NOVEL TRANSFORMING GENES IN MURINE MYELOID LEUKEMIA

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NOVEL TRANSFORMING GENES IN MURINE MYELOID LEUKEMIA

Nieuwe transformerende genen in myeloide muizenleukemie

PROEFSCHRIFT

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Anne-Marie Sophie Joosten

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Copromotor:	Dr. H.R. Delwel

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Voor Tim

Contents

Chapter 1

Introduction

I. Normal hematopoiesis

Blood cell formation in adult mammalians takes place in the bone marrow. The parental cells in the bone marrow responsible for the blood cell formation are the so-called hematopoietic stem cells. These primitive precursor cells are capable of self-renewal but may also differentiate into the distinct committed hematopoietic progenitor cells [1-3], which may finally develop into the individual blood cell types, e.g. erythrocytes, neutrophilic granulocytes, monocytes, platelets, or B- and T-lymphocytes [1, 4, 5] (Figure 1). There is considerable amplification in the system: one stem cell may be capable of producing 10^6 mature blood cells after 20 cell divisions. As the cells differentiate they lose adhesion to the bone marrow stroma and enter the blood circulation.
Stem and progenitor cell differentiation depends largely on hematopoietic growth factors (HGF) (Figure 1). HGFs regulate proliferation, differentiation and survival of progenitor cells, and may also influence the function of mature blood cells [6]. HGFs sometimes act locally at the site where they are produced, but they may also circulate in the plasma. The major sources for HGFs are T-lymphocytes, monocytes, endothelial cells, and fibroblasts [7]. The HGF erythropoietin (EPO) is synthesised in the kidney [8], whereas thrombopoietin (TPO) is mainly produced in the liver [9, 10]. The biological effects of HGFs are mediated through specific receptors on target cells. Upon binding of a HGF to its specific receptor, a cascade of signals is transmitted through the cytoplasm to the nucleus resulting in activation of DNA synthesis and regulation of transcription and translation of genes that are critical in development. The existence of primitive multipotential precursors and separate committed progenitor cells has been demonstrated *in vitro* by using semi-solidified colony culture techniques. The type of precursor that is grown in these cultures mainly depends on the growth factors added. HGFs that may stimulate multilineage growth of primitive murine bone marrow progenitor cells, either alone or in combination with other cytokines are e.g. TPO, Stem cell factor (SCF) and Interleukin 3 (IL-3). Primitive stem cells may develop into precursor cells that are committed to a particular differentiation lineage. Colonies containing neutrophils develop in cultures supplemented with granulocyte-colony stimulating factor (G-CSF). Macrophage colonies develop in the presence of macrophage-CSF (M-CSF), whereas erythroid precursors differentiate upon EPO stimulation (Figure 1).

II. Leukemia

Errors may occur in the process of hematopoietic development, as the result of mutations in the DNA of hematopoietic precursor cells, causing inappropriate expression of genes critical in blood cell development. These genetic lesions may lead to overactivation of genes (proto-oncogenes), inactivation of genes (tumour suppressor genes) or expression of aberrant gene products (e.g. fusion proteins). The consequences of these defects may be multiple, e.g. increased rate of proliferation, alterations in cell survival or a block in differentiation of particular hematopoietic lineages. The combination of different genetic lesions in a specific hematopoietic progenitor may ultimately result in the development of leukemia. Leukemia is characterised by an accumulation in the bone marrow of non-functional blood cells arrested at a particular stage of differentiation.

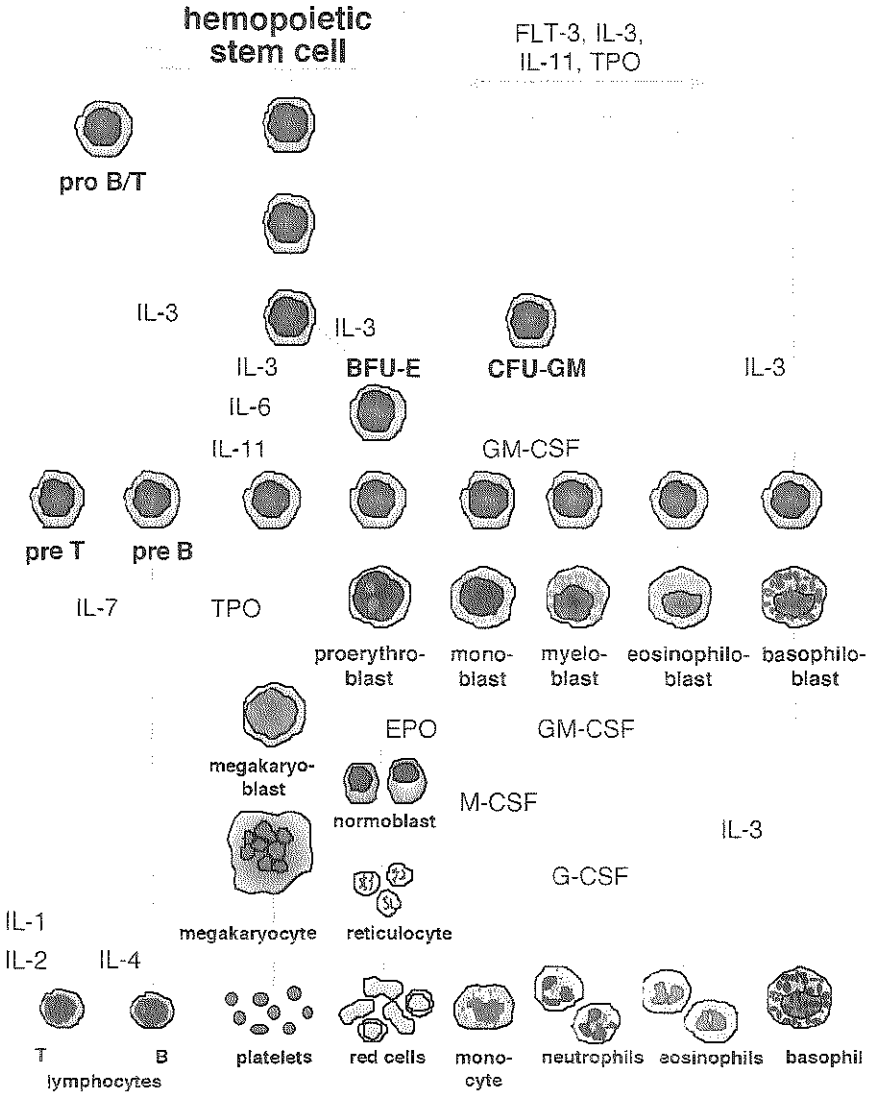

Figure 1. Hematopoiesis.
After a number of cell divisions and differentiation steps a pluripotent stem cell gives rise to a common lymphoid stem cell, as well as to a series of progenitor cells for three main marrow cell lines: (a) erythroid, (b) granulocytic and monocytic, and (c) megakaryocytic. These processes are mediated by hematopoietic growth factors (IL = interleukin; TPO = thrombopoietin; GM-CSF = granulocyte/macrophage-colony stimulating factor; EPO = erythropoietin; M-CSF = macrophage-colony stimulating factor; G-CSF = granulocyte-colony stimulating factor). *In vitro* these progenitor cells can be identified by in semi-solid culture medium by the type of colony they form. (BFU_E = burst-forming unit, erythroid; CFU = colony forming unit; GM = granulocyte-monocyte).

IIa. Leukemia: classification by morphology

Chronic as well as acute leukemias exist, which are both subdivided into myeloid or lymphoid forms. In acute myeloid leukemia (AML), primitive myeloblasts pile up in the marrow, whereas in acute lymphoblastic leukemias (ALL), immature B-lymphoid (B-ALL) or T-lymphoid cells (T-ALL) accumulate in the hematopoietic system. Among the chronic leukemias we recognise various types, e.g. chronic myeloid leukemia (CML), chronic lymphocytic leukemia (CLL), hairy cell leukemia, prolymphocytic leukemia and various leukemia/lymphoma syndromes.

Myeloid leukemias can be distinguished from lymphoid disease by morphologic analysis, immunophenotyping, cytogenetics and/or molecular diagnosis (see next paragraph). Acute myeloid leukemias may be classified according to the varying degrees of granulocytic, monocytic, erythroid or megakaryocytic differentiation of the leukemia cells. AML is subdivided into 8 variants based on morphology and cytochemistry (FAB-classification, M_0-M_7) [11, 12].

Patients with CML present with a high amount of granulocytic cells in marrow and blood. In the majority of CML patients this chronic phase will be followed by an acute phase with high numbers of primitive myeloid cells in blood and marrow.

Another group of diseases that frequently develop into AML is formed by the myelodysplastic syndromes (MDS). MDS is commonly found in the elderly and characterised by bone marrow failure. Most cases occur *de novo*, but an increasing number arises because of previously given chemo-or radiotherapy. CML as well as MDS are examples of myeloid diseases that following variable time intervals may transform into acute leukemia indicating that leukemia may be a multigenetic disease and that at least two events are required to obtain full AML. Development towards AML may also occur in patients suffering from particular hematopoietic disorders, such as neutropenia with underlying G-CSF-receptor mutations [13], Diamond-Blackfan anemia [14, 15], or the autosomal recessive disorder Fanconi Anemia (FA) [16]. Patients are at high risk to developing myeloid leukemia, again suggesting that multiple genetic defects in those patients give rise to clinically apparent leukemia.

The identification of the genetic lesions that are responsible for dysregulation of hematopoiesis in humans and in animal models has been one of the main subjects in leukemia-research in the past two decades and forms the central theme of this thesis. For this purpose a large panel of retrovirally induced leukemias was generated, which were classified using morphological and immunological analyses (Chapter 2). The ultimate goal of these studies was the identification and cloning of novel disease genes (Chapters 3 and 4).

IIb. Cytogenetics and molecular analysis of disease genes

Leukemia is the result of disordered expression of genes leading to imbalanced proliferation, differentiation, and/or survival of hematopoietic precursor cells. Certain defects may result in abnormal expression of a particular gene (proto-oncogenes), whereas due to bi-allelic inactivation so-called tumour suppressor genes may be turned down. Another class of cancer susceptibility genes consists of DNA repair genes, which are involved in maintaining genomic stability [17-20].

A number of aberrant gene products in human leukemias have been identified and isolated by chromosomal breakpoint cloning. Leukemias are frequently characterised by the appearance of non-random chromosomal translocations. In translocations, part of one chromosome is aberrantly fused to another chromosome, often resulting in the

generation of a fusion-gene and the subsequent expression of an aberrant fusion-transcript and -protein. Cytogenetic abnormalities provide useful parameters for the distinction of leukemias with distinct prognosis. Patients carrying translocations t(15;17), t(8;21) or inv(16)/del(16) are among the so-called good-risk AML cases, whereas AML cases with a abnormality involving chromosome 3q26, 11q, 17p, 20q, 21q, or with a translocation t(6;9) or t(9;22) represent the bad-risk group [21-23]. Among the group of bad-risk AML cases are also the ones with monosomy 7 or a 7q-, monosomy 5 or a 5q-, and patients with a complex karyotype (more than three cytogenetic abnormalities in a leukemic cell) [21, 24]. Genes that may be aberrantly expressed in these latter leukemias are currently unknown. AML cases with cytogenetic abnormalities other than the ones described so far (e.g. +8, -Y, +6, del(12p)) or patients without any apparent cytogenetic abnormality have an intermediate-risk [22, 23, 25]. The most frequent chromosomal abnormalities in AML and the genes involved have been summarised in Table 1. Besides the cytogenetic abnormalities that frequently result in the expression of specific breakpoint related fusion genes, a number of more subtle genetic alterations have been identified. Among these are point mutations, deletions or insertions in various genes that may be critical in hematopoietic development e.g. *AML1*, *c/EBPα*, *p53* or *RAS* [26-29].

Table 1: Translocations in AML.

translocation	genes involved	leukemia subtype	fusion protein	reference
t(8;21)(q22;q22)	*AML1/ETO*	AML-M_2	AML1-ETO	[30]
t(16;21)(q24;q22)	*AML1/MTG16*	AML	AML1-MTG16	[31]
t(3;21)(q26;q22)	*AML1/MDS1*	AML	AML1-MDS1	[32]
	AML1/EVI1		AML1-EVI1	[33]
	AML1/EAP		AML1-EAP	[34]
t(3;3) (q21;q26)	*EVI1/Riboforin*	AML/MDS	EVI1	[35, 36]
inv(3)(q21;q26)	*EVI1/Riboforin*	AML/MDS	EVI1	[35, 36]
t(3;12)(p13;q26)	*EVI1/TEL*	AML	ETV6-EVI1	[37]
t(1;3)(p36;q21)	*MEL/Riboforin*	AML	MEL1	[38]
inv(16)(p13;q22)	*CBFβ/MYH11*	AML-M_{4eo}	CBFβ-MYH11	[39]
t(15;17)(q22;q21)	*PML/RARα*	AML-M_3	PML-RARα	[40]
t(11;17)(q23;q21)	*PLZF/RARα*	AML-M_3	PLZF-RARα	[41]
t(5;17)(q35;q21)	*NPM/RARα*	AML-M_3	NPM-RARα	[42]
t(4;11)(q21;q23)	*AF4/MLL*	mixed lineage	MLL-AF4	[43]
t(9;11)(p22;q23)	*MLLT3/MLL*	AML-$M_{4,5}$	MLL-AF9	[44]
t(10;11)(p12;q23)	*MLLT10/MLL*	AML-$M_{4,5}$	MLL-AF10	[45]
t(11;15)(q23;q14)	*MLL/AF15*	AML-$M_{4,5}$	MLL-AF15	[46]
t(7;11)(p15;p15)	*HOXA9/NUP98*	AML	NUP98-HOXA9	[47]
t(16;21)(p11;q22)	*FUS/ERG*	AML	FUS-ERG	[48]
t(6;9)(p23;q34)	*DEK/CAN*	AML	DEK-CAN	[49]
t(9;22)(q34;q11)	*ABL/BCR*	AML	BCR-ABL	[50]

It is of interest that many of the genetic aberrations in leukemia involve genes that normally encode transcriptional regulators, e.g. *AML1*, *CBFβ*, *RARα*, *MLL*, *EVI1*, or *c/EBPα*. Transcription factors play a critical role in the initiation of transcription and selectively attract RNA polymerase molecules to specific promoters [6]. In most of the translocations the mutated counterpart of these genes will function as a repressor, thereby interfering with the normal function of the non-mutated gene-products [51]. This interference may cause abnormal expression of downstream target genes of these transcription regulators ultimately influencing development of the hematopoietic cells expressing these mutated transcription regulators. Therefore, it is not only of interest which genes may be mutated in leukemia, but also which down-stream target genes are affected in leukemias carrying one of the frequently occurring cytogenetic abnormalities.

