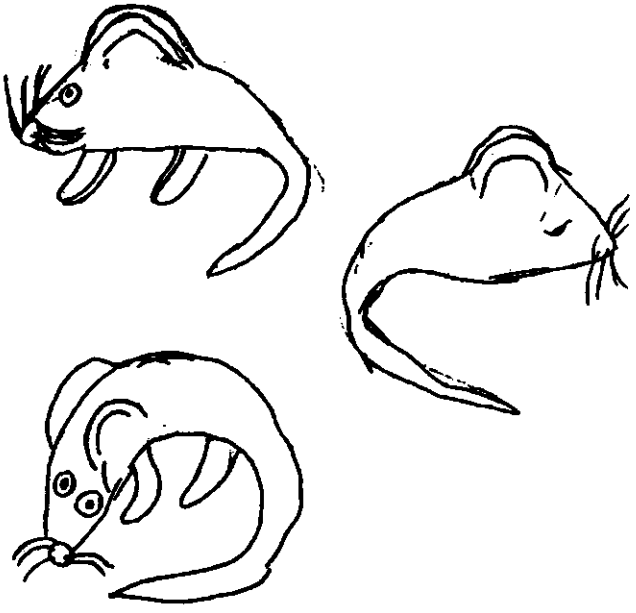

'Three mice, one is sleeping'. By Lotta (9)

CHAPTER 6

Interferon-α Treatment of P190 *BCR/ABL* Transgenic Mice

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IFN-α treatment of P190 *BCR/ABL* transgenic mice

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Abstract. Interferon-alfa (IFN-α) is one of the most effective drugs in the treatment of chronic myeloid leukemia (CML). Recently, IFN-α has also been tried in the treatment of Ph-positive acute lymphoid leukemia (ALL), a disease in part sharing the same molecular genetic lesion as CML, namely a *BCR/ABL* fusion gene. In the present study we analyzed the effect of IFN-α (rHuIFN-αA/D) on a mouse model for Ph-positive ALL - mice transgenic for the P190 *BCR/ABL* fusion gene. IFN-α treatment was started in the early leukemic phase and continued throughout the course of the disease in eight transgenic animals. No prolonged survival or altered disease pattern with regard to the development of leukemia and/or lymphoma was observed. We conclude that IFN-α, at least in a transgenic setting, does not interfere with the leukemogenic process induced by the P190 *BCR/ABL* fusion gene.

Introduction

Interferon-alfa (IFN-α) is currently one of the most potent drugs in the treatment of chronic myeloid leukemia (CML), and recent studies have shown that IFN-α is capable of inducing partial or total cytogenetic remission in 40-60% of CML patients (1). A series of studies have recently given new insights into the pathway by which IFN-α signals through its receptor to the nucleus (2-4). The mechanism of action and the reason for the high response rate in CML however remains largely unknown.

CML and a subgroup of acute lymphoid leukemia (ALL) patients (approximately 20% of adult patients) share a common cytogenetical marker, namely the Philadelphia chromosome (Ph). The Ph chromosome is the result of a reciprocal translocation, t(9;22)(q34;q11), which brings the *ABL* oncogene on chromosome 9 into the *BCR* gene on chromosome 22. This results in the synthesis of a BCR/ABL fusion protein, P210 (for review see refs. 5-7). Approximately half of the adult patients with Ph-positive ALL also have a break in the same region of the *BCR* gene (M-bcr) as CML patients. The remainder have a break further upstream in the *BCR* gene (m-bcr), resulting in a fusion protein of 190 kDa. Both P210 and P190 show a deregulated tyrosine kinase activity, believed to be of importance in the leukemogenic process (5-7).

Given the similarity between CML and Ph-positive ALL in terms of the presence of a BCR/ABL fusion protein, IFN-α treatment has lately been tried in Ph-positive ALL patients. The number of patients studied to date is limited, but some beneficial effect has been reported in ALL patients with an M-bcr rearrangement (8,9).

Recently we have established an 'ALL-type' P190 *BCR/ABL* transgenic mouse model (10-13) in which founder animals develop acute leukemia/lymphoma within 10-58 days after birth. In the present study we analyzed the effect of IFN-α treatment on the disease evolution in a line of these transgenic animals.

Materials and methods

Mice. A total of 12 mice were included in this study. All mice were heterozygous carriers of a P190 type *BCR/ABL* transgene (10) and were from a single line of mice, #623, which has been characterized extensively (11-13). Lymphoblastic leukemia/lymphoma invariably develops in this line, with death following around 68 days on an average (13). The average age at death has slightly increased in successive generations of this line (unpublished observations). Mice initially have no detectable BCR/ABL expressing cells in their peripheral blood (PB). As they grow older, such cells can be detected in the peripheral blood and this heralds the onset of overt disease (12).

Mice were genotyped at 10 days of age, and control and treatment group mice were housed in the same cage and were of comparable age at the start of the experiment. A blood sample of 5-10 µl was withdrawn from the tail artery of each mouse every 10 days. At the first detection of BCR/ABL expression in PB, mice were randomly assigned to a control or treatment group.

The animals were monitored by peripheral white blood cell (WBC) count twice a month and for signs of lymphoma

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Table I. Disease characteristics of control and IFN-α treated mice.

Animal no.	BCR/ABL expression detected at day	IFN-α (units)	Terminal WBC ($x10^9$/l)[a]	Lymphoma burden[b]	Spleen weight (g)[a]	Survival (days)[c]
1	51	none	70	+	0.30	103
2	59	none	51	-	0.60	129
3	36	none	3	++	0.15	225
4	51	none	12	-	0.13	241
5	51	10^4	11	+++	0.48	89
6	36	10^4	24	-	0.20	124
7	30	10^4	ND	-	ND	90
8	61	10^4	28	+++	1.00	98
9	70	10^4	63	+++	0.36	92
10	78	10^4	26	-	0.22	165
11	102	$5x10^4$	60	++	0.62	169
12	99	$5x10^4$	28	-	0.33	222

[a]Normal mean values in mice: WBC, $8x10^9$/l; spleen weight, 0.1g; ND, not determined; [b](-) no, (+) low, (++) medium, (+++) high; [c]Animal no. 7 was found dead at the age of 90 days. No autopsy was performed.

daily. Animals were sacrificed when terminally ill. Autopsy was performed and the degree of splenomegaly and lymphoma recorded.

Injection of mice with interferon. Control group mice (4 total) received an intraperitoneal injection of 100 µl 0.15 M NaCl five times per week. Six mice received 100 µl injections of 10^4 units rHuIFN-αA/D in 0.15 M NaCl five times per week, whereas two animals received $5x10^4$ units. rHuIFN-αA/D was a generous gift from Hoffmann-La Roche Inc., Nutley, NJ. This hybrid protein is active on mouse cells *in vivo* and *in vitro* (14). To verify its biological activity, a control mouse was injected with 10^4 units of rHuIFN-αA/D, and RNA was subsequently isolated using the acid guanidinium thiocyanate-phenol-chloroform method (15). Total RNA (10 µg) was run on formaldehyde/formamide gels, blotted as described (16), and subsequently hybridized to the IFN inducible probe #204, kindly provided by Dr P. Lengyel, Yale University (17). This gene was induced 2-3 fold as compared to a non-treated control.

Expression of BCR/ABL. To detect BCR/ABL expression in PB, RT/PCR was performed as described (10) on a small sample of PB withdrawn from the tail artery. All samples were tested for suitability in the RT/PCR reaction using actin amplimers. Samples were run on agarose gels, and hybridized to an oligonucleotide specific for the P190 BCR/ABL translocation junction as described (10). In addition, the presence of the BCR/ABL P190 protein was confirmed using Western blot analysis on splenic samples taken at sacrifice from animal 6, 10, and 11 (Table I).

Results

As determined by RT/PCR, all animals started to express the BCR/ABL fusion messenger in the range between 30-102 days (Table I). At this stage all animals had normal WBC counts and no signs of lymphoma. IFN-α treatment was well tolerated based on the observation that no difference in behavior between control and treated mice was apparent. All control animals (n=4) developed leukemia and/or lymphoma; the average lifespan was 174 days (range 103-241 days). The average time from first detection of BCR/ABL expression to death in this group was 125 days (range 50-190 days). Leukemia and/or lymphoma also developed in the group of mice treated with 10^4 (n=6) and $5x10^6$ (n=2) units of IFN-α. The average lifespan in this group was 131 days (range 89-222 days) (Table I). The average time from first detection of BCR/ABL expression to death was 65 days (range 22-123 days). Thus, in summary, no prolonged lifespan or altered disease pattern was observed in the IFN-α treated group when compared with untreated animals.

Discussion

CML and Ph-positive ALL are in part characterized by the same molecular genetic lesion, a *BCR/ABL* fusion gene, believed to be of major importance in the initiation of the leukemogenic process. IFN-α has been shown to induce partial or total cytogenetic response in a large fraction of patients with CML (1). The detailed mechanism of how IFN-α induces hematologic and cytogenetic remission in CML is however unclear, and it remains controversial whether the suppression of the leukemic cells through IFN-α is a result of

a direct effect on the leukemic cells or an indirect effect mediated through the normal host cells (18-20).

Transgenic animal models offer a unique possibility to study the influence of various treatment regimens on specific diseases. Disease course and treatment protocols can be monitored strictly, and drugs can be administered in the very early disease phase, before secondary genetic changes have accumulated. A potential disadvantage, however, is that cure, in a strict sense, might not be achieved, since all cells in these animals carry the transgene. Also, transgenic constructs are usually regulated by an artificial promoter not present in the normal gene. This could result in a lack of effect if the drug were to interfere with the regulation of the natural promoter. Nevertheless, if a drug would interfere with the signal transduction pathway used by, e.g., BCR/ABL to induce leukemia, prolonged survival and/or altered disease pattern could be expected. In this study we investigated whether IFN-α, instituted in the very early leukemic phase, is capable of prolonging the lifespan, or change the characteristic disease pattern in P190 *BCR/ABL* transgenic animals. The average lifespan in eight transgenic animals treated with IFN-α was 131 days, compared to 174 days in four control animals. Moreover, the disease pattern with regard to development of lymphoblastic leukemia and/or lymphoma was similar in the two groups. Despite the relatively small number of animals investigated, we find it reasonable to assume that IFN-α is not capable of prolonging the lifespan in these animals, and thus does not interfere with the leukemogenic process induced by the P190 *BCR/ABL* fusion gene in this transgenic mouse model.

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'A mouse that looks like a rat'. By Nisha (9)

CHAPTER 7

Increased Neutrophil Respiratory Burst in *bcr* Null Mutants

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Increased Neutrophil Respiratory Burst in *bcr*-Null Mutants

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Summary

Philadelphia (Ph)-positive leukemias invariably contain a chromosomal translocation fusing *BCR* to *ABL*. The BCR–ABL protein is responsible for leukemogenesis. Here we show that exposure of *bcr*-null mutant mice to gram-negative endotoxin led to severe septic shock and increased tissue injury by neutrophils. Neutrophils of *bcr* (−/−) mice showed a pronounced increase in reactive oxygen metabolite production upon activation and were more sensitive to priming stimuli. Activated (−/−) neutrophils displayed a 3-fold increased $p21^{rac2}$ membrane translocation compared with (+/+) neutrophils. These results connect Bcr in vivo with the regulation of Rac-mediated superoxide production by the NADPH-oxidase system of leukocytes and suggest a link between Bcr function and the cell type affected in Ph-positive leukemia.

Introduction

Chronic myelogenous leukemia (CML) is characterized cytogenetically by the presence of the Philadelphia chromosome and clinically by a clonal expansion of neutrophils and their myeloid precursors. Philadelphia chromosome (Ph)-positive leukemias have a specific reciprocal translocation, [t(9;22)(q34;q11)], which fuses the *ABL* proto-oncogene to *BCR* (Heisterkamp et al., 1983; Groffen et al., 1985). *BCR–ABL* transgenic mice and retroviruses have been used to show that the resulting fusion protein, BCR–ABL, is directly responsible for the generation of leukemia (reviewed by Van Etten, 1993; Heisterkamp et al., 1993a).

The product of the *ABL* gene is a ubiquitously expressed tyrosine kinase, and its activation in *BCR–ABL* by the BCR moiety is essential for the development of leukemia (reviewed by Van Etten, 1993). The normal function of *ABL* in vivo is unclear. *abl*-null mutant mice are runted and die 1–2 weeks after birth. In addition, many show B and T cell lymphopenia, the cause of which remains unknown (Tybulewic et al., 1991; Schwartzberg et al., 1991).

The 160 kDa product of the *BCR* gene has several distinct domains. The segment encoded by exon 1 has serine/threonine kinase activity in vitro and is responsible for activating *ABL* in the chimeric *BCR–ABL* gene product (Maru and Witte, 1991; McWhirter and Wang, 1991; Muller et al., 1991). *BCR* exons 3–10 have sequence homology to the *DBL* proto-oncogene, a guanine nucleotide exchange factor for human CDC42 (Eva and Aaronson, 1985; Hart et al., 1991), suggesting a similar catalytic activity for Bcr.

In vitro, the C-terminal end of $p160^{bcr}$ has GTPase-activating protein (GAP) activity toward the small GTP-binding proteins Rac1 and Rac2, and CDC42 (Diekmann et al., 1991; Hart et al., 1992). Rac1 and Rac2 belong to the Rho family of small $p21^{ras}$-like GTPases, members of which are involved in cytoskeletal organization. Rac1 is required for growth factor–induced membrane ruffling of Swiss 3T3 fibroblasts. Bcr can regulate this Rac1-induced ruffling in vitro, since microinjection of the purified Bcr GAP domain abolishes this process (Ridley et al., 1993). A number of other proteins are also GAPs toward Rac1 or Rac2 in vitro, including rhoGAP, β-chimaerin, n-chimaerin, ABR, and p190 (Diekmann et al., 1991; Leung et al., 1994; Ahmed et al., 1994; Heisterkamp et al., 1993b; Ridley et al., 1993). Because of the multitude of proteins implicated in the in vitro regulation of Rac, the in vivo importance of the Bcr GAP activity toward Rac is unclear.