The identification of novel transforming genes as described in the studies presented in this thesis may serve several goals. Novel disease genes identified using our leukemia models may represent 1) breakpoint genes not previously identified, 2) disease genes that may be abnormally expressed in human leukemia as the result of mutations other than chromosomal translocations, 3) genes that are downstream of any of the abnormally expressed genes in AML or 4) genetic lesions that are specific for virus induced mouse leukemias.

III. Identification of disease genes by means of retroviral insertional mutagenesis

Since leukemia is believed to be a multigenetic disease, abnormal expression of a particular gene is assumed to be one event among multiple defective genes within a certain leukemia. Therefore, even in cases in which a disease gene has been identified, it is most likely that other genetic defects resulting in aberrant gene expression are required to obtain full leukemic transformation [52, 53]. Furthermore, in approximately 50% of the clinically diagnosed myeloid leukemias no cytogenetic abnormalities have been detected, whereas in other cases that do carry a cytogenetic abnormality the genes located near the breakpoints are still unknown [54-56]. An alternative approach to find as yet undisclosed leukemia disease genes is the identification and cloning of common virus integration sites (VIS). This approach has proven to be a sensitive tool to identify novel proto-oncogenes (e.g. *N-myc*, *Evi1* [57, 58]) as well as tumour suppressor genes (e.g.*p53*, *Nf1* [59]).

In fact, a number of genes located on chromosomal breakpoints or otherwise aberrantly expressed in human hematopoietic malignancies have been identified through retroviral insertional mutagenesis in murine leukemias. Examples of these are *Evi1* [58, 60], *Evi2(Nf1)* [61-63], *Evi6(Hoxa9)* [64, 65], *Bcl1(Cyclin D1)* [66, 67], *N-Myc* [57, 68], *and Erg* [69].

The molecular basis of retroviral insertional mutagenesis in the development of leukemias is the activation of proto-oncogenes or inactivation of tumour suppressor genes as a result of proviral integration into the host genome. In mice the development of lymphoid, myeloid or erythroid leukemias is dependent on the combination of mouse strain and retrovirus. Friend MuLV gives rise to mainly erythroid leukemias [70], whereas Moloney MuLV in Balb/c mice is able to induce mainly lymphoid leukemias [71]. Although Cas-Br-M MuLV is able to generate all

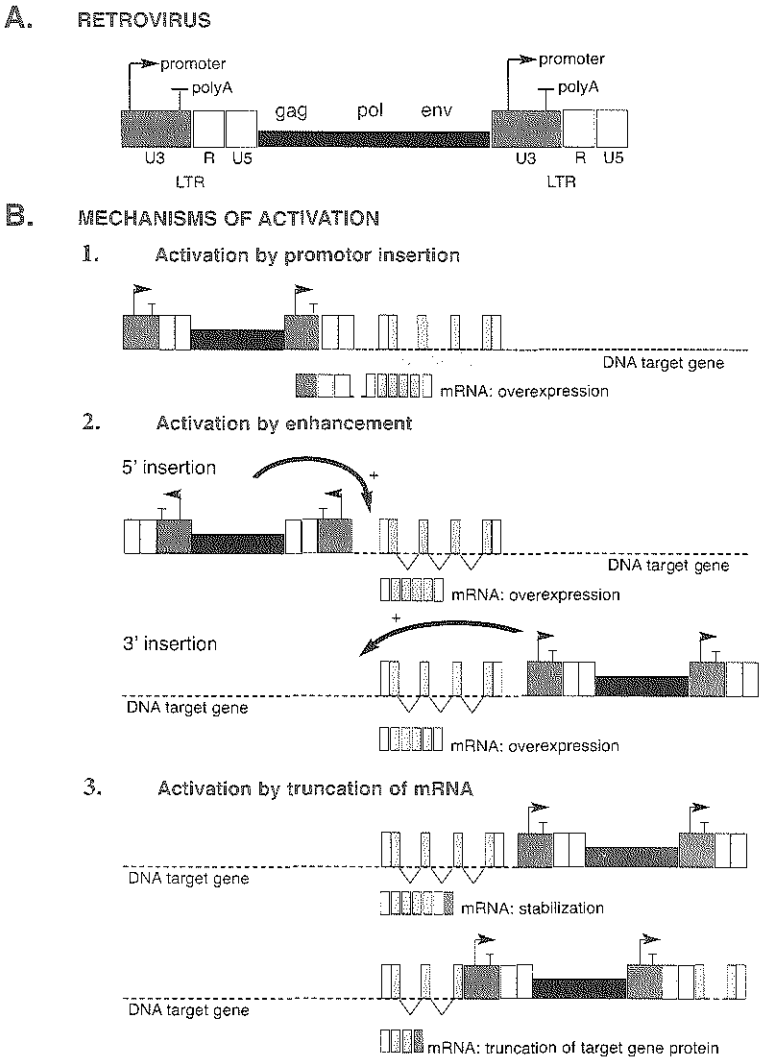

Figure 2. Retroviral insertional mutagenesis of host genes.

A. Structure of the proviral genome. The retrovirus contains *gag*, *pol*, and *env* genes, flanked by a long terminal repeat (LTR) on both sides. The LTR includes the regulatory sequences, and is constructed from a U_3 (containing a promoter and a poly-A signal), an R, and a U_5 region.

B. Mechanisms of activation by retroviral insertion:
   1. Activation by promoter insertion: transcription initiates from the viral promoter in either the 5' or 3' LTR, thereby replacing the function of the normal promoter.
   2. Activation by enhancement: transcriptional enhancement is probably the most frequent mechanism of gene activation by insertional mutagenesis. Activation by enhancement allows more flexibility with respect to the proviral orientation, and distance between provirus and target gene.
   3. Activation by mRNA truncation: this is possible by removing mRNA destabilising sequences in the 3'UTR, or by integration of the provirus into the coding sequence of a gene.

three leukemic phenotypes in NIH-Swiss mice [72], the majority of the leukemias are of myeloid origin.
Generally, expression of proto-oncogenes located in the vicinity of an inserted provirus is elevated by retroviral promoter or enhancer sequences. However, retroviral integration within the protein-coding region of a cellular target gene resulting in the expression of truncated proteins has also been reported. Provirus that is integrated into the host genome may subsequently activate a proto-oncogene or inactivate a tumour suppressor gene. This may occur by promoter insertion, enhancement, removal of mRNA destabilising motifs, or by transcription termination. Transcriptional enhancement is probably the most frequent mechanism of gene activation by insertional mutagenesis, whereas termination of transcription is the least frequent [73] (Figure 2).

The cloning of common VISs is an attractive approach leading to the identification of leukemia disease genes. Until recently the cloning of VISs consisted of time-consuming and laborious procedures of genomic cloning, Southern blotting and complicated strategies to identify potential target genes within a locus. The isolation of common VISs and potential disease genes on large scale requires a more efficient strategy. In Chapters 3 and 4 we developed and used two PCR based methods to clone large numbers of virus integration sites in retrovirally induced myeloid leukemias. The availability of sophisticated databases allows for a rapid identification of target "disease" genes.

IV. Models to study the effect of disease genes on myeloid development

Most of the genes that we have found by retroviral insertional mutagenesis in the studies presented in this thesis had not previously been identified as disease genes in leukemia. To investigate the role of each of those genes in normal development and in transformation representative *in vitro* and *in vivo* models could be useful.

IVa. In vitro models

Study of genes of interest *in vitro* requires cellular models with full capability to differentiate. The 32D murine myeloid differentiation system is a suitable cell line that allows for investigating gene expression in myeloid transformation *in vitro*. The cells are fully growth factor (IL-3) dependent and in the presence of the G-CSF-receptor, they are capable of terminal differentiation towards neutrophils [74]. Thus the introduction of a candidate disease gene, the effect of that gene on growth factor dependent proliferation, cell survival or neutrophilic maturation may be assessed. For instance introduction of a truncated G-CSF-receptor, representative of a preleukemic genetic abnormality, into 32D cells showed that the cells no longer developed towards neutrophils following G-CSF stimulation [75]. Other genes that were successfully introduced into 32D cells and converted a leukemic phenotype are e.g. *Evi1*, *DR-nm23*, *CBFβ*, and *Notch1* [76-79].
Another growth factor dependent *in vitro* model is the erythroid mouse cell line I/11 [80, 81]. In analogy with 32D cells, these erythroid precursor cells are maintained in culture at an immature stage using a combination of two growth factors EPO and SCF and will differentiate when stimulated with EPO alone. Whether genes may easily be

introduced into these cells to study leukemic transformation *in vitro* has not been studied yet.
In vitro differentiation models, such as 32D or I/11 may also be of use to study normal expression of genes during differentiation. Genes normally expressed in normal immature progenitors, but turned down during differentiation may become transforming when they are aberrantly regulated. For instance, the identification of genes expressed in primitive cells but not turned down during differentiation cause a differentiation block and lead to leukemia. Genes which specific maturation stage dependent expression patterns and located in a common VIS may represent disease genes involved in the block of myeloid differentiation.

Comparative cDNA was isolated and prepared from I/11 cells unstimulated versus stimulated with EPO and SCF and used for hybridisation of Atlas™-filters, to identify genes or gene-combinations specific for certain differentiation stages. The gene nm23-M2 was identified as a virus integration in a retrovirally induced myeloid leukemia (Chapters 4 and 5). In Chapter 6 it is shown that normal expression of the nm23-M2 gene in I/11 cells is regulated by growth factors. Moreover, the gene appeared to be highly expressed in primitive cells but it was downregulated during differentiation. A potential novel mechanism of expression regulation is discussed.

IVb. In vivo models

To determine the effect of overexpression of a gene in question on hematopoiesis *in vivo* and to investigate its possible role in hematopoietic malignancies, transgenic mice may be generated. Since expression of the gene should occur at a specific stage of the cellular developmental pathway, a proper choice of promoter should be made. To study the role in hematopoietic development, the promoter of the *Sca-1* (Ly-6E.1) gene may be suitable. Genes controlled by regulatory elements of the *Sca-1* gene are expressed in primitive hematopoietic cells, as has previously been shown for *LacZ* [82, 83]. Transgenic mice have been created for the proto-oncogene *Evi1* [84], in which the transgene was under the control of this *Sca-1* promoter. Although the presence of *Evi1* transgene product was demonstrated, it was concluded that after a follow-up of more than one year these mice did not develop leukemia. In fact similar findings were reported for studies using *Aml1-Eto* mice [85].
Since leukemia development is believed to be a multigenetic disease, it is not unexpected that these animals did not develop any disease. Several pieces of evidence have been put forward to show that additional hits were required to obtain full leukemic transformation. Injection of *Evi1* transgenic mice with Cas-Br-M MuLV demonstrated that significantly more transgenic mice developed leukemia than virus treated control littermates [84]. *Aml1-Eto* mice did not generate leukemia either unless exposed to secondary stimuli [86]. A model in which mice develop a myeloid proliferative disease is the *Pml/Rar* transgenic, with the transgene under the control of Cathepsin-G promoter. Interestingly, these animals show increased abnormal myeloid development, when the reciprocal *Rar/Pml* fusion gene is expressed as well [87]. These studies demonstrate that transgenic models are valuable tools to identify additional disease genes that may cooperate with any gene of interest.

In Cas-Br-M induced leukemias virus integrations were frequently found in the gene encoding the peripheral cannabinoid receptor Cb2. In Chapter 7 we describe the

generation of two Cb2-transgenic lines, with the transgene under the control of Sca-1 promoter. The effect of the transgene on hematopoiesis and the role in leukemia development was studied using retroviral treatment of the transgenic mice.

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Chapter 2

Phenotyping of *Evi1*, *Evi11/Cb2*, and *Evi12* transformed leukemias isolated from a novel panel of Cas-Br-M MuLV-infected mice

Marieke Joosten, Peter Valk, Yolanda Vankan, Nico de Both, Bob Löwenberg, and Ruud Delwel

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Abstract

Cas-Br-M murine leukemia virus (MuLV) is a slow-transforming retrovirus, which potently induces leukemias in mice and is therefore well suited for retroviral insertional mutagenesis. We used Cas-Br-M MuLV in NIH/Swiss mice to establish a new panel of mainly myeloid leukemias. All tumours found in leukemic animals were classified by gross pathology, morphology, immunophenotype as well as the incidence of known common virus integration sites (VISs) in MuLV-induced myeloid malignancies, i.e., *Evi1*, *Evi1l/Cb2*, *Evi12*, *Fli1* and c-*Myb*. Interestingly, male mice were more susceptible than females to the induction of leukemia by Cas-Br-M MuLV. Seventy-four of the Cas-Br-M MuLV-inoculated mice developed a severe splenomegaly, sometimes in association with a thymoma. Although most of the immunophenotyped Cas-Br-M MuLV tumours were of myeloid origin (58%), numerous T-cell leukemias (21%) and mixed myeloid/T-cell leukemias (21%) were found. The myeloid leukemias and myeloid compartment of the mixed leukemias were further characterised by immunophenotyping with stem cell-, myeloid- and erythroid-specific antibodies. The known Cas-Br-M MuLV common VISs *Evi1*, *Evi1l/Cb2* and *Evi12* were demonstrated in 19%, 12% and 20% of the cases, respectively, while no *Fli1* and c-*Myb* rearrangements were found. Integrations into *Evi1* were restricted to myeloid leukemias, whereas those in *Evi1l/Cb2* and *Evi12* were identified in myeloid as well as T-lymphoid leukemias. This panel of well-characterised Cas-Br-M MuLV-induced hematopoietic tumours may be useful for the isolation and characterisation of new proto-oncogenes involved in myeloid or T-cell leukemias.

Introduction

Slow-transforming retroviruses that do not contain oncogenes in their genome induce tumours by means of insertional mutagenesis [1-3]. The identification of common virus integration sites in retrovirally-induced tumours provides a powerful strategy to isolate novel transforming genes from leukemias and lymphomas [1-3]. Moreover, genes located on chromosomal breakpoint regions or aberrantly expressed in human hematopoietic malignancies have frequently been identified through retroviral insertional mutagenesis in murine leukemias, e.g., *Evi1* [4, 5], *Evi2 (NF1)* [6-8], *Evi6 (Hoxa9)* [9, 10], *Bcl1 (CyclinD1)* [11, 12], N-*Myc* [13, 14] and *Erg* [15, 16]. The molecular basis of retroviral insertional mutagenesis in the development of leukemias is the activation of proto-oncogenes or inactivation of tumour suppressor genes as a result of proviral integration into the host genome. Generally, expression of proto-oncogenes located in the vicinity of an inserted provirus is elevated by retroviral promoter or enhancer sequences. However, retroviral integration within the protein-coding region of a cellular target gene resulting in the expression of truncated products has also been observed [2, 3].

Cas-Br-M MuLV leukemia virus (also referred to as Cas-Br-E MuLV) is a naturally occurring ecotropic virus that induces a wide variety of hematologic diseases in NFS mice [17] and non-B, non-T-cell leukemias in NIH/Swiss mice [18]. A number of recurrent targets have been observed: *Evi1* [5, 19, 20], *Evi11/Cb2* [21], *Evi12* [22] and *Fli1* [18] or for Moloney and Friend MuLV, i.e., c-*Myb* [23-25]. *Evi1* encodes a zinc-finger transcription factor [5] and overexpression of *Evi1* has been shown to interfere in granulocytic [26] as well as erythroid differentiation [27]. *Evi11* and *Evi12* are two relatively novel common VISs that we recently identified in Cas-Br-M MuLV-induced IL-3 dependent myeloid cell line NFS107 and the Cas-Br-M MuLV-induced primary tumours described in this manuscript [21, 22]. *Evi11* is located on mouse chromosome 4 and the target proto-oncogene in this locus is the gene encoding the hematopoietic peripheral cannabinoid receptor Cb2 [21]. The candidate proto-oncogene in *Evi12*, which was mapped on mouse chromosome 10 [22], is currently unknown. The ets-transcription factor *Fli1* was originally identified by cloning proviral insertions from Friend MuLV-induced erythroid leukemias [28], but proviruses in Fli1 were later also shown in non-B, non-T-cell leukemias [18]. The transcription factor c-*Myb* is one of the best-studied proto-oncogenes in myeloid disease [29] and has been shown to be a target for proviral insertions in myeloid leukemias [3].

To isolate novel common VISs and identify new target proto-oncogenes and cooperating proto-oncogenes [22] involved in myeloid leukemia development, we have generated a novel panel of Cas-Br-M MuLV-induced leukemias in NIH/Swiss mice. We have characterised the hematologic malignancies by gross pathology, cytology and immunophenotyping using a panel of monoclonal antibodies directed to cell surface antigens of various blood cell lineages. Seventy-four of the 91 animals, presenting with a hematopoietic disease were leukemic, manifested by a splenomegaly and/or a thymoma. While the majority of the leukemias were of myeloid origin (59%), a significant number of the leukemias expressed a T-cell phenotype (21%) or a mixed myeloid/T-cell phenotype (21%). We also determined the frequencies of proviral insertion in loci that have previously been shown to be recurrent targets in myeloid malignancies for Cas-Br-M MuLV. Here we demonstrate frequent retroviral insertion in *Evi1*, *Evi11* and *Evi12*, but not in *Fli1* and c-*Myb*. Retroviral insertion in relation to the immunophenotype of the Cas-Br-M MuLV-induced leukemias has been investigated.

This well characterised panel of retrovirally-induced leukemias provides a powerful

source for the isolation of novel transforming proto-oncogenes involved in myeloid and T-cell leukemia development.

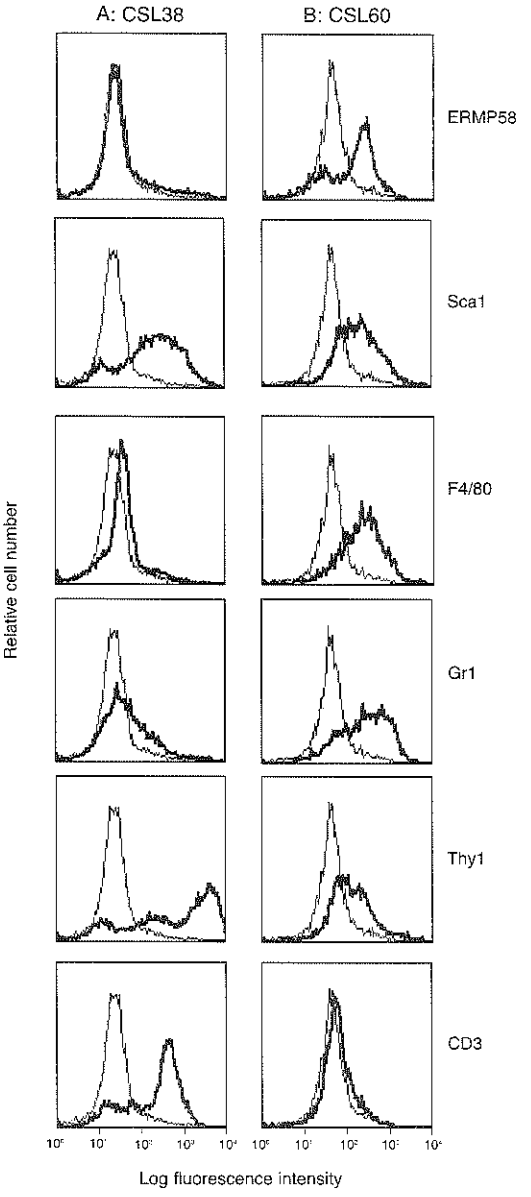

Figure 1.
Immunophenotyping of Cas-Br-M MuLV-induced primary CSL tumours.
Representatives of leukemias with a T-cell phenotype ($Thy1^{+}$ and $CD3^{+/-}$) (**A.** CSL38) and with a non-T-cell phenotype($CD3^{-}$ and $Thy1^{-or+}$) (**B.** CSL60). CSL60 is a typical example of a leukemiatype IV (Table 5), i.e., blasts with granulocytic and monocytic differentiation.

Materials and Methods

Mice, viruses and pathology of tumours

NIH/Swiss mice were obtained from Harlan (Horst, The Netherlands). Cas-Br-M MuLV-producing NIH 3T3 cells (donated by Dr. Janet W. Hartley from the National Institutes of Health, Bethesda, MA) [30] were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Gent, Belgium) containing 10% fetal calf serum (FCS). To obtain virus a 70% sub-confluent culture of Cas-Br-M MuLV-producing NIH 3T3 cells was incubated in 5 ml of culture medium for 18 hours at 37 ^{0} C, in a 75cm^2 culture flask. Newborn NIH/Swiss mice were subcutaneously injected with 100 µl of viral supernatant, made cell free by filtration (pore size, 0.22 µm; Nucleopore Corp., Pleasanton, CA). Leukemias appeared 5 to 13 months after inoculation, with mice sacrificed when moribund. Leukemic mice had enlarged spleens, frequently accompanied with gross thymus enlargements. Diagnosis was based on gross pathology, cytological staining with May-Grünwald Giemsa and flow-cytometric analysis using monoclonal antibodies directed to membrane surface antigens (Table 1). Leukemias were also characterised for DNA rearrangements within immunoglobulin heavy chain (IgH) genes. Cells were cryo-preserved in aliquots of 50 x 10^6 cells in 7.5% DMSO and 20% FCS using a controlled freezing apparatus, and storage in liquid nitrogen.

Table 1: Monoclonal antibodies.

Antibody	Antigen	Specificity	References
ER-MP54	ER-MP54 Ag	myeloid cells	[31]
ER-MP58	ER-MP58 Ag	myeloid cells	[31]
M1/70	Mac1	non-fixed macrophages granulocytes natural killer cells	[32]
F4/80	F4/80 Ag	macrophages	[33]
RB6-8C5	Gr1	granulocytes	[34]
ER-MP21	Transferrin Receptor	cells in cycle erythroid cells	[35]
TER119	Glycophorin A	mature erythroid cells	[36]
59-AD2.2	Thy1	T-cells hematopoietic stem cells myeloid cells	[37]
KT3	CD3	mature functional T-cells	[38]
RA3 6B2	B220	B-cells myeloid cells	[39]
E13 161-7	Sca1	hematopoietic precursors T-cells	[40]

Labelling of cells with monoclonal antibodies and flow-cytometry

Cells were labelled sequentially with rat monoclonal antibodies (MoAbs) followed by GαRa (Goat anti Rat)-FITC (Nordic, Tilburg, The Netherlands). All incubations were carried out 30 minutes on ice in phosphate buffered saline supplemented with 1% bovine serum albumin. The specifications of the MoAbs used in this study are described in Table 1. Cell surface fluorescence was analysed using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). A sample was considered positive for a particular marker when 30% or more of the cells showed positive fluorescence (Figure 1). In the case of Thy1 expression, a discrimination between intermediate positive (Thy1$^+$; not

more than 1 log difference in fluorescence intensity between positive and the negative control) and strongly positive (Thy1^{++}; 2 log difference or more between positive and negative) (Figure 1) was made.

DNA isolation, Southern blot analysis and probes

Isolation of genomic DNA and Southern blot analysis was carried out exactly as described previously [21]. The immunoglobulin heavy chain region was analysed with an *Eco*RI-*Bgl*II (2.5 kb) J_h probe from clone H24 [41] (a gift from Dr. T.Honjo, Kyoto University, Japan). The T-cell-receptor β-chain gene was analysed with a 4 kb *Hin*dIII $J_{\beta 1}$ probe [42]. Rearrangements in the *Evi1* locus were detected using the 535 bp cDNA fragment that we recently described [16], while those in the *Evi1/Cb2* locus were studied with a 1.2 kb *Eco*RI-*Bam*HI *Cb2* cDNA fragment [21]. *Evi12* rearrangements were studied with the 503 probe obtained with a RT-PCR based strategy to isolate complementary DNA fragments flanking retrovirus integration sites [16]. Rearrangements in the c-*Myb* gene were determined with a 450 bp *Nco*I-*Eco*RI and a 500bp *Pst*I-*Pst*I cDNA probe spanning exons 3,4,5 and 6 of the gene [43]. *Fli1* insertions were studied using a 1.7 kb *Eco*RI cDNA probe [28].

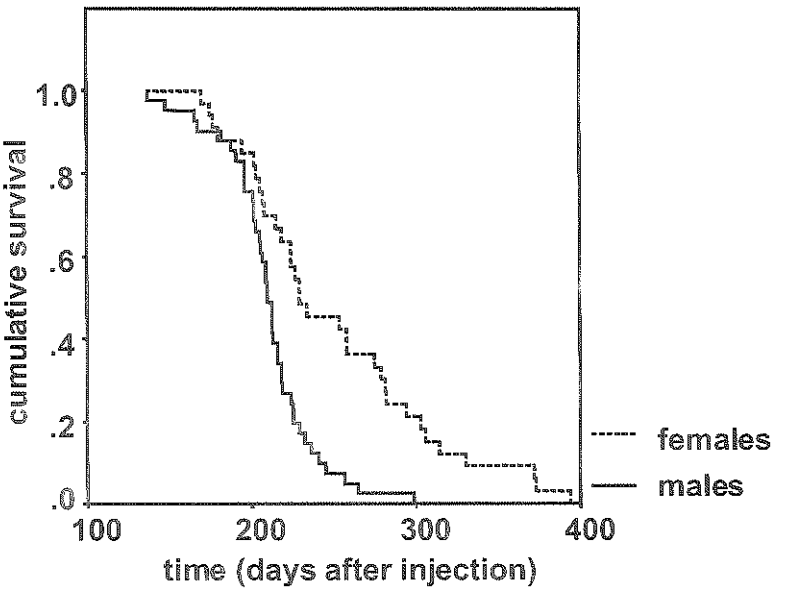

Figure 2.

Survival curves of male (—) and female (---) NIH/Swiss mice developing leukemia after inoculation with Cas-Br-M MuLV.

A log-rank test was performed, based on Kaplan Meier analysis. Males died significantly earlier than females ($p<0.001$). The x-axis represents the time at which the mice developed leukemia after injection with the Cas-Br-M MuLV.

Results

Leukemia development and gross pathology

Tumour incidence, gross pathology and histology - Newborn NIH/Swiss mice were inoculated with Cas-Br-M MuLV-containing culture supernatant. Ninety-one of the 116 mice inoculated developed hematologic malignancies, with 74 of these becoming severely sick with signs of splenomegaly and/or a thymoma (Table 2). The other 17 mice developed a mild disease, with a moderate increase (two-fold) in spleen size. Eighteen of the 25 mice without evidence of hematological disease died of unknown causes. The remaining 7 mice (females) were sacrificed at the end of the experiment, i.e., after 394 days, without signs of leukemia or any other disease.

Table 2: Frequency of Cas-Br-M MuLV-induced leukemias in NIH/Swiss mice.

	No. of mice	No. of leukemias	Frequency (%)
Males	54	41	76
Females	62	33	53
Total	116	74	64

Cytological analysis of mice with splenomegaly alone revealed immature blast cells (46%), myeloblasts with granulocytic features (27%) or lymphocytes (27%). In 82% of the mice with a thymoma histology the presence of a high percentage of lymphocytes was observed, whereas the remaining (18%) contained immature blast cells without signs of granulocytic differentiation.

Differences between male and female mice - In this study we focused on the 74 cases with severe hematologic malignancies. Interestingly, only 33 out of 62 (53%) female mice developed severe leukemia, whereas tumour formation in male mice was evident in 41 of 54 (76%) cases. Furthermore, male mice developed leukemia more rapidly then female mice (Figure 2). Thus, male NIH/Swiss mice appear to be more susceptible to leukemia induction by Cas-Br-M MuLV than female animals. In contrast, gross pathology revealed no significant differences between sexes in the leukemia types found (Table 3). Thirty-one out of 41 male mice (71%) and 24 out of 33 females (73%) manifested leukemia with a major spleen enlargement, while in 10 of 41 males (24%) and 7 of 33 females (21%) the mice also developed a thymoma. Two females developed thymoma without splenomegaly.

Table 3: Gross pathology of Cas-Br-M MuLV-induced leukemias.