On the basis of the data described above, Bcr seems likely to play a role in signal transduction. However, no naturally occurring mutants have been identified, nor has Bcr directly been shown to participate in signal transduction in vivo. Therefore, we have generated *bcr*-null mutant mice to examine the function of Bcr in normal and leukemic cells within the context of a whole organism. Our results connect Bcr for the first time with the regulatory control of Rac in vivo during neutrophil priming and activation and suggest a correlation with the cell types involved in Ph-positive leukemia.

Results

bcr Gene Targeting

A segment of the genomic murine *bcr* gene containing exons 2, 3, and 4 was isolated from a $B_6CBA/F1$ mouse genomic library (Figure 1b). A targeting vector, containing a large 15.5 kb genomic segment, was constructed in which exon 2 was partially replaced by insertion of the positive neomycin resistance selection marker (Figure 1a). The construct was electroporated into embryonic stem (ES)-D3 cells, and correct targeting, as confirmed by Southern blot analysis, was obtained.

Clone p11.2, which contained a correctly targeted *bcr* gene, was injected into recipient C57BL/6 blastocysts and chimeric and *bcr* (+/−) animals obtained. The line was test-bred to C57BL/6, CF-1, and $B_6CBA/F1$ females.

Mutant *bcr* (+/−) animals appeared phenotypically normal, and matings between heterozygous animals yielded the expected Mendelian ratio of wild-type, heterozygote,

†Both authors made an equal contribution.

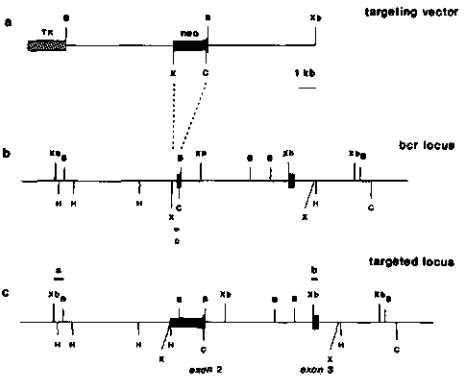


Figure 1. Targeted Disruption of the Mouse *bcr* Gene

(a) The positive/negative selection targeting vector pBEKO-2.

(b) Part of the wild-type *bcr* locus.

(c) Structure of the interrupted *bcr* gene following homologous recombination. *bcr* exons 2 and 3 are shown as closed boxes, the PGK-*neo* gene as a hatched box. B, BamHI; C, ClaI; H, HindIII; Xb, XbaI; X, XhoI. Individual ES cell clones were screened for homologous recombination with external probes (a and b in [c]) and internal probe (c in [b]).

and homozygote offspring. Fertility of null mutant males and females was normal.

Northern blot analysis with a *bcr* exon 1–specific probe did not reveal any stable messenger RNA(s) in (−/−) mouse brain (data not shown), which has the highest level of *bcr* expression in wild type (Heisterkamp et al., 1993b). To confirm that no *bcr* gene products were being synthesized in the mutants, Western blot analysis was performed. *bcr* (+/−) animals expressed levels of *bcr* approximately half of that of wild type (Figure 2; compare lanes 1 and 2), whereas the *bcr* (−/−) mice produced no $p160^{bcr}$ (Figure 2, lanes 3 and 4). Together, these results demonstrate that the mutant (−/−) mice indeed produce no Bcr.

Hematopoietic Development and Lymphoid Function Are Intact in *bcr* (−/−) Mice

Because of the involvement of BCR in leukemia and its suggested role in myeloid development (Bedi et al., 1993; Wetzler et al., 1993), composition, and functionality of mutant hematopoietic compartments were examined in detail. In wild-type animals, different hematopoietic tissues and cells, including splenocytes, thymocytes, total bone marrow, and neutrophils, express significant amounts of $p160^{bcr}$ (data not shown). However, mutant and wild-type hemograms were alike. Size, cellularity, histology, and composition of spleen, thymus, and bone marrow were similar in (−/−) and wild-type tissues (data not shown). Isolated (−/−) splenocytes responded normally to T and B cell mitogen stimulation in vitro as determined by [^{3}H]thymidine incorporation (data not shown). Functional adequacy of the humoral immune response was confirmed in vivo by challenge with sheep erythrocytes (SRBC): anti-SRBC antibody titers in *bcr* (−/−) and *bcr* (+/+) serum were

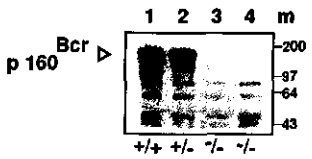


Figure 2. Western Blot Analysis of *bcr* Expression Levels in Wild-Type (+/+), Heterozygote (+/−), and Homozygote (−/−) Brain Extracts

In mice, brain has the highest level of *bcr* expression. The location of $p160^{bcr}$ is as indicated to the left. Lanes 1–3 were loaded with 40 μg of protein extract; lane 4 contains 100 μg of brain extract.

similar (data not shown). Amounts of myeloid differentiation antigen–positive (GR-1$^+$) cells in hematopoietic tissues (bone marrow, spleen, and thymus) were comparable for wild-type and mutant animals, and peripheral neutrophil counts yielded similar numbers for (+/+) and (−/−) animals (respectively, 19.5% ± 9.3%, n = 17, and 19.4% ± 5.7%, n = 22). This shows that Bcr plays no clear role in these aspects of hematopoietic regulation and function.

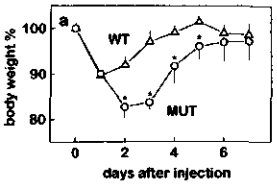
Microfilament Reorganization in *bcr* (−/−) Cells

Because of the GAP activity of Bcr toward members of the Rho family in vitro, we examined microfilament reorganization in *bcr* (−/−) cells. Three independent primary skin fibroblast cultures were established from both (+/+) and (−/−) genotypes. In vitro growth characteristics of *bcr* (−/−) fibroblasts were similar to those of (+/+) fibroblasts. Cells were stimulated with recombinant human platelet-derived growth factor (PDGF). No apparent effect of the *bcr*-null mutation was found on the appearance and degree of PDGF-induced membrane ruffling in primary fibroblast cultures (data not shown).

Neutrophils undergo extensive morphological changes when activated. Two key functions of neutrophils, chemotaxis and phagocytosis, involve microfilament reorganization, and we compared these activities in *bcr* (−/−) and *bcr* (+/+) polymorphonuclear neutrophils (PMNs). Phagocytic ingestion of latex beads by isolated (−/−) PMNs in vitro was not impaired; the percentage of cells involved in phagocytosis and the number of microspheres ingested by neutrophils of both genotypes were comparable (data not shown). In vivo chemotactic ability of *bcr* (−/−) neutrophils was confirmed by their presence in peritoneal exudates: (+/+) and (−/−) lavage yielded equal amounts of PMNs. Wild-type and mutant PMNs spread equally well on fibronectin-coated slides, and F-actin distribution was comparable for wild-type and mutant neutrophils (data not shown), demonstrating lack of gross abnormalities in cytoskeletal reorganization in (−/−) cells.

Experimental Endotoxemia Causes Septic Shock and Severe Tissue Damage in *bcr* (−/−) Mice

To test for possible abnormalities of *bcr* (−/−) neutrophils in inflammatory processes in vivo, experimental endotoxemia was induced with a sublethal dose of bacterial endotoxin (lipopolysaccharide [LPS]) in *bcr* (−/−) and *bcr* (+/+) animals. Under these conditions, wild-type mice devel-



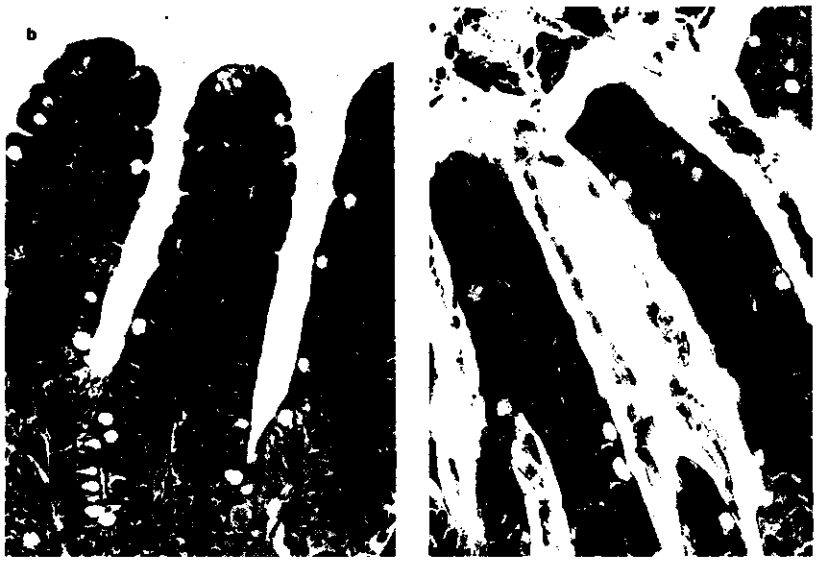


Figure 3. Aggravated Tissue Damage in *bcr*-Null Animals in Experimental Endotoxemia

(a) Body weight loss and regain of wild-type (WT) and mutant (MUT) animals after experimental induction of endotoxemia. Data are expressed as percentage of control (n = 5 animals for each genotype; from t = 4 on, n = 4). Asterisk, $P < 0.05$ versus the wild-type group at the same time point.

(b) Degenerative changes in the small bowel of (−/−) animals at 24 hr after injection of endotoxin. Left, (+/+); right, (−/−).

oped mild symptoms that disappeared within 12–24 hr. In sharp contrast, (−/−) mice gave clear evidence of serious distress within hours of endotoxin injection. The clinical condition of the mutant mice deteriorated rapidly and developed into septic shock, which was characterized by decreased spontaneous movements in the cage, increased respiratory rates, piloerection, shivering, and diarrhea; this condition persisted in (−/−) mice for at least 4 days. Null mutant animals displayed a sustained loss of body weight and delayed recovery from endotoxemia as compared with wild-type mice (Figure 3a). The pathological condition of (−/−) animals was accompanied by increased intestinal edema (n = 4): wet weight/dry weight ratios were notably increased in null mutant mice (data not shown).

Animals were also sacrificed at 2, 6, 24, 48, and 72 hr after endotoxin injection. Histological analysis of wild-type and *bcr*-null mutant tissues revealed extensive damage to multiple organs only in the *bcr* (−/−) mice. Degenerative changes in the small bowel of (−/−) animals were obvious at 24 hr after LPS injection. While the ileum of a (+/+) animal at 24 hr revealed normal histology (Figure 3b, left), the presence of cellular debris and mucus plugs in the intestinal lumen and in between microvilli of a (−/−) animal indicated marked necrosis of mucosal epithelial cells (Figure 3b, right). Extensive vacuolization in proximal convoluted tubules and cortical necrosis was obvious in the kidney of (−/−) animals from 24 hr to 72 hr (data not shown); collapse of glomeruli (diffusely cellular, undistended capillaries) was apparent as early as 6 hr in (−/−) kidneys, at which time leukocytosis was most prominent. Liver sections of (−/−) mice at 6 hr also showed marked leukocytosis: margination in portal veins clearly involved neutrophils, and presence of PMNs in hepatic sinuses indicated an active neutrophil recruitment; (−/−) liver parenchyma displayed distinct fatty vacuolization at 72 hr (data not shown). At this time, hematopoiesis in (−/−) bone marrow presented a distinct shift toward granulopoiesis as compared with (+/+) bone marrow (data not shown).

In other experiments, changes in total numbers of circu-

lating leukocytes and specifically neutrophils were monitored during the course of LPS-induced endotoxemia. Both (+/+) and (−/−) animals responded to LPS injection with decreased leukocyte counts and a relative neutrophilia that developed within 6 hr, 55.1% ± 12.6% (+/+) and 85.0% ± 11.1% (−/−), respectively (percentage ratio of PMNs to leukocytes). While the total number of circulating neutrophils in wild-type animals did not change notably within the first 24 hr, the total number of circulating neutrophils had increased 2- to 3-fold in (−/−) animals within 6 hr (data not shown). Neutrophilia was more pronounced and sustained in null mutants; the percentage of neutrophils was still markedly elevated over wild-type levels at 48 hr, 26.6% ± 13.0% (+/+) and 56.4% ± 1.0% (−/−) (percentage ratio of PMNs to leukocytes). Neutrophilia was confirmed by flow cytometric analysis of whole blood samples and coincided with an altered body weight loss–regain curve (data not shown).

Histological time course analysis of the experimental endotoxemia showed that at 6 hr, neutrophil margination and infiltration were significantly more prominent in (−/−) tissues than in (+/+) counterparts (data not shown). This distinct granulocytosis was not as obvious at early stages (1–2 hr) in either wild-type or mutant tissues, suggesting that neutrophil infiltration per se was not elevated in null mutant animals at initiation of the inflammatory response.

Tissue Damage in Endotoxemic *bcr* (−/−) Mice Is Mediated by Neutrophils

The experiments above showed that (−/−) mice developed septic shock and significantly more pronounced inflammatory tissue injury than (+/+) animals and suggested that this was caused by neutrophils. However, although the induction of experimental endotoxemia with LPS rapidly results in the recruitment of massive amounts of neutrophils, this process is mediated by the concerted effort of, among others, macrophages, endothelial cells, and neutrophils, as well as multiple cytokines (Edwards, 1994). Therefore, the possibility existed that lack of *bcr* augmented the inflammatory response through mechanisms unrelated to neutrophils.