Sex	Gross pathology	No. of mice	%	Latency period (days)	mean (days)
Male	splenomegaly	31	76	166-299	215
	thymus enlargement	0	0		
	splenomegaly and thymus enlargement	10	24	137-236	197
	total	41	100	137-299	211
Female	splenomegaly	24	73	170-394	263
	thymus enlargement	2	6	175-224	
	splenomegaly and thymus enlargement	7	21	177-314	245
	total	33	100	170-394	245

Phenotype of Cas-Br-M MuLV-induced leukemias
Leukemic cells from spleen, thymus or bone marrow of 63 of the 74 cases with severe disease could be immunophenotyped by flow-cytometry using monoclonal antibodies (Table 1).
T-cell leukemias - Thirteen cases displayed a solely T-cell phenotype, i.e., $CD3^{++}$/ $Thy1^{++}$ (11 cases), $CD3^{++}$/ $Thy1^{-}$ (one case) or $CD3^{-}$/ $Thy1^{++}$ (one case), without expression of myeloid markers (Figure 1 and Table 4). In ten of those mice the T-cell origin of the leukemia was substantiated by the presence of a thymoma (Table 4). In 13 cases the leukemic cells expressed T-cell as well as myeloid markers, with eight of these displaying a thymus enlargement as well.
Myeloid leukemias - A $CD3^{-}$/$Thy1^{-}$ or $CD3^{-}$/$Thy1^{+}$ non-T-cell phenotype (Figure 1 and Table 4) was found in 37 cases. Thirty-six of those 37 cases displayed a major spleen enlargement without a thymoma. These leukemias and the non-lymphoid compartment of the 13 mixed leukemias, containing both $CD3^{++}$/$Thy1^{++}$ and $CD3^{-}$ cells, were further immunophenotyped using the complete panel of monoclonal antibodies (Table 1), which revealed heterogeneity among the cases. Six cases (5 non-T-cell and 1 mixed leukemia) showed an immature myeloid phenotype, i.e., Imm^{+} (Sca^{+}, $Thy1^{+}$, ER-$MP58^{+}$ and/or ER-$MP54^{+}$), $Gr1^{-}$, $Mac1^{-}$ and $F4/80^{-}$ (Table 5). Four cases revealed a myeloid phenotype with neutrophil characteristics, i.e., Imm^{+}, Gr^{+}, ($Mac1^{+}$) but $F4/80^{-}$. Myeloid leukemias expressing monocyte/macrophage differentiation markers, i.e., Imm^{+}, $F4/80^{+}$, $Mac1^{+}$, but $Gr1^{-}$ were identified in 16 (11 non-T-cell and 5 mixed leukemias) cases. Twelve (9 non-T-cell and 3 mixed leukemias) cases expressed monocytic as well as granulocytic differentiation markers, i.e., Imm^{+}, $F4/80^{+}$, $Mac1^{+}$ and $Gr1^{+}$. In 10 cases myeloid and erythroid differentiation markers (ER-$MP21^{+}$ and $Ter119^{+}$) were identified. In 2 out of the 37 non-T-cell leukemia cases, mainly $B220^{+}$ lymphocytes were found. Although these data suggest that these leukemias are of B-lymphocyte origin, no rearrangements in the immunoglobulin heavy chain genes were found using a J_H-probe on *Eco*RI digested genomic DNAs of these cells (data not shown). In fact, no rearrangements in the IgH gene were found in any of the other cases (data not shown).

Retroviral integrations in Cas-Br-M MuLV induced leukemias
Cas-Br-M MuLV-associated common VISs - Loci previously shown to be targets for Cas-Br-M provirus are *Evi1* [5, 19, 20, 44], *Evi1/Cb2* [21], *Evi12* [22] and *Fli1* [18] while Moloney- and Friend MuLV also target c-*Myb* [23-25]. To study the frequencies of rearrangements in these loci, Southern blot analysis was carried out on spleen and/or thymus DNA obtained from the 74 mice with severe disease. Rearrangements in *Evi1*, *Evi11* or *Evi12* were found in 28 cases (Figure 3). No abnormalities in *Fli1* or c-*Myb* were observed. In 48 out of 74 cases no retroviral insertions in any of these regions could be identified.
Evi1 - Rearrangements in the *Evi1* gene were found in 14 out of 74 cases (Figure 3 and 4A). Those novel retroviral insertions in the *Evi1* locus are situated in the same region of the *Evi1* locus, as the previously described Cas-Br-M MuLV *Evi1* insertions (Figure 4B) [16, 20, 45]. *Evi1* rearrangements were demonstrated in non-T-cell or mixed lineage leukemias, and not in T-cell leukemias (Table 6). Two leukemias expressed an immature immunophenotype, one leukemia showed monocytic differentiation, a fourth leukemia expressed granulocytic markers and two other leukemias coexpressed monocytic as well as granulocytic characteristics. Interestingly, 5 leukemias showed an erythroid phenotype, i.e., the cells were ER-MP21 and TER119 positive (Table 5). Three mice, from which the tumours afterwards were shown to have *Evi1* rearrangements, died before

Table 4. Immunophenotypingof Cas-Br-M MuLV-inducedleukemias:T-cell versus non-T-cellleukemias.

	Immunophenotype	Splenomegaly	Thymoma[a]	Total
T-cell leukemias	$CD3^{+}/Thy1^{+-}$	3[b]	10[c]	13
Non-T-cell leukemias[d]	$CD3^{-}/Thy1^{-or+}$	36	1	37
Mixed leukemias[e]	$CD3^{-+}/Thy1^{-+}$ and $CD3^{-}/Thy1^{-or-}$	5	8	13
Not evaluable		11	0	11
Total		55	19	74

[a] Mice with a thymoma include cases with or without a splenomegaly.
[b] In one case (CSL70) $CD3^{++}/Thy1^{-}$ lymphocytes were found.
[c] In one case (CSL54) $CD3^{-}/Thy1^{++}$ lymphocytes were observed.
[d] $CD3^{-}$ non-T-cell leukemias Thy^{-} or $Thy1^{-}$ but not $Thy1^{++}$ cells.
[e] Mixed leukemias contain $CD3^{++}/Thy1^{++}$ T-lymphocytes as well as $CD3^{-}$ leukemia cells.

Table 5. Immunophenotyping of non-T-cell leukemias and number of *Evi1* positive tumours among these.

	Leukemia type	Immunophenotype[a]	Non-T-cell leukemias	Mixed leukemias[b]	Evi1-+ leukemias
I	Immature blast cells	$Gr1^{-}$, $F4/80^{-}$, $Mac1^{-}$ and Imm^{+}	5	1	2
II	Blasts with neutrophilic differentiation	$Gr1^{++}$ $F4/80^{-}$, $(Mac^{+})^{c}$ and Imm^{+}	4	0	1
III	Blasts with monocytic differentiation	$Gr1^{-}$, $F4/80^{+}$, $Mac1^{+}$ and Imm^{+}	11	5	1
IV	Blasts with granulocytic and monocytic differentiation	$Gr1^{+}$, $F4/80^{-}$, $Mac1^{+}$ and Imm^{-}	9	3	2
V	Blasts with myeloid and erythroid differentiation	ER-$MP21^{+}$ $TER119^{+}$, Imm^{+}, $(Mac1^{+})$, $(F4/80^{-})$ and $(Gr1^{-})$	6	4	5
Vi	B-lymphocytic	$B220^{+}$ and other[c]	2	0	0
Total			37	13	11

Leukemias were immunophenotyped using the monoclonal antibodies indicated in Table 1.
[a] (Imm^{+}) indicates that cells may express a combination of Sca1, ER-MP58, ER-MP54, and /or Thy1.
[b] Mixed leukemias contain T lymphocytic as well as non T-cell leukemia cells. In this case the non-T-cell compartment is analysed. [c] expression markers between brackets may be positive or negative

any viable cell could be harvested and immunophenotyped.

Evi1/Cb2 - The target gene in *Evi1* has been shown to be *Cb2*. Retroviral insertions in *Cb2* occur either 5' or 3' of the protein-coding region [21, 22]. Rearrangements in *Evi1/Cb2* were found in 9 of the 74 cases studied (Figure 3). The exact positions of these *Evi1* retroviral insertion sites have been documented recently [22]. *Evi1* rearrangements were found in T-cell, myeloid or mixed leukemias (Table 6). Three *Evi1* rearrangements were found in leukemic spleens obtained from mice that died before immunological analysis could be carried out. Interestingly, in addition to the 9 leukemic cases, Southern blot analysis revealed rearrangements in the *Evi1/Cb2* locus in splenic DNA from 2 of the 17 cases with a mild disease, i.e. CSL74 and CSL75.

Evi12 - *Evi12* rearrangements occurred in 15 of the 74 leukemic cases (Figure 3). Those proviral insertions are all present in a relatively small 1600 bp region, located upstream of the *Tra1/Grp94* gene and have been described recently [22]. The target gene in *Evi12* is currently unknown. As with *Evi11/Cb2* rearrangements, retroviral insertions in *Evi12* have been found in T-cell (3 cases), non-T-cell (2 cases) and mixed leukemias (4 cases) (Table 6). Out of the 74 mice that developed a severe leukemia, 5 died before any viable cell could be harvested. Interestingly, all five mice carried a retroviral insertion in *Evi12*. *Tumours with multiple rearrangements* - Coincidence of *Evi1* with other integrations appeared to be rare: *Evi1* and *Evi11/Cb2* in 1 case, *Evi1* and *Evi12* in 2 cases, while *Evi1*, *Evi11/Cb2* and *Evi12* was also observed in 1 case (Figure 3). Interestingly, six of the *Evi11/Cb2* rearranged cases also harboured *Evi12* virus integrations (Figure 3). These data may suggest cooperation in leukemia development between *Cb2* and the target gene in the *Evi12* locus. Only three of those cases could be analysed phenotypically. One leukemia had a T-cell phenotype (CSL14) and 2 mixed leukemias expressed T-cell as well as myeloid markers (CSL12 and -16).

Table 6. Common virus integration sites in Cas-Br-M MuLV-induced tumours: immunologic characterisation.

Leukemia type	Evi1	Evi11/Cb2	Evi12
T-cell leukemia	0	2	3
Non-T- non-B-cell leukemia	9	1	2
Mixed leukemia[a]	2	3	4
Dead[b]	3	3	5
Total	14	9	15

[a] Mixed leukemias contain T lymphocytic as well as non T-cell leukemia cells.
[b] Immunophenotype could not be determined due to the high percentage of dead cells

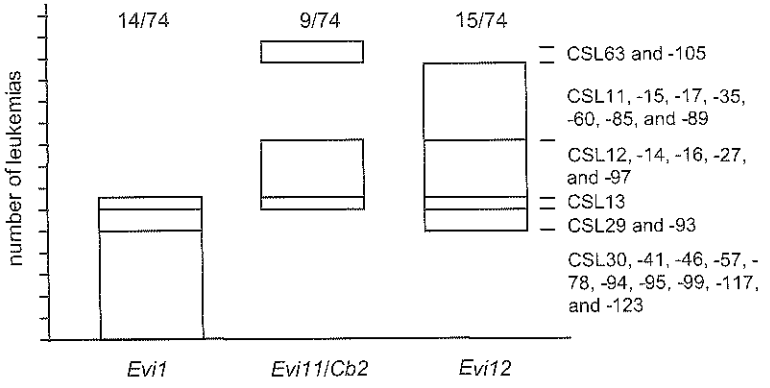

Figure 3.
Common virus integration sites *Evi1*, *Evi11/Cb2* and *Evi12* in primary CSL tumours
Diagram representing the different provirus insertions in *Evi1*, *Evi11/Cb2* and *Evi12* in the primary CSL tumours. Overlapping boxes symbolise coincidence of provirus insertions in two or three common VISs, i.e., *Evi1*, *Evi11/Cb2* and/or *Evi12*. The CSL leukemia numbers are depicted. The y-axis represents the sum of the leukemias.

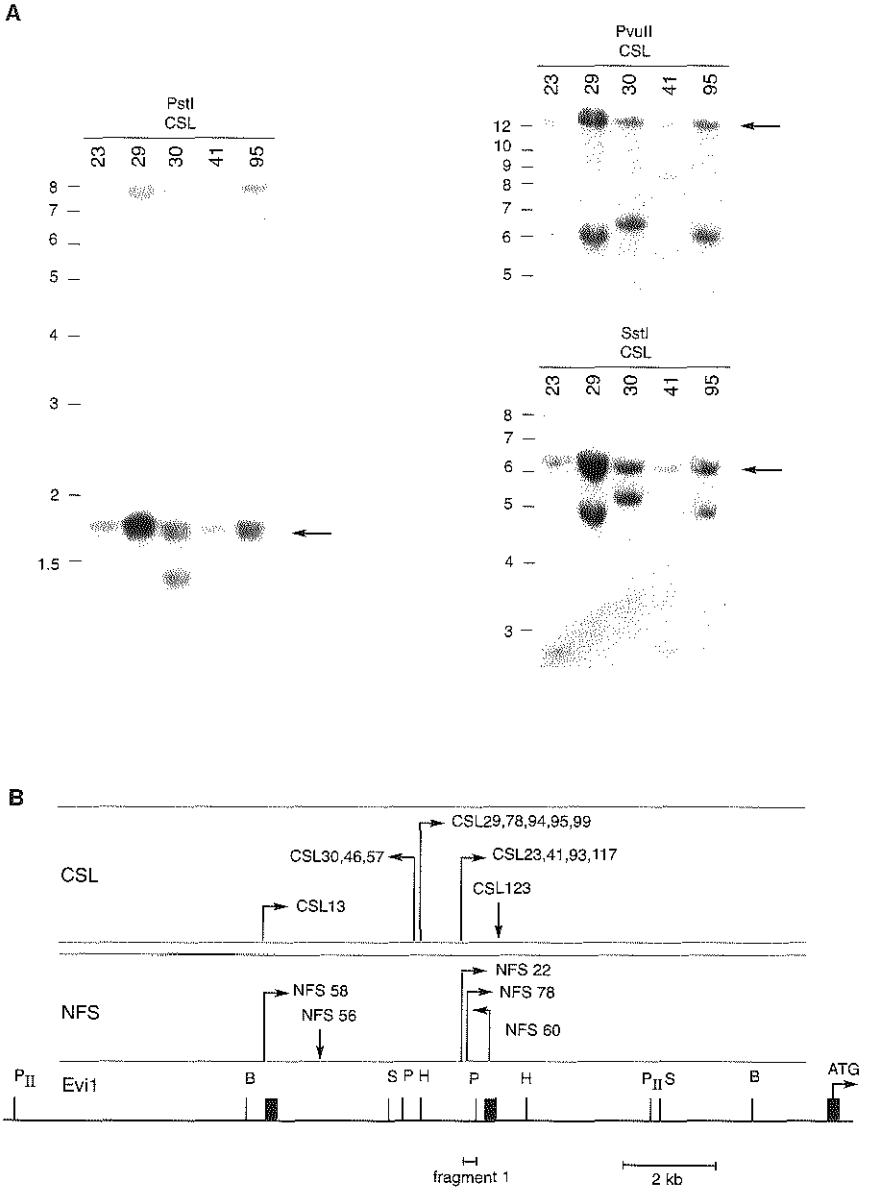

Figure 4.