We tested this by inducing neutropenia, as described by others in experiments linking multiple system organ failure to neutrophils (Hewett et al., 1992; Simpson et al., 1993; Wakiyama et al., 1993). Wild-type and mutant mice were first rendered neutropenic by cyclophosphamide (CTX) treatment. The animals were then injected with LPS to induce endotoxemia. Of the four experimental groups, only the control null mutant animals (LPS alone) displayed the earlier-described clinical manifestations associated with severe septic shock. Neutropenic animals ([+/+] and [−/−]) recovered as fast as wild-type control animals (LPS alone) from experimental endotoxemia, which was also reflected by the progression of body weight loss and regain (data not shown).

Generalized and specific tissue damage was assessed by plasma lactate dehydrogenase (LDH) and plasma aspartate and alanine aminotransferase (AST and ALT), plasma creatinine, and blood urea nitrogen (BUN) level measurements at 24 or 48 hr after LPS or CTX plus LPS injection. In concordance with histological findings, null mutant mice injected with LPS only had considerably higher levels of plasma LDH and AST (Figures 4a and 4b) and ALT, BUN, and creatinine (data not shown) than the wild-type animals, indicative of considerable damage to the hepatic and renal organ systems, among others. The combined treatment with CTX and LPS was somewhat more damaging than injection with LPS alone (in wild-type animals), but no significant differences were measured between neutropenic animals of either genetic constitution.

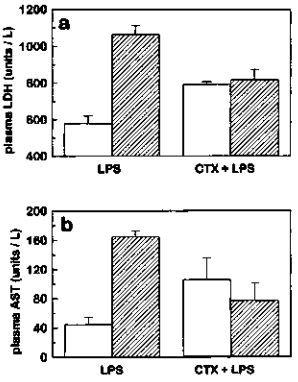


Figure 4. Neutropenia Abolishes LPS-Induced Tissue Damage in *bcr* (−/−) Mice

Plasma LDH and AST levels 48 hr after injection of LPS or LPS plus CTX. Hatched bars indicate *bcr* (−/−) values, open bars wild-type values. Three animals of each genotype were used. Data are shown as ± SEM.

At 48 hr, the degenerative histological characteristics associated with renal system organ failure in (−/−) kidney were absent from (−/−) mice treated with LPS plus CTX, and no differences were noted between ileum and liver of either wild-type (LPS), wild-type (LPS plus CTX) or mutant (LPS plus CTX) mice (data not shown). Thus, the biochemical and clinical differences between neutropenic and normal (−/−) animals were unambiguous: preestablished neutropenia prevented the distress caused by the experimental septic condition. Taken together, these results demonstrate that neutrophils are responsible for the severely aggravated pathological condition observed in *bcr* (−/−) mice.

Superoxide Production by *bcr* (−/−) Neutrophils Is Elevated

During the respiratory burst, neutrophils generate reactive oxygen metabolites via the nicotinamide adenine dinucleotide phosphate (NADPH)–oxidase system. These metabolites are the major mechanism for killing microorganisms. Bone marrow (BM) neutrophils, circulating neutrophils, and elicited (e.g., through peritoneal injection of casein) peritoneal exudate (PE) neutrophils can all perform the respiratory burst if appropriately activated, although they differ in their degree of biological maturation.

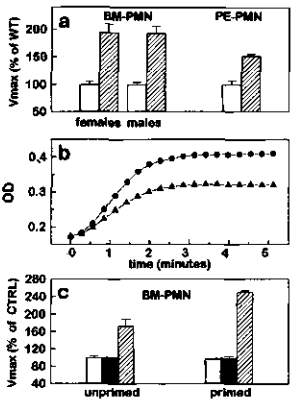


Figure 5. The Production of Reactive Oxygen Metabolites Is Elevated in *bcr* (−/−) Neutrophils

(a) O_2^- production by BM-PMNs and by PE-PMNs. The data are normalized for wild-type values. Combined data are displayed for two female and three male mice per histogram in three independent experiments. Open bars, (+/+) mice; hatched bars, (−/−) mice. BM-PMNs were stimulated with PMA, PE-PMNs with fMLP.

(b) Cytochrome C reduction by (−/−) mutant (circles) and wild-type (triangles) PE-PMNs after activation with fMLP.

(c) Priming of BM-PMNs with a low concentration of the phorbol ester PMA enhances the superoxide production by (−/−) PMNs. Data from two independent experiments are combined. Open bars, (+/+) mice; closed bars, (+/−) mice; hatched bars, (−/−) mice. V_{max} values are expressed as percentage of wild-type activity at t = 0.

BM granulocytes of (−/−) and (+/+) mice were purified through density gradient centrifugation. Upon activation with phorbol myristate acetate (PMA), *bcr* (−/−) neutrophils showed an almost 100% increased respiratory burst compared with that of wild-type neutrophils (Figure 5a). Consistent with this result, the respiratory burst evoked in vitro by both non-receptor- and receptor-mediated stimuli (PMA and N-formyl-Met-Leu-Phe [fMLP], respectively) in (−/−) PE neutrophils was also higher than that of corresponding wild-type cells (Figure 5a). Since the duration of respiratory burst was similar for both (+/+) and (−/−) neutrophils, the increased production rate resulted in a correspondingly 2-fold higher endpoint O_2^- production by (−/−) neutrophils under in vitro conditions (Figure 5b).

bcr (−/−) Neutrophils Are More Easily Primed and Activated

Priming of neutrophils by very low concentrations of agonists (which, by themselves, do not activate the neutrophil) causes an up-regulation of their function upon subsequent activation. This includes the oxidative burst (Edwards, 1994). We used BM-PMNs to investigate whether *bcr* (−/−) neutrophils were more readily primed than wild type. BM-PMNs were left undisturbed or were treated with concentrations of PMA (as described by Shaafi et al., 1988; Lofgren et al., 1993) that were too low to cause priming of (+/+) neutrophils. Interestingly, this same concentration of PMA did prime (−/−) PMNs, resulting in superoxide production that was 260% of that of (+/+) PMNs (Figure 5c),

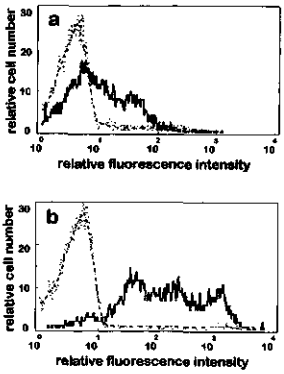


Figure 6. Oxidative Burst Activity in (−/−) Circulating Neutrophils

Oxidation of the nonfluorescent intracellular DCFH to the highly fluorescent DCF was examined by flow cytometry; cell populations were gated for granulocytes. Peripheral blood neutrophils from wild-type (broken line) and bcr (−/−) (solid line) mice are compared.

(a) Animals were treated with ≤2 mg LPS/kg of body weight (intraperitoneally) 1 hr before isolation of blood.

(b) *Animals had received 20–22 mg LPS/kg of body weight 48 hr before* a blood sample was obtained.

while resting levels of superoxide production of primed (−/−) and of (+/+) neutrophils were negligible (data not shown). This suggested that *bcr* (−/−) BM-PMNs were also more sensitive to priming stimuli than wild-type PMNs. Heterozygous (+/−) and wild-type responses were alike (Figure 5c).

We determined whether (−/−) neutrophils in circulation also had an increased sensitivity to stimuli, or showed elevated superoxide production in vivo, or both. Peripheral blood neutrophils were allowed to take up 2'7'-dichlorodihydrofluorescein diacetate (DCFH-DA), and intracellular oxidation of DCFH to fluorescent dichlorofluorescein (DCF) by reactive oxygen metabolites was measured. Resting neutrophils in whole peripheral blood samples of (+/+) and (−/−) mice showed a similar baseline fluorescence (data not shown). Circulating neutrophils were then harvested from mice after a brief (60 min) exposure to a low (<2 mg/kg body weight) dose of LPS. This dose is more than 10-fold lower than that used to induce experimental endotoxemia. Without additional (in vitro) activation, over 50% of the *bcr* (−/−) neutrophils showed a high level of spontaneous cytoplasmic fluorescence, as compared with an identically treated wild-type control (Figure 6a).

The circulating neutrophils of *bcr* (−/−) animals in severe septic shock and from similarly treated control (+/+) mice were also examined 48 hr after LPS injection. Neutrophils isolated from (−/−) mice showed extensive spontaneous (i.e., no additional activator was added in vitro) respiratory burst, while the neutrophils of similarly treated wild-type mice again showed only baseline fluorescence (Figure 6b). This demonstrated that the severe septic shock exhibited by the *bcr* (−/−) mice was accompanied by a massive production of reactive oxygen metabolites by activated PMNs.

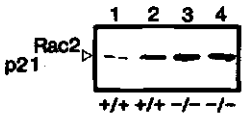


Figure 7. Western Blot Analysis of Rac2 in *bcr* (−/−) and Wild-Type Neutrophil Membrane Fraction

Lanes were loaded with 40 μg of protein. Lanes 1 and 2, wild-type membrane fraction; lanes 3 and 4, *bcr* (−/−) membrane fraction. Neutrophils in lanes 2 and 4 were additionally activated in vitro by treatment with 10 ng/ml PMA for 8 min.

Increased Membrane Translocation of $p21^{rac2}$ in (−/−) Neutrophils

Apart from the more generalized function for Rac1 in membrane ruffling, Rac1 and Rac2 also play an important specialized role in the NADPH–oxidase system of phagocytic cells (Knaus et al., 1991; Dorseuil et al., 1992; Abo et al., 1991; Heyworth et al., 1993). Since Bcr is a GTPase-activating protein for Rac in vitro, we asked whether the deregulated production of oxygen radicals in *bcr* (−/−) neutrophils was related to Rac.

PE neutrophils were isolated from *bcr* (−/−) and *bcr* (+/+) mice. These neutrophils had been activated in vivo. One portion was left without further treatment, the other additionally activated in vitro. Membrane and cytoplasmic fractions were assayed quantitatively for Rac2 by Western blotting and densitometry. The total amount of Rac2 recovered (membrane plus cytoplasmic fractions) was approximately equal for *bcr* (−/−) and *bcr* (+/+) neutrophils. In wild-type mice, the amount of Rac2 present in the membrane fraction after in vivo activation was initially low (Figure 7, lane 1), but increased nearly 2-fold upon further (in vitro) activation (lane 2). Similar data were previously obtained by Quinn et al. (1993) with human neutrophils. In *bcr* (−/−) mice, a substantially higher amount of Rac2 was associated with the membrane (Figure 7, lane 3): a 3-fold increase was measured in comparison with wild-type cells (lane 1). In vitro stimulation of the *bcr* (−/−) neutrophils did not further increase the level of membrane-associated Rac2 (Figure 7, lane 4).

Discussion

Lack of Correlation between *bcr* Expression and Mutant Phenotype

The *bcr* gene is expressed in many different types of tissues in the mouse. During development, it is detectable from the zygote stage on, and expression continues throughout embryogenesis (unpublished data). The absence of a functional mouse *bcr* gene, however, has no apparent effect on fertility or development. A role of BCR in myeloid development (Bedi et al., 1993; Wetzler et al., 1993) has also been suggested. Although *bcr* is expressed in all mature hematopoietic cell types tested, size, cellularity, histology, and composition of the major hematopoietic organs (bone marrow, spleen, thymus, and blood) were similar in wild-type and *bcr*-null mutant mice. We conclude that lack of Bcr alone is not sufficient to perturb myeloid and lymphoid development or T and B lymphoid function in vitro and in vivo. However, it is possible that disturbances, if any, will become apparent with advanced age.

To explain the preferential involvement of the maternal allele of *BCR* in the Philadelphia translocation, it has been suggested that the *BCR* gene may be imprinted (Haas et al., 1992). In our *bcr* mutant mice, the remaining *bcr* allele is actively expressed in heterozygote brain (Figure 2) and in blood cells of heterozygotes (+/−), regardless of whether the allele is from paternal ([+/−] male × [−/−] female) or maternal origin ([+/−] female × [−/−] male) (our unpublished data). This is in agreement with experiments performed with human peripheral blood (Fioretos et al., 1994). In all experiments in which neutrophil function was tested, *bcr* heterozygote (+/−) responses were found to be comparable to those in wild type, which would seem to eliminate the possibility of a gene dosage effect of monoallelic loss of *bcr*.

Activities of Bcr in Vitro and in Vivo

Similar to several other proteins, Bcr has GAP activity toward $p21^{rac1/rac2}$ and CDC42Hs, members of the Rho family of small GTP-binding signal transduction molecules. One function identified for $p21^{rac1}$ is that it mediates mitogen-induced membrane ruffling in fibroblasts (Ridley et al., 1992). When Swiss 3T3 mouse fibroblasts are microinjected with the GAP domain of Bcr, PDGF-induced membrane ruffling is abolished (Ridley et al., 1993). Mouse *bcr* was abundantly expressed in our wild-type skin fibroblast cultures and in neutrophils (unpublished data). We examined both membrane ruffling and cytoskeletal reorganization in isolated *bcr* (−/−) cells but were unable to find any differences between (−/−) and (+/+) cells. We conclude that there must be a functionally redundant mechanism that compensates for the lack of Bcr, such as one involving the Bcr-homologous protein $p93^{abr}$ (Heisterkamp et al., 1993b), in membrane ruffling. Alternatively, Bcr does not interact with the Rac that is involved in this process. Adhesion and cell spreading of neutrophils did not appear affected by lack of $p160^{bcr}$, in agreement with earlier observations (Ridley et al., 1992) that demonstrated that Bcr does not stimulate GTP hydrolysis by $p21^{rho}$, a regulator of focal adhesion and stress fiber formation, in vitro (Ridley and Hall, 1992).

Bcr Regulates Oxygen Radical Production in Neutrophils

Neutrophils are key mediators in the defense against invading micro-organisms, and inherited or acquired impairment of the production of reactive oxygen metabolites by neutrophils can lead to life-threatening infections. Although the primary function of neutrophil- and macrophage-produced oxidants is microbicidal, paradoxically, the reactive oxygen metabolites they produce can also cause extensive injury to surrounding tissue. This is of great clinical importance; generalized inflammation in gram-negative sepsis is perpetuated by phagocytic superoxide release and may evolve into septic shock and multi-

ple organ system failure (Shasby et al., 1982; Demling, 1990; Shiratori et al., 1990; Yoshikawa et al., 1994). Therefore, the production of toxic oxygen radicals must be tightly regulated: biologically appropriate amounts must be produced only at the site of inflammation, and only after proper activation.

Our experiments show that *bcr* is important for at least two potentially overlapping aspects of this regulation. First, activated neutrophils isolated from *bcr* (−/−) bone marrow showed up to 100% increased production of reactive oxidants in vitro as compared with wild-type neutrophils. Similarly, *bcr* (−/−) exudative neutrophils showed an elevated superoxide production. Circulating neutrophils isolated from severely endotoxemic *bcr* (−/−) animals produced reactive oxygen metabolites without further additional in vitro activation. Thus, the regulation of absolute amounts of superoxide production is impaired in *bcr* (−/−) neutrophils.

Second, (−/−) neutrophils are more sensitive toward priming stimuli. After activation, primed (−/−) neutrophils from the bone marrow produced an even larger excess of superoxide than similarly treated (+/+) neutrophils. Circulating neutrophils of *bcr* (−/−) mice injected with a low dose of LPS showed a significant increase in DCF fluorescence after uptake of DCFH-DA in comparison with identically treated control animals. This is indicative of the intracellular production of reactive oxygen metabolites. We conclude that *bcr* (−/−) neutrophils are more readily primed in comparison with wild-type neutrophils.

Circulating neutrophils are expected to be exposed to priming substances in vivo. Regulation of neutrophil activity at this level appears biologically quite important in vivo, as is dramatically illustrated by the severe pathological reaction of *bcr* (−/−) mice to experimentally induced endotoxemia. Experimental endotoxemia led to extensive tissue damage in *bcr* (−/−) animals, as measured by clinical, biochemical, and histological parameters; (−/−) mice became lethargic and overtly sick and in some instances even died.

In conclusion, Bcr normally has a function in the negative regulation of the oxidative burst in neutrophils. This is consistent with the negative regulatory effect of Rac GAPs in in vitro experiments (Heyworth et al., 1993). In particular, our data point to a specialized role of Bcr in the protection of the organism against premature activation of the oxidative burst.

Regulation of Rac2 Is Affected by Lack of *Bcr*

$P21^{rac1/rac2}$ is an essential part of the activated respiratory burst (NADPH)–oxidase present in neutrophils, macrophages, and B cells (Abo et al., 1991; Quinn et al., 1993; Knaus et al., 1991; Bokoch and Knaus, 1994; Benna et al., 1994). Activation of the NADPH–oxidase in a cell-free assay is completely dependent upon the presence of GTP, and nonhydrolyzable GTP analogs potentiate O_2^- production (Bokoch and Knaus, 1994, and references therein). Inhibition of Rac function blocks superoxide (O_2^-) production (Knaus et al., 1991; Dorseuil et al., 1992). Upon neutrophil activation, Rac undergoes a GDP–GTP exchange and translocates from the cytosol to the plasma membrane (Bokoch et al., 1994). This translocation correlates with oxidase activation and the generation of reactive oxygen metabolites (Quinn et al., 1993). Several proteins exhibit regulatory activity as a GAP toward Rac1 and Rac2 in vitro (Diekmann et al., 1991; Heisterkamp et al., 1993b; Ridley et al., 1993; Ahmed et al., 1994; Leung et al., 1994), including Bcr. We therefore examined Rac translocation in *bcr* (−/−) neutrophils.

Although the total amount of Rac2 was similar in *bcr* (−/−) and (+/+) neutrophils, its subcellular distribution in PE neutrophils, which were activated in vivo, was different. Purified membrane fractions of elicited PE *bcr* (−/−) neutrophils showed a significantly increased translocation of $p21^{rac2}$ to the membrane, providing evidence for a direct link between Bcr and Rac2 in the biochemical processes that lead to superoxide production. It is of interest that further in vitro activation of *bcr* (−/−) neutrophils had no effect on the level of Rac2 translocation. This suggests that there is a maximum to the amount of Rac2 that can be membrane associated. In vitro, the GAP function of Bcr stimulates GTP hydrolysis by Rac (Diekmann et al., 1991), thereby rendering Rac inactive. Because of this, it is tempting to speculate that relatively more $p21^{rac}$ is present in a GTP-bound state in primed or activated neutrophils that lack cellular Bcr, and that this causes an increase in membrane association. Since the process of GTP loading by itself is sufficient to cause Rac to translocate to the membrane (Philips et al., 1993; Bokoch et al., 1994), the increased membrane association of Rac2 in the *bcr* (−/−) neutrophils would correspond with the presence of increased levels of Rac–GTP in the cell.

BCR in Disease

These studies were initiated to explore the normal cellular function of a gene that is involved in Ph-positive leukemia in humans. In the leukemic cells of such patients, only one intact *BCR* allele remains; the other is rearranged as a result of fusion to *ABL*. The BCR–ABL fusion protein consists of *BCR* exon 1–encoded sequences and often also includes the DBL homology domain. The carboxy-terminal end of BCR–ABL contains almost all of the ABL protein. The BCR GAP domain is translocated to chromosome 9 (reviewed by Van Etten, 1993; Heisterkamp et al., 1993a) and may be transcribed as a chimeric mRNA from the *ABL* promoter (Melo et al., 1993).

Our data define BCR as a protein that functions as a regulator of the respiratory burst in certain hematopoietic cells; phagocytes (neutrophils, macrophages) and B cells are all specialized hematopoietic cells with an active NADPH-associated oxidative burst complex (Edwards, 1994). It is unlikely to be a coincidence that these cell types are also involved in Ph-positive leukemia.

There appear to be a number of discrete mechanisms through which *BCR* contributes to leukemogenesis. One is at the level of transcription: the *BCR* promoter controls expression of both the remaining nonrearranged *BCR* allele and the *BCR–ABL* oncogene in human leukemia.

Apart from the structural influence on *ABL* in *BCR–ABL*

(Maru and Witte, 1991; McWhirter and Wang, 1991; Muller et al., 1991), *BCR* may contribute to leukemia on another level: the presence of a functional $p160^{bcr}$ was proposed as a requirement for malignant transformation by *BCR–ABL* (Liu et al., 1993; Lu et al., 1993). We are now able to test this hypothesis in *BCR–ABL*-transgenic *bcr* (−/−) mice.

Independent of the exact mechanism, it seems reasonable to conclude from our data that there is a correlation between the cell type affected in Ph-positive leukemia and Bcr function. In addition, the findings presented here connect $p160^{bcr}$ with the regulatory control of the cellular Rac-GTP/Rac-GDP pool in vivo during neutrophil priming and activation. Although its exact function remains to be established, it is clear that Bcr has a previously unsuspected role as a regulator of the oxidative burst. Because of the clinical importance of neutrophils in, among others, adult *respiratory distress syndrome*, *bacterial septicemia*, and rheumatoid arthritis, it will be of interest to examine Bcr regulation and activity in these clinical conditions as well as in normal cells.

Experimental Procedures

Construction of *bcr* Targeting Vector and Generation of Null Mutant Mice

The mouse *bcr* locus was cloned from a partial MboI B_6CBA/F1 genomic library. Targeting vector pBEKO-2 was constructed by replacing a 500 bp XhoI–ClaI fragment, spanning part of *bcr* intron 1 and exon 2, with a 1.7 kb XhoI–ClaI fragment that contained a PGK-neo-poly(A) cassette. Negative selection was with a herpes simplex virus thymidine kinase gene. pBEKO-2 DNA (25 μg) was linearized with NotI and used to electroporate 2.5×10^6 ES-D3 cells. ES cultures were maintained *on STO feeder layers in medium supplemented with 70% buffalo rat* liver cell–conditioned medium. DNA from individual colonies was digested with ClaI–XbaI or HindIII–XhoI (Figure 1) and hybridized to external probes. Of 35 doubly resistant clones screened, 10 had undergone homologous recombination. Injected blastocysts were cultured overnight and then reimplanted into 2.5 days pseudopregnant females.

Western Blot Analysis

Tissues and organs were homogenized and analyzed by immunoblotting procedures essentially as described (Harlow and Lane, 1988). The *bcr* (AB-2) anti-human BCR monoclonal antibody (Oncogene Science, Incorporated) is directed against an epitope in exon 8. Rac2 antiserum was as described (Quinn et al., 1993).

Membrane Ruffling

Primary skin fibroblast cultures were established from term embryos or newborn pups. Membrane ruffling was studied in chamber slides (Nunc, Incorporated). Confluent wild-type and mutant fibroblast cultures were stimulated with 6 ng/ml PDGF-BB (human recombinant; GIBCO BRL) in serum-free medium at 37°C, 100% humidity, 5% carbon dioxide as described (Ridley et al., 1992). *Durations of stimulation* were 0, 2, 5, 10, and 30 min. Cells were fixed in 7.4% formaldehyde and stained with a NBD–phallacidin solution (Molecular Probes, Incorporated) containing lysophosphatidylcholine (Sigma) to permeabilize the cells. Ruffle formation was studied by using Nomarsky and fluorescent microscopy. The extent of ruffling by wild-type and mutant fibroblasts in response to PDGF was assessed by blinded analysis.

Neutrophil Spreading

Neutrophils were allowed to attach to fibronectin-coated slides for up to 45 min at 37°C, 5% carbon dioxide, and 100% humidity. Cells were fixed with 7.4% formaldehyde in PBS and permeabilized and stained for F-actin with a lysophosphatidylcholine, NBD–phallacidin solution (Molecular Probes, Incorporated). Comparison of cell spreading, focal adhesion, and lamellipod formation of wild-type and mutant neutrophils was done by blinded analysis.

Phagocytosis Assay

Exudative neutrophils and BM neutrophils were incubated at a density of 1×10^6 cells/ml in KRPG buffer in siliconized tubes. A 50-fold excess of fluorescent latex beads was added (Fluoresbrite; Polysciences, Incorporated). In some experiments, neutrophils were stimulated with 100 ng/ml PMA. Incubations were carried out at 37°C with gentle agitation and stopped at 30 min, 60 min, 90 min, or 120 min by addition of ice-cold PBS. Phagocytic ingestion was assessed by flow cytometric analysis. The amount of cells participating in phagocytosis and the amount of fluorescent microspheres ingested were evaluated.

Induction of Endotoxemia In Vivo

Age- and sex-matched animals of 23–25 g body weight were anesthetized with metofane (methoxyflurane; Pitman-Moore) and injected intraperitoneally with 20–22 mg LPS/kg body weight unless differently stated. Changes in body weight were monitored at 24 hr intervals; the general clinical condition of the animals was monitored at regular intervals during the days following induction of experimental endotoxemia. To assess acute edematous bowel injury, animals were sacrificed 2 hr after administration of LPS. Wet tissue weight was determined immediately; dry tissue weight was determined after overnight incubation at 80°C. The ratio of net wet weight to net dry weight was calculated. As reported previously (DeForge et al., 1994), no measurable edema formation was found in lung tissue at 1 or 2 hr after LPS injection.

Induction of Neutropenia

Animals were anesthetized and injected intraperitoneally with 150 or 200 mg of CTX (Cytoxan; MeadJohnson)/kg of body weight. Neutropenia was confirmed 72 hr later by manual peripheral leukocyte and differential count. Animals were then injected with 20–22 mg LPS/kg of body weight. Body weight loss and regain was monitored for two (LPS only) or four to five animals (Cytoxan plus LPS) for each genotype. Three experiments were done. Heparinized blood was centrifuged, and plasma was collected and frozen at −80°C until analysis. BUN, serum creatinine, ALT, AST, and LDH were determined at the clinical *laboratories of Childrens Hospital, Los Angeles.*

Isolation of Neutrophils from Bone Marrow and Peritoneum

Bone marrow was used as a source of unelicited PMNs (Jutila et al., 1991; Levinovitz et al., 1993). BM-PMNs were obtained by density gradient centrifugation (Watt et al., 1979). PE-PMNs were isolated by peritoneal lavage, 4 hr after injection of 2 ml of 0.05% sodium caseinate. Lavage was performed by washing the peritoneal cavity twice *with, respectively, 6 and 8 ml of ice-cold Hanks' balanced salt solution* (HBSS) buffer (GIBCO BRL) supplemented with 2 U/ml heparin. Care was taken not to cause internal bleeding while collecting exudative neutrophils. Washed neutrophils were suspended in cold Krebbe–Ringer phosphate buffer containing 1 mg/ml glucose (KRPG buffer). Mature BM-PMN phenotype was confirmed by microscopic analysis. Purity of the BM-PMN and PE-PMN populations was assessed by flow cytometry: a consistent purity of 85%–95% and 90% or higher for GR-1$^+$ cells was found for BM–PMNs and PE-PMNs, respectively. Equal levels of PE-PMN activation were measured by staining with anti-CD11b (M1/70.15) monoclonal antibodies (Caltag).

In Vitro Superoxide Production Assay

Respiratory burst was induced by stimulation of 5×10^6 mouse PMNs/250 μl with 100 ng/ml PMA (BM-PMNs and PE-PMNs) or 1×10^{-5} M fMLP (PE-PMNs), in standard KRPG buffer. O_2^- production was followed for 5–10 min in a kinetic microplate reader by inhibitable ferricytochrome C reduction (Mayo and Curnutte, 1990). The rates of fMLP- and PMA-induced superoxide production were consistent with previous observations. The range of superoxide production by PMA-stimulated wild-type BM-PMNs was 10–15 nmol O_2^-/min/10^7 cells; wild-type PE-PMN V_{max} values ranged from 35 to 45 nmol O_2^-/min/10^7 cells. Preincubation was accomplished for varying amounts of time (90–120 min) at 37°C with low (0.1–1.0 ng/ml) PMA concentrations that did not evoke measurable superoxide production in either (−/−) or (+/+) PMNs. Following preincubation, the cells were stimulated with 100 ng/ml PMA. The maximal rate of cytochrome C reduction was calculated by using the extinction coefficient 21.1 mM^{-1} cm^{-1}. All PMN exper-

iments were carried out within 2 hr of isolation. Animals used in each individual experiment were always age- and sex-matched.