Provirus insertion in the common VIS *Evi1*

A. Southern blot analysis of *Pst*I-, *Pvu*II- and *Sst*I-digested genomic DNA of primary CSL tumours. Filters were hybridised with fragment 1 (Figure 4B), a cDNA specific for the *Evi1* locus [16]. The size marker is depicted in kilobases and an arrow indicates the size of the normal allele. All tumours, i.e., CSL23, -29, -30, -41 and -95 contain rearrangements in the *Evi1* locus, representing the leukemic subpopulation.

B. Schematic representation of the genomic structure of the *Evi1* locus (PII:*Pvu*II, B:*Bam*HI, S:*Sst*I, P:*Pst*I, H:*Hin*dIII). The first three exons of the *Evi1* gene are indicated by black boxes. The location and orientation of proviruses in the Cas-Br-M MuLV-induced primary CSL tumours and NFS cell lines [17] are depicted by arrows.

Discussion

Cas-Br-M MuLV-injected newborn NIH/Swiss mice developed detectable leukemias by approximately 140 to 400 days post inoculation. Most of these were myeloid leukemias (59%), although T-cell (21%) and mixed T-cell/myeloid (21%) leukemias were found. These results differ somewhat from other studies. Cas-Br-M induced a wide spectrum of hematopoietic neoplasias in NFS/N mice, including T-, pre-B- and B-cell lymphomas as well as erythroid, myeloid and megakaryocytic leukemias [17, 46]. Bergeron *et al.* [18, 20, 44] reported the development of mainly non-T, non-B-cell lymphomas in NIH/Swiss mice after injection with Cas-Br-E MuLV. In contrast, we observed T-cell and myeloid leukemias in the same mouse strain. The major difference between the two studies is that Bergeron used a molecular clone NE-8, described by Jolicoeur *et al.* [47], whereas we inoculated a biologically cloned Cas-Br-M MuLV stock obtained by limiting dilution [30] in the same mouse strain. In contrast to our studies, Bergeron *et al.* observed frequent insertions of Cas-Br-E MuLV in *Fli1* [18, 20, 44], whereas these investigators observed no Cas-Br-E provirus insertions in either *Evi1l/Cb2* or in *Evi12* loci (personal communication E. Rassart). In fact, only Mink Cell Focus Forming retroviral integrations were identified in *Evi1l* and *Evi12* [22], suggesting the presence of amphotropic virus contaminant in the Cas-Br-M stock, which is absent in Cas-Br-E. [48, 49] In addition, no rearrangements of c-*Myb* were detected in either study, indicating that the c-*Myb* locus is a frequent target for Moloney- and Friend MuLV [3, 23-25], but not for Cas-Br-type viruses. The results from these studies together reemphasize that different retroviruses or even subclones of a particular retrovirus may result in proviral integration in distinct loci, thereby determining the development of selective types of leukemia or lymphoma [2, 3].

More males then females developed leukemia following Cas-Br-M MuLV-injection. In fact, seven female mice did not develop any hematological malignancy at all even 13 months after inoculation. Moreover, male mice developed leukemia more rapidly than females did. Gender related sensitivity to tumour inducing agents in rodents has been documented in several tumorigenic conditions before. For instance, female animals have been reported to be more sensitive to certain tumour inducing agents or viruses [50, 51], whereas other studies have demonstrated a higher tumour incidence in males following exposure to radiation [52, 53]. As an interesting parallel to our study, the radiation-induced tumours, showing greater susceptibility in male mice, represent cases of acute myeloid leukemia [52, 53]. Explanations to clarify these gender-specific differences are mainly based on speculation, including possible differences in sensitivity of certain tumour cells to gender-specific hormones [50, 53]. Although, gender-specific leukemia development is currently not one of the purposes of our investigations, the difference in sensitivity between male and female mice to Cas-Br-M MuLV-induced leukemia progression is intriguing and requires further study.

Retroviral insertions in the *Evi1* locus occurred in myeloid, erythroid or mixed myeloid/T-cell leukemias (19% (14/74)). These results are in agreement with many reports showing that *Evi1* is a proto-oncogene mainly involved in myeloid leukemia progression [5, 20, 44, 45]. In humans, *EVI1* has shown to be mutated in acute myeloid leukemias with chromosome 3q26 abnormalities [4] and in certain cases of myelodysplastic syndrome [54], a preleukemic disease characterised by a severe anemia. Overexpression of *Evi1* in 32Dcl3 cells has been shown to interfere with granulocytic differentiation of these cells when stimulated with G-CSF [26]. In addition, Kreider *et al.* [27] demonstrated a block of erythroid differentiation by *Evi1 in vitro*. Possibly, Evi1 interferes with GATA1, a

transcription factor that is indispensable for erythropoiesis [55, 56]. Recent studies with *Evi1* transgenic mice also support this hypothesis, i.e., a defective erythropoiesis as a result of *Evi1* overexpression [57]. In this study, we show that *Evi1* retroviral integrations frequently occur in leukemic blast cells expressing not only myeloid, but also erythroid specific markers, i.e., ER-MP21 and TER119. These observations support the hypothesis that one of the major effects of aberrantly expressed *Evi1* in hematopoietic precursors is a block of erythropoiesis.

In contrast to *Evi1* proviral insertions, retroviral integrations in *Evi11/Cb2* (12% (9/74)) and *Evi12* (20% (15/74)) are not lineage-restricted. The observations that *Evi11/Cb2* and *Evi12* mutations occurred in T-cell leukemias as well as in myeloid leukemias, suggest that retroviral insertions in those loci may have occurred early in hematopoiesis, i.e., in primitive hematopoietic stem cells. Subsequently, additional mutations, e.g., *Evi1* retroviral insertions, may determine whether myeloid or lymphoid leukemias evolve.

Leukemia initiation and progression involves aberrant expression of multiple genes [58]. Two or more VISs have frequently been shown to be present in one particular tumour or tumour cell line. For instance, *Hoxa* and *Pbx1*-related genes in myeloid leukemias [10], c-*Myc* and *M-CSF* in a monocyte tumour [59], *IL-3*, *SCL* and *Hoxb8* in myeloid WEHI-3B(D$^-$) cells [60], *IL-3* or *GM-CSF* and c-*Myb* in WEHI-274 cells [61], *IL-3* and *GM-CSF* in *in vivo*-passaged FDC-P1 cells [62], *p53* and *PU.1* in erythroleukemias [63], and *Fli1* and *p53* in non-T-, non-B-cell leukemias [20]. These data indicate that leukemogenesis, like oncogenesis in general [64, 65], is indeed a multistep process involving mutation of multiple oncogenes [58]. The simultaneous occurrence of different VISs in the same infected animal suggests a similar mechanism underlying murine leukemogenesis. *Evi11/Cb2* and *Evi12* insertions frequently coincided in the same leukemias suggesting that the *Cb2* in *Evi11* and the currently unknown *Evi12*-target oncogene cooperate in leukemia development. Here we demonstrate retroviral integration in *Evi1* and *Evi11/Cb2* in two cases, i.e., CSL13 and -23. In fact, aberrant expression of *Evi1* and *Evi11/Cb2*, as a result of retroviral insertions in both loci has been demonstrated in the myeloid cell line NFS78 [21]. These results suggest that in certain cases of myeloid leukemia these two proto-oncogenes may collaborate in hematopoietic transformation. This hypothesis will be directly assessed *in vivo* by cross-breeding of *Evi1*- and *Cb2*-transgenic mice that we have recently generated (unpublished results). *Evi1* also appears to collaborate with an *Evi12*-related proto-oncogene since three leukemias contain insertions in both these loci, i.e., CSL13, -29 and -93. Two common VISs, which frequently coincide are *Evi11/Cb2* and *Evi12*, i.e., rearrangements were shown in both common VISs in CSL12, -13, -16, -27 and 97. Although the target gene in *Evi12* is currently unknown, the data obtained so far are highly suggestive for collaboration of *Cb2* and a protooncogene at the *Evi12*-locus in leukemia progression. In one leukemia, CSL13, three loci, *Evi1*, *Evi11/Cb2* and *Evi12* harbour Cas-Br-M provirus, which may imply that indeed three or maybe more genetic defects are required for full leukemic transformation [64].

Interestingly, in 18 of the 26 cases retroviral insertion was observed in only one locus, i.e., *Evi1, Evi11 or Evi12* and in 48 cases we did not identify any retroviral insertion in the loci that we studied. These results suggest that many unknown candidate target genes for Cas-Br-M provirus are still waiting to be discovered in our panel of leukemias. Large-scale isolation of retroviral flanking sequences is currently being carried out in our series of leukemias using inverse PCR procedures. Nucleotide sequence analysis and differential probing to gridded high density filters from a BAC library will be carried out to identify novel common integration sites and new (collaborating) proto-oncogenes. These studies may result in the definition of complementation groups of transforming genes which may

provide insight into the defects that may occur in different signalling pathways leading to full malignant transformation of hematopoietic cells.

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Chapter 3

A rapid RT-PCR based method to isolate complementary DNA fragments flanking retrovirus integration sites

Peter J.M. Valk, Marieke Joosten, Yolanda Vankan, Bob Löwenberg, and Ruud Delwel

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Abstract

Proto-oncogenes in retrovirally induced myeloid mouse leukemias are frequently activated following retroviral insertion. The identification of common virus integration sites (VISs) and isolation of the transforming oncogene is laborious and time-consuming. We established a rapid and simple PCR based procedure, which facilitates the identification of VISs and novel proto-oncogenes. Complementary DNA fragments adjacent to retrovirus integration sites were selectively isolated by applying a reverse transcriptase reaction (RT) using an oligo(dT)-adapter primer, followed by PCR using the adapter sequence and a retrovirus long terminal repeat (LTR) specific primer. Multiple chimeric cDNA fragments suitable for Southern and Northern blot analysis were isolated.

Results and Discussion

Retroviral insertional mutagenesis is a powerful method to isolate proto-oncogenes from retrovirally induced leukemias and lymphomas (reviewed in [1]). A common VIS, which marks the position of a possible proto-oncogene, is characterised by retroviral insertions within corresponding genomic loci of various independent tumours and visualised by Southern blot analysis with probes flanking the actual VIS. Unknown flanking DNA sequences have been determined by genomic cloning [2], inverse PCR [3], biotinylated DNA labelling followed by PCR [4] and other PCR based methods [5, 6]. However, these methods carry certain disadvantages. Isolation of VIS flanking cellular DNA fragments by genomic cloning requires the establishment of DNA libraries, which is time-consuming and the libraries have to be made for every individual tumour or cell line. The PCR based methods consist of critical ligation [3], tailing [6] or biotinylating steps [4].
Several mechanisms are known by which retroviral sequences affect normal gene expression [1]. Promoter activation as well as enhancement by proviral integration within the 3' untranslated region requires that the viral LTR and the cellular gene are in the same transcriptional orientation [1]. As a result of these integrations transcription may be initiated from the retroviral LTR promoters and terminated by poly(A) signals of cellular genes (Figure 1A). Consequently, retrovirally initiated chimeric mRNA transcripts consist of a 5' leader derived from the viral LTR [2, 7] and a 3' poly(A) tail of a cellular gene. The overall RT-PCR based method to isolate these chimeric cDNAs is schematically shown in Figure 1A. Poly(A)$^+$ RNA was purified using oligo(dT) cellulose columns (Pharmacia) from a panel of Cas-Br-M MuLV-induced murine myeloid leukemia cell lines (NFS22, NFS56, NFS58, NFS60 and NFS78) [8]. First-strand cDNA was obtained by reverse transcriptase (RT) reactions at 37°C with 3μg poly(A)$^+$ RNA, 1 unit RNAguard (Pharmacia) and 100 units of SuperScript RT (Gibco) in 50mM Tris-HCl (pH 8.3), 75mM KCl, 3mM $MgCl_2$,1mM DTT, 0.5mM dNTP^s and 40mM oligo(dT)-adapter primer (5'GTC GCG AAT TCG TCG ACG $CG(dT)_{15}$3'). The integrity of the poly(A)$^-$ RNA and first-strand cDNA synthesis was verified by PCR (10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 150μM dNTP^s and 2.5 units of Taq-polymerase (Pharmacia), 1 min at 94°C, 1 min at 50°C and 3 min at 72°C (25 cycles)) with human β-actin specific primers, highly homologous to murine β-actin and located in two separate exons (MB6: 5'CTG GAC TTC GAG CAA GAG AT3' and MB7: 5'TCG TCA TAC TCC TGC TTG CT3'). Fragments of 433 bp were amplified with the β-actin primers solely in the presence of reverse transcriptase (Figure 1B). Subsequently, PCRs (1 min at 94°C, 1 min at 58°C and 3 min at 72°C (30 cycles)) were performed on the RT-reactions of the NFS cell lines by using the LTR-specific primer (pLTR1: 5'GGG TCT CCT CAG AGT GAT TG3') and the adapter primer (adapter: 5'GTC GCG AAT TCG TCG ACG CG3'). Fragments of different size, 0.1 to 2.5 kb, were detected in all cell lines tested (Figure 1B). No DNA fragments were amplified if PCR was not preceded by cDNA synthesis, indicating that only transcribed fragments were amplified. Fragments were cloned into the *Eco*RV site of pBluescript SK$^+$ (Stratagene, La Jolla, CA) and the viral origin of the cDNAs was confirmed by sequence analysis (Figure 1C). No cDNAs were detected entirely consisting of viral sequences but, as expected, 100% of the cDNAs contained LTR sequences at the 5' end. The cDNA sequences were compared with the database of the National Center for Biotechnology Information (NCBI) to identify possible homologous sequences. VISs were detected

within the murine homologue of the h*ERG* gene [9], which encodes a transcription factor, involved in AML t(16;21) [10] and within the promoter region of the mouse T-cell receptor γ chain near the Vg6 gene [11].
The basis of insertional mutagenesis to identify proto-oncogenes is the isolation of common VISs in independent tumours or tumour cell lines. Southern blot analysis of one fragment, derived from NFS22, is shown in Figure 2A. Rearrangements in various independent cell lines, i.e., NFS22, NFS58, NFS60 and NFS78, were detected. A partial restriction map and the orientations of the proviruses were established (Figure 2B) using the Southern blot data. The orientation and location of the proviral DNAs in NFS58, NFS60 and NFS78 implicated that fragment 1 was localised in the commonly rearranged *Evi1* locus [7, 12], within intron 2 of the *Evi1* gene. Northern blot analysis showed that this intron due to proviral integration is highly expressed in NFS22 and NFS78 (Figure 2C), which could be expected from the orientation of virus integrations in NFS22 and NFS78 (Figure 2B). In myeloid leukemias the *Evi1* gene is frequently activated due to viral integration within the murine gene [7] and in human AMLs due to translocation involving chromosome 3q26 [13]. Transcripts initiated from the retroviral promoter were selectively amplified. The existence of a poly(A) signal (AATAAA) (70%) and more than 15 adenine residues (55%) at the 3' end of all cDNA fragments (data not shown) may suggest that cDNA synthesis regularly initiated at mRNA poly(A) tails. It is however possible that cDNA synthesis initiates at A-rich sequences. In fact, the isolated *Evi1* cDNA represents a sequence located 5' within a cellular gene. Nevertheless, the isolation of cDNA fragments will eventually facilitate the identification of target genes in the leukemias (Figure 2C). cDNA fragments which did not show homology with known genes will be used for Southern blot screening purposes to search for new common VISs. The fact that three out of thirteen cDNAs represent known genes of which two (*Evi1* and *Erg*) have been demonstrated to be involved in myeloid leukemia [10, 13] suggests that the described method is powerful for the rapid identification of novel common VISs and proto-oncogenes.

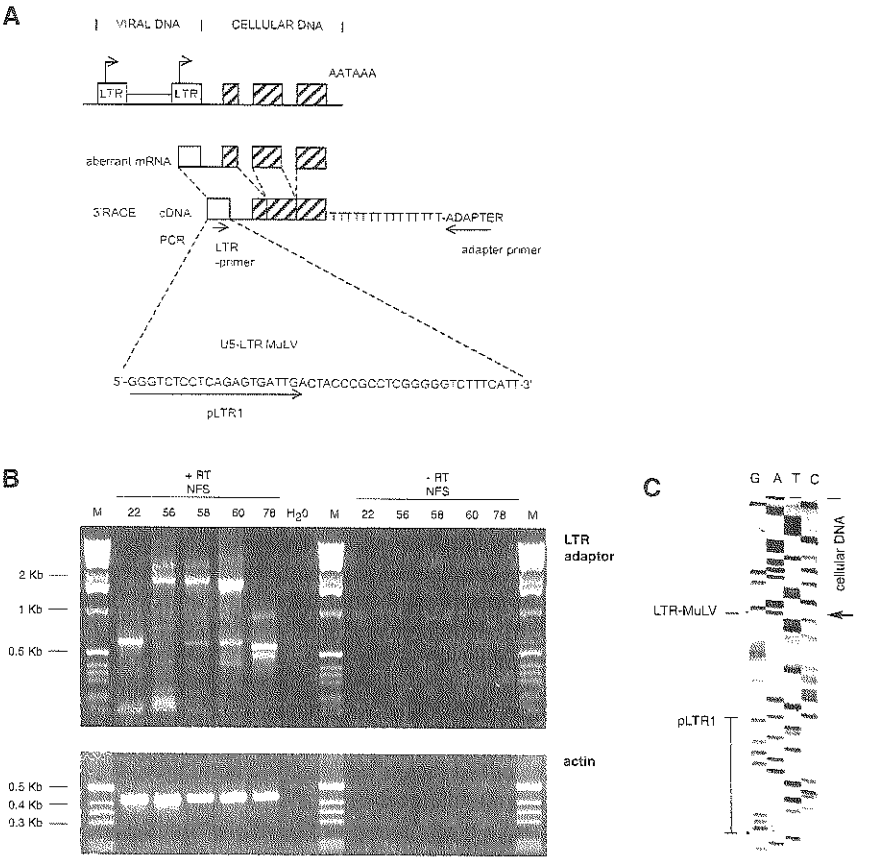
Figure 1. RT-PCR based method to isolate cDNA fragments flanking VISs.

A. Schematic representation of the strategy to rapidly amplify cDNA fragments adjacent to virus integration sites. Viral insertions in a cellular gene may generate aberrant mRNAs initiated through transcription from the viral promoter located on the long terminal repeat (LTR). The aberrant mRNA is polyadenylated downstream of the cellular polyadenylation signal (AATAAA). cDNA is synthesised by reverse transcription using an oligo(dT)-adapter primer which primes on the poly(A) tails or A rich sequences (3'RACE: 3' rapid amplification of cDNA ends). Using the adapter primer and an LTR specific primer (pLTR1) the cellular DNA fragments flanking the VIS are amplified by PCR.

B. RT-PCR (adapter/pLTR1) on poly(A)$^+$ RNA isolated from Cas-Br-M MuLV-induced myeloid leukemia cell lines NFS22, NFS56, NFS58, NFS60 and NFS78 (+RT). As a control PCR was carried out on poly(A)$^-$ RNA samples without the addition of reverse transcriptase (-RT). The integrity of the isolated poly(A)$^-$ RNA and the first-strand cDNA was confirmed by RT-PCR with β-actin primers (M:marker).

C. Sequence analysis of downstream of the LTR primer located MuLV LTR-specific sequences. The arrows (5'-TTTCA^NN-3') indicate the general fusion site of viral and cellular cDNA in both A and C. The cDNA sequence depicted in this figure is identical to the murine EST (DDBJ/EMBL/GenBank accession no. W97251).

Figure 1


A
VIRAL DNA
CELLULAR DNA
AATAAA
LTR
LTR
aberrant mRNA
3'RACE
cDNA
TTTTTTTTTTTTTT-ADAPTER
PCR
LTR
-primer
adapter primer
U5-LTR MuLV
5'-GGGTCTCCTCAGAGTGATTGACTACCCGCCTCGGGGGTCTTTCATT-3'
pLTR1
B
+ RT
NFS
- RT
NFS
M
22
56
58
60
78
H2O
M
22
56
58
60
78
M
2 Kb
1 Kb
0.6 Kb
LTR
adaptor
0.5 Kb
0.4 Kb
0.3 Kb
actin
C
G A T C
cellular DNA
LTR-MuLV
pLTR1

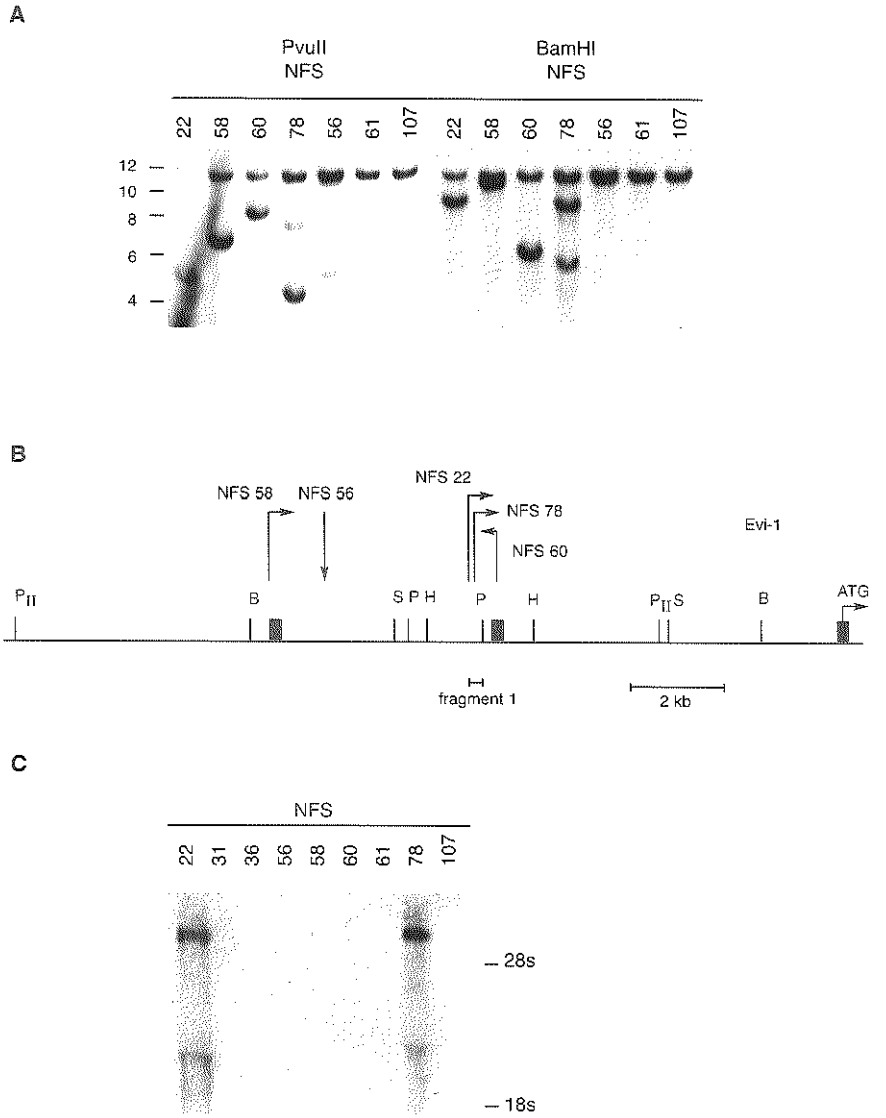


Figure 2. Fragment 1 is located in the known common VIS *Evi1*.

A. Southern blot analysis (*Pvu*II and *Bam*HI digest) of a panel of retrovirally induced leukemic cell lines NFS22, NFS58, NFS60, NFS78, NFS61 and NFS107 hybridised with fragment 1.

B. Limited restriction map of the *Evi1* locus with the site and orientation of viral integrations, indicated by arrows, in the leukemic cell lines NFS22, NFS58, NFS60 and NFS78 (PII:*Pvu*II; B:*Bam*HI, S:*Sst*I; P:*Pst*I; H:*Hin*dIII). The black boxes indicate the 5' end exon structure of the *Evi1* gene [7, 12].

C. Northern analysis of the cell lines NFS22, NFS31, NFS36, NFS56, NFS58, NFS60, NFS61, NFS78 and NFS107 probed with fragment 1.

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Chapter 4

Large-scale identification of novel potential disease loci in mouse leukemia applying an improved strategy for cloning common virus integration sites

Marieke Joosten, Yolanda Vankan-Berkhoudt, Marjolein Tas, Monja Lunghi, Yvonne Jenniskens, Evan Parganas, Peter J.M. Valk, Bob Löwenberg, Eric van den Akker, and Ruud Delwel

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Abstract

The identification of common virus integration sites (cVIS) in retrovirally induced tumours in mice provides a powerful strategy to isolate novel transforming genes. Applying virus LTR-specific inverse-PCR and RT-PCR combined with automated sequencing on Cas-Br-M Murine Leukemia Virus (MuLV) induced myeloid leukemias, 126 virus integration sites were cloned. Using locus- and LTR-specific primers, a nested-PCR/Southern-blotting procedure was developed on genomic DNA from a large panel of MuLV-induced leukemias, to analyse whether a particular virus insertion represented a cVIS. In fact 39 out of 41 integrations analysed this way appeared to represent a common virus integration. We recognised 6 previously cloned cVISs, i.e. *Evi1, Hoxa7, c-Myb, Cb2/Evi11, Evi12,* and *His1* and 33 novel common insertions, designated *Cas-Br Virus Integration Site (Casvis).* Among this group we found integrations in or near genes encoding nuclear proteins, e.g. *Dnmt-2, Nm23-M2, Ctbp1* or *Erg*, within receptor genes, e.g. *Cb2* or *mrc1,* novel putative signalling or transporter genes, the ringfinger-protein gene *Mid1* and a panel of genes encoding novel proteins with unknown function. The finding that 39 out of 41 integrations analysed represented a cVIS, suggests that the majority of the other virus insertions that were not yet analysed by the PCR/Southern- blotting method are located in a cVIS as well and may therefore also harbour novel disease genes.

Introduction

Genes responsible for leukemic transformation are frequently located near non-random chromosomal translocations [1, 2]. However, in approximately 50% of the clinically diagnosed myeloid leukemias no cytogenetic abnormalities have been detected. Furthermore, in a number of cases that do carry a cytogenetic abnormality [3-5] the genes located near the breakpoints are still unknown. Moreover, since leukemia is believed to be a multi-step process, aberrant expression of different disease genes affecting multiple pathways are required to obtain full leukemic transformation [6, 7]. An alternative procedure to identify leukemia disease genes is the cloning of common virus integration sites (cVIS). This approach has proven to be a sensitive tool to identify novel proto-oncogenes as well as tumour-suppressor genes. In fact, several genes located near chromosomal breakpoints or otherwise aberrantly expressed in human hematopoietic malignancies have been identified through retroviral insertional mutagenesis in murine leukemias or lymphomas as well, e.g. *Evi1* [8, 9], *Evi2 (NF1)* [10-12], *Evi6 (Hoxa9)* [13, 14], *Bcl1 (Cyclin D1)* [15, 16], *N-Myc* [17, 18], *and Erg* [19, 20]. A major assumption in studies of MuLV-induced leukemias is that proviral integration is essentially random and that common sites of integration are observed in tumour samples due to selection of rare cells with proviral insertions in these common sites. The selective pressure is provided by the expression of the target gene nearby [6, 7]. Although one might suspect that common as well as non-common VISs will be present in a leukemia, it is at present unclear which percentage of VISs in a particular tumour represents common sites of integration. In this study, we developed a high-throughput procedure to clone novel cVISs on a large-scale to answer this particular question.

Until recently, the identification of cVISs has been a laborious procedure. In order to isolate cVISs and potential disease genes on a large scale PCR approaches were previously established, e.g. an inverse PCR (IPCR) and a RT-PCR based procedure [20], [21]. Making use of IPCR and subsequent automated DNA sequencing investigators identified VISs on a large scale using AKXD- or BXH2-virus-induced leukemias [22, 23]. By comparison of every virus flanking fragment with sequences in the GenBank of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov, NCBI, Bethesda, MD, USA) and with the nucleotide sequences of each of the other cloned virus insertions, a significant number of novel common integration sites and potential disease genes were identified. On the other hand, a large number of virus insertions were found only once. Although these insertions had frequently taken place in key regulatory genes, commonality could not be shown using this approach. Here we present a strategy in addition to the PCR/high-throughput sequencing protocol, to not only identify large numbers of virus integration sites, but also rapidly define whether a virus insertion represents a cVIS.