Priming and Activation of Circulatory Neutrophils In Vivo

Animals were injected with lipopolysaccharide. At the indicated time points, animals were anesthetized with metofane, and 50–100 μl blood samples were taken by retro-orbital sinus bleeding. Erythrocytes were lysed, and leukocytes were washed with PBS and suspended in 500–1000 μl of PBS containing 40 μM DCFH-DA. Cell suspensions were incubated in siliconized 5 ml polypropylene tubes for 30–45 min at 37°C under reduced light, while gently agitated. Cells were then fixed by addition of 1 vol of ice-cold 2% paraformaldehyde and immediately analyzed by flow cytometry essentially as described (Himmelfarb et al., 1992).

Translocation of Rac2 to the Membrane

Exudative neutrophils were isolated and prepared as described above. Cells from 15 mice were pooled and divided into two equal portions, one for each experimental point. PMNs were treated with diisopropyl fluorophosphate (DFP) *prior to activation with 10 ng/ml PMA*. Stimulation occurred at 37°C in siliconized tubes, in the presence of superoxide dismutase, catalase, and 0.1% bovine serum albumin. Incubations were terminated after 7–8 min by addition of ice-cold reaction buffer. Cells were disrupted by nitrogen cavitation as described elsewhere (Quinn et al., 1993). Membrane fractions were obtained by centrifugation over a discontinuous sucrose gradient and examined by Western blot analysis as described (Quinn et al., 1993). Relative amounts of Rac2 were determined by video densitometry using an image analysis system.

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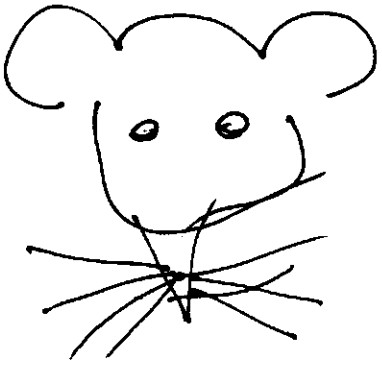

'A mouse (transgenic)'. By Vesa (30+)

CHAPTER 8

Regional Localization and Developmental Expression of the *bcr* Gene in Rodent Brain

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Regional Localization and Developmental Expression of the *bcr* Gene in Rodent Brain

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Abstract

The *BCR* gene is implicated in the development of Ph-positive leukemia through its fusion with the non-receptor tyrosine kinase gene *ABL*. The normal 160 kDa Bcr protein has several functional domains and recently one specific role for Bcr was established in the regulation of respiratory burst activity in white blood cells. *Bcr* expression levels are relatively constant throughout mouse development until adulthood in brain and in hematopoietic tissues, a pattern which is distinctly different from that of the functionally related *n-chimerin* gene. In the present study RNA in situ hybridization was used to explore the normal cellular function of Bcr in rodent brain and hematopoietic organs. The data pinpoint the high *bcr* expression in brain to the hippocampal pyramidal cell layer and the dentate gyrus, and to the piriform cortex and the olfactory nuclei, reflecting a potentially interesting function for Bcr in these highly specialized brain regions.

Introduction

The human *BCR* locus was first identified in a breakpoint cluster region on chromosome 22 in Philadelphia positive leukemia (Groffen et al., 1985; Heisterkamp et al., 1983): through a t(9;22)(q34;q11) translocation, the *BCR* gene becomes fused with the *ABL* gene, a non-receptor tyrosine kinase normally located on chromosome 9 (Heisterkamp and Groffen, 1991; Rowley et al., 1990) The leukemogenic role of *BCR/ABL* has been demonstrated experimentally in transgenic mice carrying a *BCR/ABL* fusion-transgenes and by retroviral transduction experiments in which mice receive *BCR/ABL*-positive bone marrow transplants (Daley et al., 1990; Elefanty et al., 1990; Heisterkamp et al., 1990; Voncken et al., 1992).

The *BCR* gene encodes a 160 kDa cytoplasmic protein with several functional domains and is a protein likely to participate in signal transduction. Its N-terminal domain encoded by exon 1 has a serine/threonine kinase activity, can oligomerize, and is capable of binding other protein factors, among which $p145^{Abl}$ (McWirther et al., 1993; Sawyers et al., 1991). The central part of Bcr has homology to the G-nucleotide exchange factor (GEF) *Dbl* and the carboxy terminal part is homologous to the n-chimerin protein, which is selectively expressed in neuronal tissue (Diekman et al., 1991; Ron et al., 1991). As is the case for n-chimerin, this domain of Bcr has GTP-ase activating protein (GAP) activity toward a subfamily of $p21^{Ras}$-related small GTP-binding proteins, $p21^{Rho/Rac}$ (Diekman et al., 1991). Recently, we have established at least one function for Bcr as a regulator of NADPH-oxidase mediated superoxide production in neutrophils (Voncken et al., 1995).

In the mouse, *bcr* expression is detectable as early as at the zygote stage, continues throughout embryogenesis (unpublished observations) and is found in most adult tissues examined (Heisterkamp et al., 1993). In man, *BCR* has also been shown to be ubiquitously expressed. Notwithstanding its role in normal and leukemic blood cells (Campbell et al., 1991; Collins et al., 1987; Voncken et al., 1995), the Bcr protein is found in high levels in brain. This observation has intrigued us as it suggests a possible

different function for $p160^{Bcr}$. This notion is corroborated by the seemingly highly significant function of G-proteins in brain (Hopkin, 1994). In an attempt to further delineate a putative cellular function of Bcr in the brain we have studied the regional expression of *bcr* in rodent brain by means of RNA in situ hybridization. We also report data on the expression kinetics of *bcr* during mouse development in hematopoietic organs and in brain.

Materials and methods

Tissue preparation

Paraffin embedded adult mouse brain sections were obtained from Novagen Inc. (Madison, WI). Adult Sprague-Dawley rats were decapitated rapidly and brains were removed onto dry ice and stored at -80°C. Coronal sections (20 μm) were mounted on gelatine-coated slides and stored at -80°C.

Probes

A 400 bp fragment of mouse *bcr* was amplified by RT/PCR with the primers ALLE (Kawasaki et al., 1988) and BEX3 (5' AACCCACTTTCTCATCT-CCAG 3') as previously described (Heisterkamp et al., 1990). The RT/PCR product was gel purified and digested with EcoRI and ClaI yielding a 251 bp fragment which was subcloned directionally into pSK (Stratagene). The identity of the cloned fragment was confirmed by sequencing both strands using the Sequenase 2.0 system (USB). To obtain antisense and sense RNA probes, the construct was linearized with BamHI (antisense) or XhoI (sense) and transcribed in vitro with α-^{35}S-UTP using T7 and T3 RNA polymerase respectively, essentially as described by the manufacturer (Novagen Inc., Madison WI). A second *bcr* antisense RNA probe was produced by digesting a 1.8kb HindIII/EcoRI genomic fragment (Zhu et al., 1990) cloned in pSK with NarI and transcription with T7 RNA polymerase. Probes were labeled to high specific activity, typically 2.0 x 10^8 cpm/μg. The oligonucleotide probe BEX2 (5' GAGGAAGAAGGTGAATCATCG 3') was labeled on the 3' end with ^{35}S-dATP using terminal deoxynucleotidyl transferase (Bethesda Research Lab.). An oligonucleotide probe for an unrelated gene, CRH, was similarly generated and labeled as specificity control (Baram and Schultz, 1991; Yi et al., 1993).

In situ hybridization procedures

In situ hybridization on paraffin embedded mouse tissues was performed using the SureSiteII System from Novagen. Briefly, paraffin was removed by three washes in xylene and rehydrated through a graded ethanol series. Tissue pretreatment consisted of ribosome disruption in 0.2 N HCl for 5 minutes, deproteinization in 1 μg/ml proteinase K for 5 minutes, followed by acetylation in 0.1 M triethanolamine-HCl, pH 8.0, containing 0.25% (v/v) acetic anhydride for 5 minutes. Prehybridization, hybridization with antisense and sense probes on parallel sections, and subsequent washings were performed according to the manufacturers instructions. Slides were apposed to XAR2 films for 1-2 days, then dipped into Kodak NTB-2 emulsion, exposed in a sealed container for 2-3 weeks at +4°C, and finally developed and fixed using Kodak developer D-19 and Kodak fixer respectively. Slides were stained in 5% aqueous Giemsa, examined and photographed with bright and darkfield optics using an Olympus BH-2 microscope equipped with an Olympus C-35AD-2 camera. In situ hybridization on rat brain sections using the oligonucleotide probes described above was performed essentially as previously described (Baram and Schultz, 1991; Baram and Schultz, 1992; Yi et al., 1993). After prehybridization for 1 hr and hybridization for 20 hr at 40°C in a humidity chamber, serial washes were performed (4 washes at 2xSSC for 15 min at 40°C, followed by one wash in 1xSSC and one in 1xSSC at room temperature), followed by dehydration through ethanol-0.3M NH_4Ac and apposition to X-ray film (Hyperfilm B-max, Amersham) for 24-48 hrs.

Northern blot analysis

Tissues from C57Bl/6 x B6CBA F1 mice were minced in a polytron and total RNA was extracted using the acid guanidinium thiocyanate phenol-chloroform method (Chomczynski and Sacchi, 1987). At embryonic day E15, E17, and E20 the head

region was separated from the remainder of the embryo. At parturition (P0) and postnatal day 1, 5, 10, 15, 20, 25, 30, and 35, the entire brain including cerebellum was dissected. In order to compare the developmental expression of *bcr* in brain with *bcr* expression in hematopoietic tissue, RNA was prepared from liver at E15, E17, E20, at parturition, and from spleen at postnatal day 1, 5, 10, 15, 20, 25, 30, and 35. Samples (10 μg) were run on formaldehyde/formamide gels, blotted as described (Sambrook et al., 1989) and subsequently hybridized to the 1.8kb HindIII/EcoRI probe containing coding sequences from mouse *bcr* exon 1. Filters were washed at low stringency (2.5xSSC, 65°C for 20 min). At this stringency the probe also cross hybridizes to the 28S and 18S ribosomal bands making it possible to check for equal loading of the lanes. Densitometric scanning was performed using an in-house manufactured densitometric device.

Results

In situ hybridization patterns

Coronal sections of adult mouse brain revealed specific hybridization mainly localized to cells of neuronal morphology when hybridized with either of the two (non-overlapping) antisense *bcr* probes described above. The sense probe did not produce any specific signal in any of the experiments performed. High concentration of *bcr* mRNA was present in hippocampus, and to a somewhat lower degree in the cerebral cortex. In hippocampus and dentate gyrus, region CA1-CA3, the pyramidal cell and granular cell layers were strongly labeled (Figs. 1a and 1b). In cortex, neurons in layers II-VI were moderately labeled, whereas a stronger labelling was noted in the piriform cortex. Some thalamic nuclei showed a weak hybridization. Neurons in amygdala and hypothalamus were not labeled above background hybridization. The hybridization pattern in cerebellum was not investigated. In rat, in situ hybridization with the labeled oligonucleotide revealed similar expression patterns: dentate gyrus granular cells, hippocampal pyramidal layer and piriform cortex clearly displayed a specific hybridization pattern, diminishing in intensity, in the order indicated above (Fig 2.). The anterior olfactory nucleus also displayed a specific hybridization pattern (not shown).

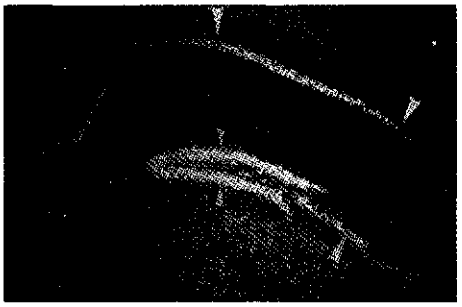

Figure 1a. *Darkfield photograph of a coronal section of a mouse brain showing strong hybridization to the hippocampus (large arrow-heads) and dentate gyrus (small arrow-heads) when hybridized with the antisense bcr probe.*

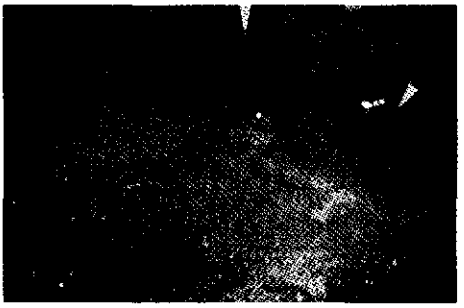

Figure 1b. *A parallel section of the section presented in figure 1a showing only weak background hybridization to the hippocampus (large arrow-heads) and no hybridization signal to the dentate gyrus when hybridized with the sense bcr probe.*

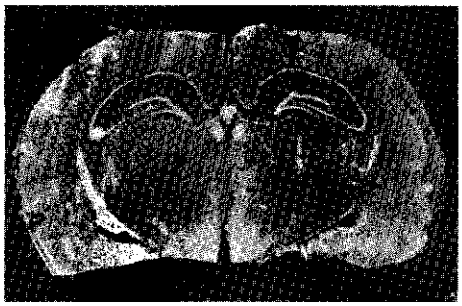

Figure 2. Coronal section of rat brain showing strong hybridization to hippocampus (large arrow-heads) and dentate gyrus (small arrow-heads) when hybridized to the bcr oligonucleotide (see: Material and Methods).

Gene expression throughout development

Northern blot analysis of *bcr* expression in mouse brain showed a transcript of approximately 7 kb which was present at E15 and remained constant throughout the embryonal period of development, as well as through postnatal days 1-35 (Fig. 3). Densitometric scanning revealed no clear changes in *bcr* expression level over time, when corrected for unequal loading on the basis of background hybridization to 18 and 28S ribosomal RNA. The role of Bcr in blood cells has recently been validated (Voncken et al., 1995). Northern blot analysis of liver and spleen samples, both organs, that have a distinct hematopoietic function during embryogenesis and for some period after birth, showed a lower level of *bcr* expression throughout the same developmental stages, but similar to *bcr* expression in brain, the level remained constant throughout pre- and postnatal development (data not shown).

Discussion

The Bcr protein is a multidomain protein and is involved in Ph-positive leukemogenesis through its fusion to the non-receptor tyrosine kinase Abl (Heisterkamp and Groffen, 1991; Van Etten, 1993). Although we have recently established a function for Bcr in neutrophils, the reason why *bcr* is highly expressed in brain remains elusive. We have used in situ hybridization to study *bcr* expression patterns in rodent brain. Our data show that *bcr* is highly expressed in the hippocampus and dentate gyrus, and to a lower level in the piriform cortex and anterior olfactory nuclei.

Granule cells of the dentate gyrus are primarily generated postnatally. In the mouse and the rat, a small percentage of these cells is constantly generated in the adult (Gage, 1994; Gould et al., 1994; and references therein). Since *bcr* expression appears highest in these structures, we cannot entirely exclude the possibility that Bcr is directly or indirectly associated with cell proliferation. Regulatory molecules for small GTP-binding proteins have been increasingly implicated in cell growth and division over the recent years. The *DBL* gene, a G-nucleotide exchange factor, is an oncogene (Eva et al., 1985), and tissue directed expression of *DBL*-transgenes in animals causes specific metaplasias (Eva et al., 1991). *BCR* is implicated in human myeloid and lymphoid leukemias and a region on chromosome band 17p

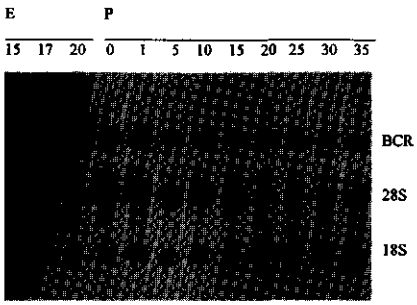

Figure 3. Autoradiogram showing a constant expression of brain bcr from embryonal day 15 (E15) to postnatal day 35 (P35). The bcr transcript of approximately 7 kb and the 28 and 18S ribosomal bands are indicated.

which contains the *BCR*-related *ABR* gene, appears to be deleted in particular cancers (Isomura et al., 1994; Morris et al., 1995; Saxena et al., 1992). Recently, a novel etiological mechanism was described for the development of malignant Schwannomas in von Recklinghausen neurofibromatosis: not $p21^{RAS}$ itself, of which mutated forms are frequently implicated in human neoplasias, but one of its regulators, *neurofibromin*, a GTP-ase activating protein (GAP) for RAS, appeared deactivated through loss of both functional *neurofibromin* alleles (Basu et al., 1992; Bollag and McCormick, 1992; DeClue et al., 1992). Loss of *neurofibromin* also contributes to the formation of malignant myeloid diseases in children (Shannon et al., 1992). The resulting constitutive activation of $p21^{RAS}$ in neurofibromatosis illustrates an obvious role for small GTP-binding proteins as well as their regulatory molecules (i.e. GAPs and GEFs) in processes that control cellular growth in the brain.

The C-terminal part of Bcr has a high degree of homology with that of n-chimerin, which, like Bcr, possesses GTP-ase activity toward $p21^{rac}$, a member of the RAS superfamily. The regional expression pattern of *bcr* in brain overlaps in part that of n-chimerin. *N-chimerin* expression is restricted to neurons, with the highest levels of expression in hippocampal pyramidal cells, granulae cells of the dentate gyrus, in cortical neurons, and in the Purkinje cells of the cerebellum (Lim et al., 1992). The developmental expression of *bcr*, however, displays different dynamics than that of *n-chimerin*: whereas *n-chimerin* expression rises sharply after birth to maximal levels at 20 days postpartum (Lim et al., 1992), a period coinciding with cellular differentiation and synaptogenesis, *bcr* expression remained constant from E15 through day 35 after birth. Northern blot analysis of hematopoietic organs (liver and spleen) also revealed a constant level of *bcr* expression from embryonal stage E15 throughout development until adulthood.

It is difficult to assess the significance of the relatively high level of *bcr* expression in the hippocampal region of the brain. Recently, n-chimerin expression in the canary forebrain was connected with seasonal changes in song learning and behavior; the conserved structure and pattern of n-chimerin expression in the song circuit suggested a fundamental function for this gene in the vertebrate forebrain, and a role in the regulation of neural plasticity (George and Clayton, 1992). In the young mouse brain, the developmental expression pattern of *n-chimerin* seems to disclose a potential role in development of particular cognitive functions (Lim et al., 1992). The finding that *bcr* expression is more or less constant throughout brain maturation would seem to point to a regulatory role of a more fundamental character in cellular processes.

In rodent brain, *bcr* mRNA in hippocampus is most abundant in dentate gyrus cells, lower in CA3 and lowest in CA1. The functional organization of hippocampus is such that input through the perforant path reaches granule cell layers. The granular cells, through axons (mossy fibers) synapse on CA3 neurons, which then impinge on CA1. The gradient of *bcr* expression levels along this functional neuronal path may reflect a potentially interesting, yet presently poorly understood function of Bcr in these highly specialized structures.

A growing number of GTPases and GAPs has been implicated in brain biology over the most recent years. Often these proteins (i.e. n-chimerin, α2-chimerin, Bcr, Abr) display a brain specific expression pattern (Hall et al., 1993; Leung et al., 1994; Lim et al., 1992;; Tan et al., 1993), or are, as described above, implicated in brain physiology (n-chimerin) or neoplasia (neurofibromin). Members of the extended family of GTPases have also been implicated in synaptic vesicle transport in nerve terminals (Liu et al., 1994; Von Gersdorff and Matthews, 1994), furthermore indicating the importance of such protein factors in fundamental brain related processes. Future investigations will undoubtedly identify additional regulatory molecules for G-proteins (i.e. GAPs and GEFs) that play key roles in brain biology.

Acknowledgements

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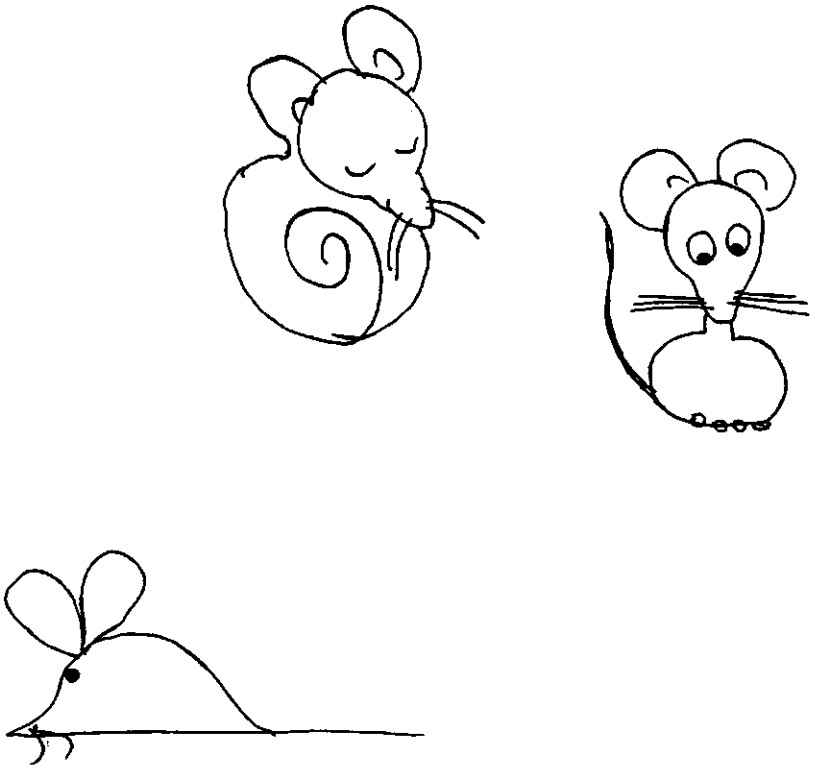

'Two mice and a ..... mouse'. By Nora and John (30-something)

CHAPTER 9

Discussion

• **Discussion**

In 1990, the group of Groffen and Heisterkamp reported on a transgenic mouse model for Philadelphia-positive acute lymphoblastic leukemia (ALL). The disease that developed in the *BCR/ABL* P190 transgenic mice resembled the human disease closely: most founder animals died of acute pre-B lymphoblastic leukemia.

In subsequent studies, the transgenic animal model for Ph^+ ALL was characterized in detail and used to examine processes that accompany tumor onset and progression (reviewed by: Groffen et al., 1992; Groffen et al., 1993).

Analysis for *BCR/ABL* expression in other tissues, than those of hematopoietic origin, demonstrated that the transgene was expressed in all tissues examined (Chapter 4). This observation is important in view of tumor etiology: since *BCR/ABL* expression affects bone marrow cells, it would seem, that in these cells, the appropriate factors (effector molecules, affector molecules) are present for mediating the effects of the Bcr/Abl protein. This view is consistent with the demonstrated critical role for the normal c-*abl* proto-oncogene in a restricted population of cells in the immune system (Tybulewic et al., 1991; Schwartzberg et al., 1991). Also, *bcr* gene ablation in mice affects cells of myeloid and lymphoid origin (Chapter 7). The combined results indicate that cellular factors and/or signal transduction pathways in cells of hematopoietic origin, that involve the parental molecules (Bcr and Abl), are quite possibly implemented in Bcr/Abl mediated leukemogenesis as well. Not only would this explain the restricted oncogenicity of Bcr/Abl, the observations would also explain why the *Ph*-chromosome, which one would expect to arise by chance in many proliferating tissues, has so far been found in blood cancers only.

Such a scenario would, however, question the relevance of *in vitro* experimentation for example in rodent fibroblasts or kidney cells. How can transformation by *BCR/ABL* occur in cell lines, which bear no apparent biological relevance to disease in human or mouse? A possible answer is found in studies, that scrutinized the establishment of *BCR/ABL* mediated growth factor independence in myeloid cells *in vitro*. It was speculated that, because additional mutations are required to *BCR/ABL* expression for abrogation of growth factor dependence *in vivo*, such cell lines may harbour co-operating mutations (such as for example the co-operation of *BCR/ABL* with *myc* in transformation of Rat-1 cells; Lugo and Witte, 1990) already (Karabinowski et al., 1994). Alternatively, overexpression of an activated kinase in Bcr/Abl could cause immediate (reversible) epigenetic alterations in these assay systems. Neither human Philadelphia-positive hematopoietic progenitors nor chronic phase CML cells loose their growth factor requirement and normal proliferative responses to growth factors (Moore et al., 1973; Goldman et al., 1974; Metcalf et al., 1974; Lansdorp et al., 1985). Also, analysis of our mouse model suggests, that over-expression of the *BCR/ABL* transgene is not a prerequisite for leukemic transformation (Chapters 3 and 2), indicating that the chosen *in vitro* assay conditions are not necessarily relevant to human leukemia.

Karyotypic analysis of leukemic bone marrow of a significant number of *BCR/ABL* P190 transgenic mice demonstrated that leukemic cells undergo a clonal development and karyotype evolution toward a more aggressive tumor: a high frequency of aneuploidy was found in advanced leukemia, as occurs in human leukemia (Sandberg, 1990; Williams et al., 1990), with a preference for gain of chromosomes 10, 12, 14 and 17 (Chapter 2). Tumor advancement toward a more aggressive genotype, through accumulation of multiple genetic mutations, was illustrated by serial leukemic bone marrow transplants, that revealed a gain of clinical malignancy of the cancer in irradiated recipient mice (Chapter 2).

It has been argued, that the initial development of *Ph*-positive leukemia in man is unrelated to the presence of the *Ph*-chromosome (i.e. *BCR/ABL*): primary *Ph*-negative clone(s) would have an increased susceptibility, of unknown nature, to acquire chromosomal abnormalities (Fialkow et al., 1967; Fialkow, 1977). The data obtained by analysis of karyotypic abnormalities and clonal evolution, as it develops in

leukemic clones in our *BCR/ABL* P190 transgenic mice, seem to argue against such scenario (Chapter 2). The expression of *BCR/ABL*, the consequence of the *Ph*-translocation, appears to be correlated with an increased risk of developing chromosomal abnormalities. Although it can be argued that this phenomenon is simply the consequence of expanded pool of B-cell precursors and would therefore be unrelated to *BCR/ABL* expression, this explanation could not readily account for the random extra chromosomes seen in some of the early leukemic mice which did not clearly exhibit a packed bone marrow. It was concluded, that in the *BCR/ABL* P190 transgenic mice, the presence of the *BCR/ABL* gene product is directly or indirectly responsible for the development of chromosomal abnormalities (Chapter 2).

This does, however, not imply that *BCR/ABL* on its own, without the contribution of any other gene is sufficient to cause leukemia. It is possible that *BCR/ABL* expression predisposes the cells to secondary events (e.g. additional gene mutations and/or chromosomal translocations). An ever increasing number of genes is being implicated in cancer, including, among other broad categories, tumor suppressor genes, genes for growth factors and their receptors, for intracellular signal transducing proteins, transcription factors, and for proteins affecting programmed cell death and cell cycle control. Any of these categories is likely to be affected by mutation which will allow the leukemia to gain in malignancy. In human CML, the t(9;22) somehow predisposes to further malignant progression of the leukemia in almost all patients. A large number of additional chromosomal abnormalities have been observed as CML progresses from the chronic phase to an accelerated or blastic phase (Sandberg, 1990). Karyotypically, additional non-random changes are found in some CML patients, but not in all. This could yield clues as to which additional genes are involved. Rearrangements of the p53 gene have been noted frequently during transition to blast crisis, as have been mutations in *RAS* or deletions of interferon genes (Ahuja et al., 1990; Marshal et al., 1990; Liu et al., 1988; Neubauer, 1990).