Swiss mice infected with Cas-Br-M MuLV may develop myeloid or lymphoid malignancies as the result of retroviral insertion in disease genes [24, 25]. Classical cloning procedures resulted in the identification of several disease genes in Cas-Br-M MuLV-derived tumours, e.g. *Evi1*, *Evi11/Cb2* or *Evi12* [8, 26, 27]. Large scale IPCR and RT-PCR was performed utilising DNA or mRNA from 28 primary Cas-Br-M MuLV induced myeloid leukemias in Swiss mice (CSL) [28] and 15 retrovirally induced myeloid cell lines [29, 30]. Virus-flanking fragments were cloned, sequenced and subsequently compared to the Celera Discovery System (Contents of May 2002) (www.celera.com, Celera Genomics, Rockville, MD, USA) and to NCBI Genbank. To investigate whether these sites represented cVISs, we developed a nested PCR

strategy on genomic DNA from 107 leukemias, by applying LTR-specific and fragment-specific primers followed by a Southern blotting protocol with an additional set of radio-labelled LTR- and fragment-specific oligonucleotides. Applying this approach, we were capable of defining whether and how frequent a virus insertion occurred in other leukemias. In addition to six previously identified cVISs we identified a large number of novel common virus insertions. The exact mouse and human chromosomal localisation were defined and in most of the cases the potential target genes in these loci could be identified.

Materials and Methods

Primary leukemias and cell lines

The NIH-Swiss derived Cas-Br-M MuLV induced primary tumours used in this study are: for IPCR: CSL 13, 16, 20, 22, 26, 30-33, 65, 71, 78, 82, 90, 91, 93, 111, 117, 123, 201, 203, 204, 212, 221, 227, 228, 237 and 239; and for RT-PCR: CSL 201 and 203. [28]. Cells isolated from leukemic spleens were stored vitally in aliquots in liquid nitrogen. The following leukemia cell lines were utilised in this study: for IPCR: DA 24, and NFS 22, 36, 56, 58, 60, 61, 78, and 124; and for RT-PCR: DA 1, 2, 3, 8, 33, and NFS 22, 36, 58, 61, 78, 107, and 124. [31]). These cell lines were cultured in RPMI plus 10 % fetal calves' serum (FCS) (GIBCO Life Technologies Inc., Gent, Belgium) and 10 Units of mouse IL3. The frequency of virus integrations was determined both on cell lines (DA 1-3, 8, 13, 24, 25, 28, 29, 31, 33, and NFS 22, 36, 56, 58, 60, 61, 78, 107, and 124), and primary leukemias (CSL 11-17, 19, 21-23, 25-27, 29, 31-33, 35, 36, 38, 39, 41, 43, 45-51, 53-61, 63-72, 74-83, 85, 87-109, 114, 117, and 123).

IPCR and RT-PCR

Isolation of genomic DNA was carried out exactly as described previously [27]. Five µg of genomic DNA was digested with *Sau3A, PvuII* or *SstI* (GIBCO Life Technologies Inc., Gent, Belgium). The products were treated with T4-ligase (GIBCO Life Technologies Inc.), which resulted in the formation of circularised products. Subsequently we performed an IPCR strategy using primers specific for the Cas-Br-M MuLV LTR. For the *Sau3A* digested/ligated fragments, the first PCR reaction was carried out with primers pLTR4 (5'-CCG AAA CTG CTT ACC ACA-3') and pLTR3 (5'-GGT CTC CTC AGA GTG ATT-3'), followed by a nested PCR using pLTR5 (5'-ACC ACA GAT ATC CTG TTT-3') and pLTR6 (5'-GTG ATT GAC TAC CCG CTT-3') Cycle conditions for both PCRs were 15" at 94°C, 30" at 57°C, and 2' at 72°C for 10 cycles, and an additional 20 cycles following the conditions 15" at 94°C, 30" at 57°C, and 2'30" at 72°C. Reactions were performed using Expand High Fidelity PCR System (Roche, Mannheim, Germany). For *PvuII* and *SstI* digested genomic DNA, the circularised DNA was amplified using primers pLTR7 (5'-GAC TCA GTC TAT CGG AGG AC-3') and pLTR1 (5'-CTT GCT GTT GCA TCC GAC TGG-3'), and pLTR8 (5'-GTG AGG GGT TGT GTG CTC-3') and 2 (5'-GTC TCG CTG TTC CTT GGG AGG-3') respectively. The first PCR was performed for 30 cycles 30" at 94°C, 1' at 54°C, and 3' at 72°C. The reaction was carried out with Expand High Fidelity PCRsystem (Roche). Nested PCR conditions were 30 cycles of 30" at 94°C, 1' at 58°C, and 1' at 72°C. This reaction was performed with Taq polymerase (Amersham Pharmacia Biotech, Roosendaal, The Netherlands). RT-PCR was carried out as described previously by Valk *et al.* [20]. Briefly, total RNA was isolated through a CsCl gradient. First strand cDNA was obtained by reverse transcriptase (RT) reactions with an oligo(dT)-adapter primer (5'-GTC GCG AAT TCG TCG ACG $CG(dT)_{15}$-3') at 37°C with 5µg RNA, using the Superscript™ Preamplification System (Life Technologies, Breda, The Netherlands) according to the instructions of the manufacturer. Subsequently, PCRs (1' at 94°C, 1' at 58°C, 3' at 72°C (30 cycles)) were performed on the RT reactions of the leukemias by using the LTR specific primer pLTR6 and the adapter primer (5'-GTC GCG AAT TCG TCG ACG CG-3'). PCR products were directly cloned into pCR2.1 (Invitrogen, Breda, The Netherlands) according to the instructions of the manufacturer and subjected to

nucleotide sequencing. Nucleotide sequences were compared to the NCBI database for analysis.

PCR analysis and Southern blot hybridisation

One μg of genomic DNA isolated from primary leukemias or cell lines was subjected to PCR with primer pLTR1 and a locus specific primer A (Figure 1). Locus specific primers of 17 to 21 nucleotides were designed specific for each of the sequences of the fragments generated by Inverse- or RT-PCR. Primers were purchased from Life Technologies. One μl of PCR product was transferred to a nested PCR reaction using primer pLTR2 and a nested locus specific primer B. Cycle conditions for both reactions were 1 cycle 5' 94°C, 30 cycles 30"94°C, 1' Tm, 1'30"72°C, 1 cycle 5'72°C. Tm was specific for each primer and was between 48°C and 62°C. PCR was carried out using Taq polymerase (Amersham Pharmacia Biotech). PCR products were electrophoresed in a 1.5% agarose gel and transferred to Hybond-N^+ nylon membranes (Amersham Pharmacia Biotech) with 0.25 M NaOH/1.5 M NaCl. Membranes were hybridised with a ^{32}P-end-labelled locus specific primer C. Labelling was carried out using T_4kinase (USB, Cleveland OH, USA) according to the instructions of the provider. Subsequently, blots were stripped in 0.4M NaOH for 30 minutes at 45°C, neutralised using 0.2M Tris-HCl, 0.2% SDS, and 0.1xSSC for 15 minutes at 45°C and hybridised with ^{32}P-end-labelled Cas-Br-M MuLV specific primer pLTR3. Blots were exposed for autoradiography with a KODAK film and an intensifying screen. After 15 minutes of exposure films were developed and analysed.

Sequencing analysis

Samples were prepared using the Bd-sequencing kit according to instructions from the provider (PE Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) and nucleotide sequencing was carried out on an ABI 310 automatic sequencer (PE Biosystems) using primers M13forward (5'-GTA AAA CGA CGG CCA GT-3') and M13reverse (5'-GGA AAC AGC TAT GAC CAT G-3'). Sequences isolated by IPCR or RT-PCR were compared to the data present in the Celera Discovery System (Celera Genomics, Rockville, MD, USA, database contents May 2002) and in the GenBank of the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA). The exact site of integration was determined. For insertions found outside a gene, the distance between the integration and the most nearby gene was calculated. The location on the mouse chromosome was established and the human equivalent was deduced by using the human databases of Celera Discovery System (May 2002), LocusLink or human mouse homology maps (both www.ncbi.nlm.nih.gov).

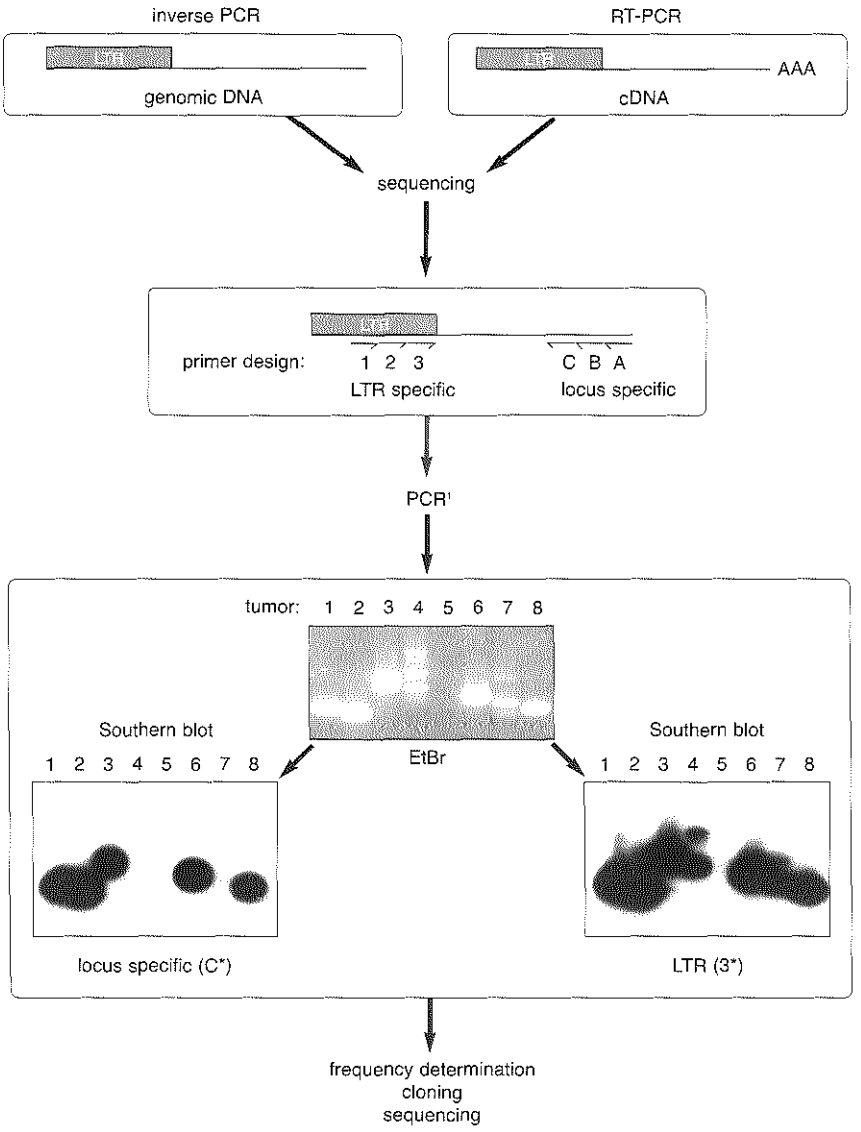

Figure 1.
IPCR or RT-PCR was performed on RNA or DNA from cell lines (DA and NFS), or CSL leukemias. The resulting virus flanking fragments were subjected to sequence analysis. LTR- and locus-specific primers were designed and used in a nested PCR strategy (*), i.e. LTR-1/primer-A PCR, followed by a LTR-2/primer-B amplification using genomic DNA from a panel of Cas-Br-M MuLV induced leukemias. PCR products were electrophoresed on a 1.5% agarose gel and subsequently blotted. Blots were first hybridised with locus-specific primer C and exposed to film, and after stripping rehybridised with primer LTR3. Bands hybridising with the LTR3 as well as the locus specific primer C (Lanes 1,2,3,6, and 8) were considered positive, i.e. tumours carrying this particular cVIS. Hybridisation with one primer only (lanes 4 and 7) are false positives. In each experiment two or three positive fragments were cloned and nucleotide sequenced to confirm specificity.

Results

To clone VISs on a large scale, a virus-LTR specific IPCR as well as a RT-PCR were applied as complementary approaches using DNA or RNA from 43 myeloid leukemias. The IPCR method was carried out on 28 primary leukemias and nine cell lines, whereas the RT-PCR based technique was performed on 12 cell lines and two primary leukemias. The fragments were cloned, subjected to nucleotide sequencing and compared to each other and to those present in the Celera and NCBI databases. In total 126 unique virus integration sites were identified of which some were found more than once in distinct leukemias. Database analyses revealed three different groups of virus integrations: i.e. (I) in a gene (65/126), (II) in a known genomic locus in the vicinity of a gene (57/126), and (III) in a unknown sequence (4/126) (Table 1).
We next determined for 41 inserted loci whether they represented a cVIS. This concerned 20 integrations from group I and 21 sequences from group II (Table 1). We designed locus specific primers for each of the virus insertions and carried out a nested PCR reaction using these primers in combination with LTR specific primers on genomic DNA isolated from 107 tumours, i.e. 87 primary CSLs, 11 DA cell lines and nine NFS cell lines (see Figure 1). The PCR products were run on an ethidium bromide stained 1.5% agarose gel, subjected to Southern blot analysis and hybridised with locus specific primer-C and pLTR3 (Figure 1). Fragments that hybridised to both primers were considered positive. In each experiment, two or more of those positive fragments were cloned and sequenced to confirm specificity and to acquire more nucleotide sequence information of the loci (Figure 1).
PCR/Southern strategy was applied on 20 of the 65 integrations from Group I (Insertion in a gene). It appeared that 19 of the 20 integrations analysed represented a common VIS. These 19 loci, including their frequency, the tumours from which the virus integrations were originally isolated, the murine and human chromosomal localisation, the NCBI accession numbers of the viral integrations, the Celera protein accession numbers, and the (putative) function of the gene products are summarised in Table 2. Using the PCR/Southern strategy we identified several previously cloned cVISs, i.e. *Evi1, Hoxa7, c-Myb, Evi11/Cb2, Evi12* and *His1*. Of the other 13 cVISs, i.e. *Casvis 1-13* a known or a novel putative gene was identified. Three of them, *Casvis 1,2,* and *3*, were located in genes encoding nuclear proteins, i.e. *Transcription elongation factor II, Dnmt2,* and *Nm23-M2* respectively. We found integrations in two genes encoding membrane receptors, i.e. *Cb2* and *mrc1*. Recurrent viral integrations were also found in genes encoding the enzyme *Hmox2*, a tyrosine kinase. Furthermore, genes encoding the carrier proteins Slc7a11 and Slc17a5, being sodium-dependent phosphate transporter-related proteins and the ringfinger protein Midline-1 were very often inserted by retroviral DNA. Finally a number of genes encoding novel putative proteins were identified as frequent common virus integration sites *(Casvis 11-13* and *Evi12)*.
In Table 3 common insertion sites are listed in which virus DNA had integrated into a genomic sequence at a relative distance from a potential target gene (Group II). Using PCR/Southern procedure 19 out of 20 integrations from Group II were identified as cVIS *(Casvis 14-33)*. Frequent proviral insertion was found in a locus harbouring the transcription regulators Erg and LIM domain binding protein 1, the signalling molecule PKCε, the TGFβ receptor and the Rap guanine nucleotide exchange factor. In 11 cases the candidate genes encode unknown putative proteins.
Since our data demonstrate that the majority of virus insertion sites analysed by PCR/Southern represent common VISs, suggest that the majority of the remaining

integrations should be considered as disease loci as well. Therefore, the remaining 45 integrations from Group I (insertions in a gene) and 36 insertions from Group II (insertion in the vicinity of a potential target gene) are summarised in Tables 4 and 5 respectively. Of these remaining virus integrations, eight were found more than once within the distinct leukemias and were therefore designated *Casvis 34-41* (Tables 5 and 6).