In view of the above, it is unlikely that the expression of the *BCR/ABL* oncogene is the only factor necessary to realize malignant leukemogenesis. Based on the analysis of our P190 transgenic mice, we hypothesize that *BCR/ABL* expression is the prime event initiating *Ph*-positive leukemia. Prolonged exposure to the biological action of Bcr/Abl deregulates the normal process of signal transduction and cell division. The resulting genetic instability sets the stage for accumulation of mutations, and malignant progression of the leukemia, would either be an indirect effect of this prime event, or the direct result of continued *BCR/ABL* expression (Chapter 2). Indeed, it has been postulated, that discordant maturation of cytoplasma and nucleus explains disruption of cell cycle regulation in *Ph*-positive CML (Clarkson and Strife, 1991): non-disjunction and aneuploidy would be a direct or indirect effect of *BCR/ABL* expression. Evidence that *BCR/ABL* upsets regulation of the cell cycle also comes from *in vitro* observations: *BCR/ABL* expression in (murine) myeloid cell line leads to an increase in the percentage of cells in the G2/M phase of the cell cycle (Laneuville, 1991).

In human P190 appears to be associated mainly with *Ph*-positive ALL, whereas P210 is found both in *Ph*-positive ALL and in CML (reviewed by: Kurzrock et al., 1988; Sawyers et al., 1991; Heisterkamp and Groffen, 1991). The longer latency time of P210 associated tumorigenesis, would seem to indicate an intrinsic variation in biochemical properties between the two distinct oncoproteins. This notion was corroborated by *in vitro* experiments with retroviral *BCR/ABL* P190 and P210 constructs: the transforming potential of *BCR/ABL* P190 was higher, a property which correlated with an intrinsically higher auto-phosphorylation activities in cultured fibroblast cell lines (Lugo et al., 1990). In transgenic mice, the P190 and P210 forms were found to cause clinically distinct conditions (Chapter 4). In human, about 50% of *Ph*-positive ALL cases carry the *BCR/ABL* P190 translocation, the other 50% the P210. Consequently, the issue whether Ph+ ALL is actually CML in blastic phase has been controversial for some time. Recently, however, clinical and molecular studies have established that indeed Ph+ ALL and acute phase

CML are two distinct diseases (reviewed by Berger, 1993). The finding, that *BCR/ABL* P190 and P210 cause distinct disease in the transgenic setting, supports this idea.

To date, all animals models for human *Ph*-positive leukemia, whether generated through retroviral (Elefanty et al., 1990; Daley et al. 1990; Kelliher et al., 1991) or transgenic technologies (Hariharan et al., 1989; Heisterkamp et al., 1990; Chapter 5), develop predominantly lymphoid malignancy of B and T type. All experimentally derived *BCR/ABL* lymphoid tumors were generated with regulatory sequences other than the *BCR* promoter. Although hematologic tumors in *Ph*-positive ALL are principally of the pre-B phenotype, B lymphoid involvement occurs only in about 25% of cases of human CML in blast crisis and T cell involvement is rarely seen in human. One explanation for this discrepancy could be that regulatory sequences within the *BCR* gene are important for the type of disease found in human and mouse. This concept contrasts with the postulate, that neither *BCR* sequences nor myeloid cell promoter elements are essential for myeloid tumorigenic transformation (Kelliher et al., 1990) and, that the *BCR* sequences that distinguish *BCR/ABL* P190 and *BCR/ABL* P210 do not control the selection of particular types of hematopoietic cells (Kelliher et al., 1991). Recently, the Bcr function was connected with the regulation of superoxide production by the NADPH-oxidase system of leukocytes *in vivo* (Chapter 7). Interestingly, *bcr* gene ablation affects specifically those cell types in mice affected by *BCR/ABL* in man and mouse. It seems reasonable to conclude from our data that a correlation exists between the cell type affected in *Ph*-positive leukemia and Bcr function.

Although data concerning the normal cellular function of Bcr are accumulating, the precise role(s) of this gene in normal cell physiology remains unclear. In the mouse, *bcr* expression is detectable as early as at the zygote stage, continues throughout embryogenesis (unpublished observations) and is found in most adult tissues examined. In man, *BCR* has also been shown to be ubiquitously expressed (Heisterkamp et al., 1993b). Notwithstanding its function in hematopoietic cells, the Bcr protein is found in high levels in brain. This observation has intrigued us as it suggests a possible different function for $p160^{Bcr}$. This notion is corroborated by the significant function of G-proteins in brain (Liu et al., 1994; Von Gersdorff and Matthews, 1994).

In the mouse and the rat, a small percentage of granule cells of the dentate gyrus is constantly generated in the adult animal (Gould et al., 1994; Gage et al., 1994 and references therein). Since *bcr* expression appears highest in these structures, the possibility that Bcr is directly or indirectly associated with cell proliferation cannot entirely be excluded. The involvement of *DBL* gene, a G-nucleotide exchange factor (GEF), in oncogenesis (Eva et al., 1985), and the implication of *RAS* and neurofibromin, a GTPase activating protein (GAP) for *RAS*, in malignant neurofibromatosis (Bollag and McCormick, 1992; DeClue et al., 1992; Basu et al., 1992) and myeloid disease in children (Shannon et al., 1992) illustrate a role for small GTP-binding proteins, as well as their regulatory molecules (i.e. GAPs and GEFs) in processes that control cellular growth, among other tissues, in the brain.

In mouse brain, the expression pattern of *bcr* shows some overlap with that of the biochemically related n-chimerin gene (Chapter 8). The kinetics of *bcr* expression are, however, different from n-chimerin, of which expression peaks at around 2 weeks of age; *bcr* mRNA levels are constant throughout late development and the first weeks after birth. N-chimerin may be involved in development of particular cognitive functions in the young mouse brain (Lim et al., 1992). Bcr is highly expressed in the hippocampal pyramidal cell layer and the dentate gyrus, and at a lower level in the piriform cortex and the anterior olfactory nuclei. The gradual change in expression levels along these structures, may reflect a potentially interesting function for Bcr in these highly specialized areas of the brain (Chapter 8).

• **Future directions**

The study of Philadelphia-positive leukemia is ultimately aimed at formulating successful treatment protocols to fight the disease. The availability of a mouse model for Ph^+ ALL will have a positive impact on the accuracy and the pace at which new anti-cancer drugs can be tested. Studies in this direction have been undertaken already: we have tested the effectiveness of interferon-α (IFN-α) in the transgenic setting (Chapter 6), unfortunately without promising results. IFN-α was shown an effective drug in the treatment of human CML (Kantarjian et al., 1993); recently a similar strategy was tried in *Ph*-positive ALL patients (Haas et al., 1988; Ohyashiki et al., 1991) some promising results were obtained in ALL patients with an M-*bcr* breakpoint. Treatment of *BCR/ABL* P190 bone marrow recipient mice with dihydroepiandosteron (DHEA) was shown to have a adverse effect on tumor progression and prolong animal survival (Catalina et al., 1995). DHEA is an adrenal steroid hormone, which, among other preventative actions, inhibits development of cancer, atherosclerosis, autoimmune disease and viral infections. Future investigations will, besides an evaluation of (yet to be discovered) anti-cancer drugs in their effectiveness against leukemia, center around the development of drugs specifically designed to inhibit *BCR/ABL* expression (e.g. hammerhead ribozymes, antisense constructs) or prevent biological functioning of the oncogene product (e.g. synthetically designed zincfinger proteins; *trans*-inhibitory factors). Collaborations in this area are well underway at this time.

At a more fundamental level, it has now become possible to investigate the nature of cytoplasmic and nuclear factors, and signal transduction pathways, that contribute to the development and malignant progression of Ph^+ leukemia in our transgenic mice. The etiological significance of cellular factors in leukemogenesis, that interact with either the *BCR/ABL* oncoproteins and/or with either one of both parental proteins, can now be analyzed. A transgenic setting is particularly well suited for the *in vivo* evaluation of mechanistic hypotheses which were based on *in vitro* observations. For example, mechanistic models that involve interaction with critical amino acid residues in Bcr/Abl oncoproteins, can be evaluated in *vivo* by using mutated Bcr/Abl genes in a transgenic setting.

Although animal models have provided strong evidence linking the development of leukemia specifically to the presence of a chimaeric *BCR/ABL* gene, attempts to generate a dependable experimental *in vivo* model system to study human CML have been largely unsuccessful to date. The need for a reliable animal model to study CML is still clear. Future research will focus on using endogenous *bcr* regulatory sequences in combination with *BCR/ABL* transgenes in mice. The design of *BCR/ABL* P210 transgenes will include repressible or inducible regulatory elements. Future experimental strategies may combine transgenic and embryonic stem cell technology.

CHAPTER 10

Summary

Summary

Human Philadelphia-positive leukemia results from a balanced chromosomal translocation, which fuses the *BCR* gene on chromosome 22 to the *ABL* proto-oncogene on chromosome 9. The understanding of *Ph*-positive leukemogenesis has advanced enormously over the last few decades. Although *in vitro* assay systems currently used, are not always relevant to human tumor biology, much can and has been learned from studies, employing cell cultures and overexpression of *BCR/ABL* oncogenes.

Another restriction in leukemia research is the availability of primary human tumor material for study. Moreover, such tissues often represent terminally advanced stages of tumorigenesis. Therefore, the importance of *in vivo* models to study Philadelphia-positive leukemia is manifold. A well defined transgenic mouse model allows for tumorigenesis to be studied from its earliest stages onward and factors and mechanisms that eventually contribute to malignant progression of the leukemic cells can be uncovered. Besides an 'unlimited' provision of tumor material for analysis, more importantly, the availability of a transgenic mouse model provides a means by which cancer treatment regimes can be tested. In addition, identification of cellular components and/or pathways that contribute to the onset or progression of leukemia may eventually lead to the discovery and development of new drugs.

In 1990, Heisterkamp and co-workers reported on a transgenic mouse model for Philadelphia-positive acute lymphoblastic leukemia (ALL). Since most of the transgenic animals of an earlier study had succumbed to leukemia, part of the aim of this thesis was to generate *BCR/ABL* P190 transgenic founder animals *de novo* and to derive a transgenic animal line(s) which was to be used for future studies. In order to better understand the animal model, leukemogenesis was studied in great detail in transgenic founder animals and their progeny. In the second chapter a cytogenetic study of the mouse model for acute lymphoblastic leukemia is presented. Karyotypic analysis of leukemic bone marrow of a significant number of mice shows, that leukemic cells undergo a clonal development and karyotype evolution toward a more aggressive tumor: a high frequency of aneuploidy is found in advanced leukemia, as occurs in human leukemia, with a preference for gain of chromosomes 10, 12, 14 and 17. These findings are corroborated by experiments that reveal a gain of malignancy of the cancer upon serial transplantation of leukemic bone marrow to irradiated recipient mice and by molecular analysis of lymphomas using immunoglobulin rearrangement as an indicator for tumor clonality. The results suggest that *BCR/ABL* has a destabilizing effect on the regulation of the proces of mitosis.

In the third chapter, a correlation is described between the transcriptional status of the *BCR/ABL* P190 transgene and the development of leukemia: methylation of particular sequences in *BCR* exon-1 in the transgene is closely coupled to transgene inactivation, providing additional evidence for a direct role of *BCR/ABL* in leukemogenesis. A biological dissection of the oncogenic specificity of *BCR/ABL* is presented in the fourth chapter. Using sensitive molecular biological techniques, it is shown that, although expression of the *BCR/ABL* transgene is detectable in every tissue, from very early on in mouse development, no other neoplasias than of hematopoietic origin are found. The results strongly suggest that the oncogenicity of *BCR/ABL* is restricted to nucleated blood cells, which is very likely a reflection of cellular functions of the *BCR* and or *ABL* gene in signal transduction specific to hematopoietic lineages. The observations would also explain why the *Ph*-chromosome, which one would expect to arise by chance in many proliferating tissues, is found only in blood cancers.

An analysis of transgenic mouse models for chronic myelogenous leukemia, using *BCR/ABL* P210 transgenes is presented in the fifth chapter. The clinical disease spectrum includes differentiated and

undifferentiated T and B cell leukemias. The myeloid compartment is implicated only sporadically and rather late in the disease process. In some instances, the observed myelo-proliferation is a sequel to deregulation of cytokine production at advanced stages of leukemia. The course of P210 induced leukemia was acute rather than chronic, be it with an on average longer latency period than typical for ALL in *BCR/ABL* P190 mice. From these studies is was concluded that in the mouse, *BCR/ABL* P210 evokes a clinically different disease than *BCR/ABL* P190. Although no evidence for a chronic myeloproliferative disorder in the peripheral blood was found, an imbalance in myelopoiesis in the bone marrow suggests an effect of *BCR/ABL* P210 on primitive myeloid progenitors.

The sixth chapter summarizes an analysis of interferon-α (IFN-α) treatment of the *BCR/ABL* P190 transgenic mice. IFN-α is currently one of the most effective drugs in the treatment of CML. Recently, IFN-α was tried in the treatment of ALL. No effect of IFN-α on animal survival or disease pattern were noted when administered to the *BCR/ABL* P190 mice. The conclusion was reached that, at least in a transgenic setting, IFN-α does not interfere with *BCR/ABL* P190 mediated leukemia.

In order to study the normal cellular function of the *BCR* gene and to eventually assess its role in leukemogenesis, studies focussing on the mouse *bcr* gene function are presented in chapters 7 and 8. The seventh chapter describes the ablation of a functional mouse *bcr* gene by means of recently developed gene targeting techniques. One of two mouse *bcr* alleles was inactivated in a mouse embryonic stem cell line through gene interruption by insertional replacement vectors. This genetically altered cell line was then injected into developing mouse embryos. Through germline transmission of the mutated allele and subsequent breeding both *bcr* alleles were inactivated. Although *bcr*-null mutants are phenotypically normal, their neutrophils display impaired regulation of respiratory burst, which becomes apparent when these cells are activated *in vivo*: an overproduction of superoxide leads to significantly more oxidative tissue damage during experimental endotoxemia. The results connect Bcr *in vivo* with the regulation of superoxide production by the NADPH-oxidase system of leukocytes and suggest a link between the cell types affected by loss of Bcr function and the those involved in *Ph*-positive leukemia.

Additional information on biological processes that Bcr participates in, are described in the eighths chapter. Notwithstanding its function in hematopoietic cells, the Bcr protein is normally found in high levels in brain. The expression pattern of *bcr* in rodent brain was examined by means of *in situ* hybridization and Northern analysis. Although not directly connected with leukemogenesis, a potentially interesting role for $p160^{Bcr}$ in the brain is discussed as its expression pattern appears to coincide with the functional organization of particularly highly specialized structures in the brain.

With the availability of well defined transgenic mouse models for *BCR/ABL* positive leukemia, an opportunity is created to study the nature of cellular interactions and processes that contribute to the onset and development of *Ph*-positive leukemia. Ultimately, such investigations are aimed at designing and testing effective therapeutic drugs to fight the disease.

Samenvatting

Philadelphia-positieve leukemie in de mens ontstaat als gevolg van een translocatie tussen de chromosomen 22 en 9, waarbij het *BCR* gen en het *ABL* proto-oncogen gefuseerd worden. De kennis omtrent de etiologie van deze leukemie is de laatste jaren sterk toegenomen, mede door de informatie die uit *in vitro* experimenten met cellijnen en gezuiverde eiwitten is verkregen. Resultaten uit deze experimenten mogen echter niet zonder meer naar de *in vivo* situatie worden geëxtrapoleerd.

Een andere duidelijke belemmering bij het bestuderen van leukemie is de beschikbaarheid van patientenmateriaal: indien al beschikbaar, representeert dergelijk primair tumorweefsel vaak een terminaal stadium van de kanker. Het belang van betrouwbare diermodellen voor de studie van Philadelphia-positieve leukemie is daarom meervoudig. Zo wordt met een goed gedefinieerd transgeen muizemodel voorzien in een bron van studiemateriaal voor het bestuderen van tumorigenese vanaf de beginstadia en de ontrafeling van processen, die uiteindelijk aan kwaadaardige progressie van leukemie bijdragen. Naast een ongelimiteerde voorraad aan tumormateriaal voor analyse, biedt een muizemodel bovendien de mogelijkheid om kankertherapieën te testen. Daarbij kan de identificatie van cellulaire factoren en processen, die een rol spelen bij onstaan en progressie van leukemie, leiden tot de ontdekking en ontwikkeling van nieuwe medicijnen.

In 1990 werd door Heisterkamp en medewerkers gerapporteerd over een transgeen muizemodel voor Philadelphia-positive acute lymfoblastisch leukemie (ALL). Omdat de meeste transgene dieren in deze studie waren gestorven aan leukemie, was een gedeelte van het in dit proefschrift beschreven onderzoek gewijd aan het maken van nieuwe transgene muizen en het opzetten van muizelijnen voor verdere studie. Om een beter begrip te krijgen van de diermodellen is het proces van ontwikkeling van leukemie in de transgene muizen uitgebreid bestudeerd. In het tweede hoofdstuk wordt een cytogenetische studie van het diermodel voor acute lymfoblastische leukemie (ALL) gepresenteerd. Karyotypische analyse van leukemisch beenmerg van een groot aantal muizen laat zien, dat de leukemie een proces van klonale ontwikkeling en karyotypische evolutie doorloopt naar een meer agressieve kanker: een hoge mate van aneuploïdie kan aangetoond worden in ver gevorderde stadia, met, net als in de mens, een voorkeur voor verdubbeling van bepaalde chromosomen. Zowel een toename in kwaadaardigheid van de kanker na seriële transplantatie naar bestraalde muizen, als een tumor-klonaliteitsanalyse onderstrepen deze waarneming. De resultaten laten zien, dat het Bcr/Abl eiwit destabiliserend werkt op de normale cel- en kerndeling.

In het derde hoofdstuk wordt een verband tussen de transcriptionele status van het *BCR/ABL* P190 transgen en de ontwikkeling van leukemie in een aantal verschillende muizelijnen gelegd: methylering van sequenties in *BCR* exon 1 in het transgen is gekoppeld aan transgen-inactivatie. Deze waarneming benadrukt nogmaals de directe rol van *BCR/ABL* in het ontstaan van leukemie.

Een biologische ontrafeling van de oncogene specificiteit van *BCR/ABL* wordt gepresenteerd in hoofdstuk drie. Gebruikmakend van gevoelige moleculair biologische technieken wordt aangetoond, dat geen andere kanker dan bloedkanker gevonden wordt, ofschoon het transgen in elk weefsel tot expressie komt vanaf vroeg in de embryonale ontwikkeling van de muis. De resultaten geven aan, dat oncogeniciteit van *BCR/ABL* beperkt is tot witte bloedcellen, hetgeen zeer waarschijnlijk de cellulaire functie(s) van het Bcr en/of Abl eiwit in signaaloverdrachts processen, die specifiek zijn voor hematopoietische cellen, weerspiegelt. Deze waarneming biedt mogelijkerwijs ook een verklaring voor het uitsluitend aantreffen van het Philadelphia chromosoom in bloedkanker, terwijl verwacht mag worden, dat het bij toeval ook in andere weefsels zou kunnen ontstaan.

Een studie aan een transgeen muismodel voor chronische myelogene leukemie (CML), dat gebruik maakt van *BCR/ABL* P210 transgenen, wordt gepresenteerd in hoofdstuk vijf. In de muizen worden vooral lymfoide bloedkankers van gedifferentieerde en ongedifferentieerde T en B-origine gevonden. Daarentegen wordt een bijdrage van myelogene cellijnen aan deze kankers slechts sporadisch aangetroffen en dan bovendien vrij laat in het ziekteproces. In bepaalde gevallen kan de myelogene celexpansie verklaard worden door een ontregelde cytokine-productie in het zieke dier. Het verloop van het ziektebeeld in *BCR/ABL* P210 muizen is acuut, zij het met een langere latentietijd dan voor ALL in *BCR/ABL* P190 muizen. Vergelijking van *BCR/ABL* P190 and *BCR/ABL* P210 muizemodellen leert, dat leukemie in deze muizen via klinisch verschillende patronen verloopt. Ofschoon geen chronisch ziektebeeld in het bloed aangetoond kan worden, suggereren voorlopige resultaten wel een effect van *BCR/ABL* P210 op primitieve myeloide precursor cellen in het beenmerg-compartiment van transgene muizen.

Het zesde hoofdstuk is gewijd aan de effectiviteit van interferon-α (IFN-α) behandeling van *BCR/ABL* P190 transgene muizen. IFN-α is momenteel een van de meest effectieve medicijnen in de behandeling van CML. Recentelijk heeft men IFN-α ook toegepast in de bestrijding van ALL. In *BCR/ABL* P190 muizen werd geen effect op overleving en/of verloop van het ziektebeeld waargenomen na IFN-α behandeling. Hieruit is afgeleid dat, althans in het transgene muizemodel, IFN-α geen effect heeft op *BCR/ABL* gerelateerde leukemie.

Om de normale cellulaire functie van het *BCR* gen en de rol, die het speelt in leukemie, beter te kunnen begrijpen, worden in hoofdstuk zeven en acht een aantal studies beschreven, die specifiek het muize-*bcr* gen betreffen. Hoofdstuk zeven beschrijft de functionele uitschakeling van dit gen door middel van gen-onderbreking in embryonale stamcellen. Deze genetisch gemanipuleerde cellen zijn vervolgens geïnjekteerd in zich ontwikkelende muize-embryo's. Door middel van onderlinge kruising van heterozygote muizen zijn vervolgens beide *bcr* allelen uitgeschakeld. Hoewel deze *bcr* nulmutanten fenotypisch normaal zijn, vertonen hun neutrofiele granulocyten een verhoogde superoxide productie. Dit is gemeten na *in vivo* activering van deze cellen: onder experimenteel septische condities wordt significant meer oxidatieve weefselschade in *bcr* nulmutanten gevonden. De resultaten wijzen op een regulerende functie voor het Bcr eiwit in de superoxideproductie door het NADPH-oxidase systeem in leukocyten. De celtypen, waarvan de functie verandert door verlies van Bcr, zijn dezelfde celtypen, die betrokken zijn in *Ph*-positieve leukemie, hetgeen het veronderstelde verband tussen de normale cellulaire *bcr* functie en het onstaan van leukemie benadrukt.

Van alle onderzochte weefsels komt *bcr* het hoogst tot expressie in de hersenen. Om andere biologische processen, waarin Bcr mogelijk een rol speelt, op te helderen, wordt het expressiepatroon van *bcr* in muizehersenen beschreven in hoofdstuk acht. Hiervoor zijn *in situ* hybridisatie technieken en Northern analyse gebruikt. Ofschoon niet direct relevant voor het onstaan van leukemie, wordt een potentieel interessante rol voor het Bcr eiwit in de hersenen besproken: het expressiepatroon van *bcr* overlapt de functionele organisatie van sterk gespecialiseerde structuren in de hersenen.

Met de beschikbaarheid van goed gedefinieerde transgene diermodellen voor *BCR/ABL*-positieve leukemie, is de mogelijkheid gecreëerd om cellulaire eiwitten en processen, die bijdragen aan het onstaan en de ontwikkeling van Philadelphia-positieve leukemie in het dier, te bestuderen. Uiteindelijk is dit onderzoek gericht op het ontwikkelen en testen van effectieve medicijnen tegen deze vormen van leukemie in de mens.

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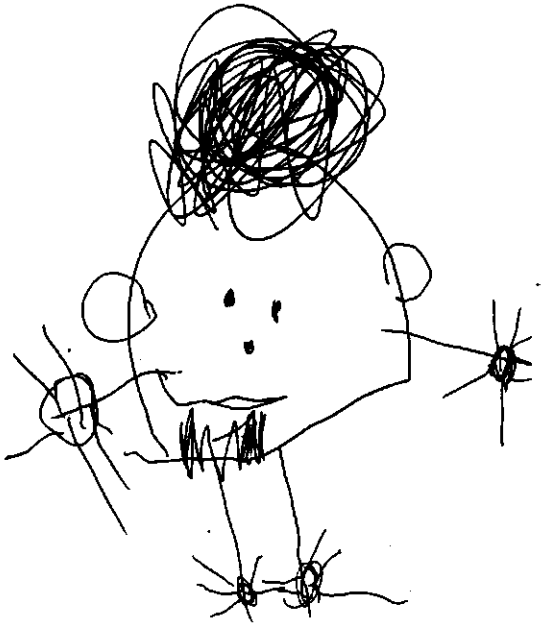

The author. By Paula (4)

Epilogue

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Los Angeles, May 1995

Curriculum Vitae

Jan Willem Voncken was born in Maastricht, 5 May 1964. He grew up in the village of Ulestraten in the southern parts of the Netherlands, where he attended elementary school and roamed the fields and forests on early sunday mornings, exploring nature with his father, Wim Voncken. He completed VWO at the St.-Maartens College in Maastricht in 1982 and went on to study molecular biology at the Wageningen Agricultural University. During his studies, he joined Dr. Theodoor Postmes, a former biology teacher of his, at the St.-Annadal Academic Hospital in Maastricht, in a research project concerning non-enzymatic glycosylation of collagen in relation to the onset and development of vascular complications in Diabetis Mellitus. It was Dr. Postmes' infectious enthusiasm for molecular biology, already while attending the St.-Maartens College, which guided Jan Willem in his resolution to continue in this field. Back in Wageningen, two additional multi-disciplinary graduation subjects were completed. A combined biochemical and toxicological study on the biotransformation of hexachlorobenzene by the liver, was conducted under the direct supervision of Dr. B. van Ommen at the departments of Biochemistry (Prof. Dr. F. Müller) and Toxicology (Prof. Dr. J.H. Koeman). His main graduation subject was conducted at the departments of Virology (Prof. Dr. R.W. Goldbach) and Process Technology (Prof. Dr. J. Tramper). With Dr. J.M. Vlak at the department of Virology as his principal tutor, he studied the long-term applicability of a baculovirus-infected continuous insect cell culture system to produce exogenous gene products. Jan Willem has always experienced the academic and scientific environment in Wageningen as very stimulating, and looks back on his education with no other than positive feelings. He completed his doctoral studies at the Wageningen Agricultural University cum laude in November 1989.

Following a practical training period in Southern California, he joined the department of Pathology Research and Laboratory Medicine at the Childrens Hospital of Los Angeles/University of Southern California in 1989. Under academic supervision of Prof. Dr. J. Groffen and Prof. Dr. N. Heisterkamp at the section of Molecular Carcinogenesis, he partook in the development of mouse models for the study of Philadelphia-positive leukemia. The research carried out at Childrens Hospital of Los Angeles is described in this dissertation. Jan Willem is currently employed by Childrens Hospital of Los Angeles, and intends to continue in the fields of oncology and developmental biology.

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Van Ommen B., Voncken J.W., Mueller F. and van Bladeren P.J.: The oxidation of tetrachloro-1,4-hydroquinone by microsomes and purified cytochrome P-450b. Implications for covalent binding to protein and involvement of reactive oxygen species. *Chem.Biol.Interactions* **65**, iss. 3 (1988): p.247-259.

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