Table 1. Numbers of unique retroviral insertions identified and studied.

	Total	**in a gene** *Group I*[b]	**near a gene** *Group II*[c]	**Sequence unknown** *Group III*[d]
Virus insertions	126[a]	65	57	4
PCR/Southern analysed/cVIS[e]	41/39	20/19	21/20	0
Not analysed[f]	85	45	36	4
Sequence comparison: cVIS[g]	8	5	3	0

[a]Number of unique virus integrations. [b]Group I: Virus integrations in a gene. [c]Group II: Virus integrations in a genomic sequence in the vicinity of a gene. [d]Group III: No sequences available in Celera or NCBI databases. [e]Number of virus integrations analysed by PCR/Southern protocol and number of integrations designated cVIS as determined by this approach. [f]Number of virus integrations not analysed by PCR/Southern. [g]Number of virus integrations that were found more than once among the remaining 85 virus insertion sites and designated cVIS as well.

Table 2. Cas-Br-M MuLV common virus integration sites (cVIS) located in a candidate gene.

name of VIS	gene product	frequency (%)	retrieved from	mouse chrom	hum chrom	NCBI acc no Casvis[a]	Celera protein acc no[c]	function
Evi1	Evi1	16[c]	NFS22	3A3	3q26	NM007963[b]	mCP2223	TR/DNA binding
Evi7	Hoxa7	58	CSL82	6B3	7p15-p14	NM010455[b]	mCP12985	TR/DNA binding
c-Myb	c-Myb	32	CSL123	10	6q22-q23	NM033597[b]	X16389[d]	TR/DNA binding
Casvis1	Transcr. elong. factor SII	58	NFS107	4C6	1p32	AF432356	mCP22410	TR/DNA binding
Casvis2	Dnmt2	35	CSL16	2A1	10p15.1	AF432357	mCP2357	TR/DNA binding
Casvis3	nm23-M2	9	CSL22	11D	17q21.3	AF432358	mCP14111	TR/DNA binding
Casvis4	Tyrosine kinase	24	CSL30	11E2	17q25.3	AF432370	mCP18961	signalling
Evi11	Cb2	11[e]	CSL78	4D3	1p36	X93168.[b]	mCP3324	receptor
Casvis5	mrc1	3	CSL111	2A1	10p13.1	AF432359	mCP2323	receptor
Casvis6	Slc7a11	15	NFS61	3C	4q28-q32	AF432387	mCP21827	transporter
Casvis7	Slc17a5	7	CSL31	9E1	6q14-q15	AF432364	mCP11743	transporter
Casvis8	drug efflux related	30	NFS22	5B1	4p16.3	AF432377	mCP3901	transporter
Casvis9	Hmox2	4	NFS78	16A1	16p13.3	AF432383	mCP86495	enzyme
Casvis10	Mid1	55	CSL20	X	Xp22	AF432365	NM010797*	ring finger protein
Evi12	putative protein	17[c]	NFS107	10C1	12q24.2	AF091114	mCP28980	unknown
His1	His1	3	NFS124	2B	2q14-q21	U10269[b]	mCP42656	unknown
Casvis11	putative protein XP051927	47	CSL26	19C1	10	AF432372	mCP2702	unknown
Casvis12	putative protein	28	CSL91	13A2	unknown	AF432366	mCP15205	unknown
Casvis13	putative protein	3	CSL117	1C1.3	unknown	AF432379	mCP74740	unknown

CVISs are ordered according to the (putative) function of the potential target genes. The frequency of integrations were determined by a PCR/Southern blotting approach (see Figure 1) in a panel of 107 leukemias. The cell lines or leukemias from which the original virus-flanking fragment was isolated are listed. The newly assigned NCBI accession numbers for the cloned viral flanking nucleotide sequences and the Celera protein accession numbers are presented.

CVISs are designated Casvis = Cas-Br-M MuLV common Virus Integration Site, TR = Transcription Regulator.

[a] The sequences in the GenBank, National Center for Biotechnology Information (NCBI), Bethesda, MD, USA: www.ncbi.nlm.nih.gov will become available through the accession numbers in May 2003. [b] These accession numbers represent the original nucleotide sequences submitted to the NCBI by others previously. [c] Accession numbers from Celera discovery system were from May 2002. It should be noted that the mouse assembly of Celera discovery system is dynamic and will be improved over time.[d]Represents a NCBI accession number. No accession number was present in the Celera Discovery System.[e]The frequency of *Evi1, Evi11* and *Evi12* was determined by Southern blotting instead of the PCR/Southern procedure [20, 27].

Table 3. Cas-Br M MuLV cVISs located in the vicinity of a candidate gene.

name of VIS	candidate gene product	frequency (%)	retrieved from	mouse chrom	hum chrom	NCBI acc no Casvis	Celera protein acc no	function	distance
Casvis14	Erg	26	NFS22	16C4	21q22.2	AF432389	mCP17313	TR/DNA binding	50kb
Casvis15	LIM domain binding 1	24	CSL90	19C3	10q24	AF432373	mCP15411	TR/DNA binding	8kb
Casvis16	Ctbp1	14	DA1	5B1	4p16	AF432388	mCP3953	TR/DNA binding	6kb
Casvis17	Calmodulin-like protein	16	NFS22	13A1	10pter-p13	AF432361	mCP19315	signalling	9kb
Casvis18	FLJ13159	13	NFS22	1A3	6q12-q13	AF432362	mCP22109	signalling	8kb
Casvis19	PKC epsilon	26	NFS22	17E4	2p21	AF432368	mCP43026	signalling	10kb
Casvis20	rap guanine nucl exchange factor	41	CSL82	11B1.3	5q31.1	AF432374	mCP81048	signalling	3kb
Casvis21	Gaba A receptor theta	19	CSL91	XA7.1	Xq28	AF432367	mCP80542	receptor	7kb
Casvis22	TGF beta receptor 1	22	CSL30	4A5	9q22	AF432381	mCP5773	receptor	7kb
Casvis23	leucin rich repeat protein	19	CSL26	9F2	3p21.33	AF432375	mCP74372	unknown	10kb
Casvis24	putative protein	31	NFS78	1H4	1	AF432390	mCP90066	unknown	8kb
Casvis25	putative protein	22	CSL31	11C	17q23.3	AF432376	mCP85151	unknown	8kb
Casvis26	putative protein	11	CSL111	10D2	2q14	AF432378	mCP43253	unknown	24kb
Casvis27	AY007814 (KIAA1199-related)	49	DA24	7D2	15q24	AF432360	mCP7266	unknown	60kb
Casvis28	NAS hypothetical protein	55	CSL22	7C	15q24-q25	AF432363	mCP89034	unknown	40kb
Casvis29	putative protein	22	CSL26	15D1	8q24.2	AF432371	mCP60729	unknown	32kb
Casvis30	NAS hypothetical protein	17	NFS36	11E1	17q24	AF432369	mCP75468	unknown	12kb
Casvis31	hypothetical prot XP072192	32	CSL30	15B3.1	unknown	AF432380	mCP43832	unknown	4kb
Casvis32	putative protein	9	CSL26	15F1	12q13	AF432382	mCP83599	unknown	9kb
Casvis33	KIAA1053	6	NFS124	14B	14	AF432391	mCP89924	unknown	15kb

Proviral integration occurred within a certain distance of a gene. Distances between proviral insertions and potential target genes are presented in the last column. For further explanations, see Table 2.

Table 4. Cas-Br-M MuLV insertions within candidate genes not further analysed by directed PCR/Southern.

name of VIS	gene product	retrieved from	mouse chrom	hum chrom	NCBI acc no	Celera protein acc no	function
	Jdp2	NFS56	12D3	14q24.3	AF432405	mCP3269	TR/DNA binding
	FLJ13479	NFS78	7F3	22q13	AF432406	mCP10439	TR/DNA binding
	Ma1-related	CSL204	17E3	2p21-22	AF517726	mCP23717	TR/DNA binding
	Satb1	CSL212	4D2.2	3p23	AF517735	mCP16222	TR/DNA binding
	Rgl1	DA1	1G1	1q24	AF432408	mCP3029	signalling
	Mapk8ip	CSL204	17A3.2	16p13.3	AF517729	mCP23126	signalling
evi2	nf1	CSL204	11B5	17q11.1	AF517717	mCP13469	signalling
	K1AA0766	CSL204	9F2	3p21.33	AF517724	mCP37661	signalling
	GGI-62-like	CSL221	3A1	8q21.11	AF517722	mCP15006	signalling
	myotonin protein kinase	CSL32	12F2	1q32	AF517703	mCP14279	signalling
	Serine/Threonine kinase	CSL65	1A1	unknown	AF517705	mCP70592	signalling
	Gng2	CSL204	14A1	14q21	AF517725	mCP4814	signalling
	Frap1	CSL212	4E1	1p36.2	AF517710	mCP19396	signalling
	Ly108	CSL13	1H2.3	1q21?	AF517697	mCP1591	Ig-receptor
	Tnfrs17	CSL239	16A1	16p13.3	AF517740	mCP6894	receptor / cell cycle regulator
	Vomeronasal receptor 1C2	DA1	7A1	19q13.4	AF432410	mCP57640	receptor
	Lysosomal ass transmembr prot 4 beta	NFS124	12E	8q22.1	AF432396	mCP50469	transporter
	Monocarboxylate transporter-related	CSL201	10B1	6q22	AF517715	mCP33645	transporter
	ATP-binding cassette C4	CSL201	14E4	13q32	AF517712	mCP80048	transporter
	Slc16a6	CSL201	11E1	17q25	AF517737	mCP11951	transporter
	ribonuclease E	CSL32	12A1.1	1p36.1	AF517704	mCP86146	enzyme
	Ran binding protein 11	CSL71	13D2.1	5p12	AF432402	mCP10629	enzyme
	Sarcosine dehydrogenase	CSL203	2A3	9q34	AF517734	mCP50903	enzyme
	Snurf	CSL237	unknown	15q11.1	AF517740	mCP7451	splicing factor
	Apobec2	CSL227	17C	6p12	AF517731	mCP12180	RNA processor
	NOLP4	NFS58	2H1	20q11.1-q11.23	AF517747	mCP18257	nucleolar protein

	Immunoglobulin heavy chain var reg	CSL31	unknown	unknown	AF517702	mCP19302	immunoglobulin
	clpx	CSL203	9B/C	15q22	AF517714	mCP11115	chaperone
	Vamp8	NFS60	6C1	2p12-p11.2	AF517748	mCP61898	membrane protein
Casvis34 *(3)*[a]	HSA-C	CSL32	10B1	6q21	AF432384	mCP11896	unknown
	BC003324	CSL31	5F	12	AF432400	mCP12901	unknown
	NAS hypothetical protein	NFS78	2F1	2	AF517753	mCP49705	unknown
	NAS hypothetical protein	NFS36	17E3	11q13.2	AF517745	mCP70914	unknown
	SH3domain interacting protein	DA33	2C2	2q24-32	AF432412	mCP19091	unknown
	Hypothetical protein XP_058389	CSL212	14A2-3	10	AF517727	mCP4840	unknown
	Ring finger protein 24, goliath-like	CSL137	2F1	20p13-p21	AF517736	mCP17978	unknown
	putative protein	CSL111	18E3	12p13	AF432404	mCP35136	unknown
	putative protein	CSL237	13D2.3	5q11	AF517718	mCP27678	unknown
	putative protein	CSL91	2H3	20q13.1-13.2	AF432403	mCP28479	unknown
Casvis 35 *(2)*[a]	putative protein	DA1	17E3	11q13.2	AF432393	mCP71055	unknown
Casvis 36 *(2)*[a]	putative protein	NFS78	2C1.1	2q23	AF432394	mCP20051	unknown
Casvis 37 *(2)*[a]	putative protein	CSL237	2B	2q22	AF517719	mCP49332	unknown
	putative protein	CSL20	1H4	1	AF517699	mCP35212	unknown
	putative protein	CSL123	14E2.1	13q14.3-q21.1	AF517696	mCP88467	unknown
	putative protein	CSL227	10A3	6q23	AF517709	mCP59156	unknown

Proviral integration occurred within certain genes. [a]Integrations in *Casvis 34-37* were found in more than one distinct leukemia. The number of independent leukemias in which these integrations were detected is between brackets in the first column. No virus insertion name was given yet for insertions found only once. For further explanations, see Table 2.

Table 5. Cas-Br-M MuLV insertions in the vicinity of a candidate gene but identified once.

name of VIS	candidate gene product	retrieved from	mouse chrom	hum chrom	NCBI acc no Casvis	Celera protein acc no	function	distance
Myc	Myc	DA24	15D1	8q24.12-q24.13	AF517716	mCP14505	TR/DNA binding	50kb
	Ddx19	NFS60	8E1	16q22.1	AF517751	mCP73351	TR/DNA binding	3kb
	Pscd1	DA8	11E2	17q25	AF432409	mCP18847	signalling	6kb
	Src homology domain containing prot	CSL212	6C1	2p11.1	AF517720	mCP61938	signalling	1k
	Pps	CSL228	11B5	17p13	AF517732	mCP13369	signalling	7kb
	Pyridoxine kinase	CSL212	17A3.3	21q22.3	AF517739	mCP5282	signalling	2kb
	Ankyrin repeat containing protein	CSL212	11B3	17p12	AF517730	mCP13813	signalling	1kb
	TNF alpha	NFS58	17B1	6p21.3	AF517743	mCP11847	cytokine	10kb
	vomeronasal receptor 1C6	NFS78	13A2	6p21.3	AF432407	mCP34098	receptor	2kb
	low density lipoprotein receptor-related	CSL212	2A1	2q24-q31	AF517711	mCP51218	receptor	27kb
	Pah	CSL123	10C1	12q22-q24.2	AF517695	mCP4545	enzyme	30kb
	Rho GTPase-activating protein	DA1	6E2	12	AF517744	mCP60910	enzyme	32kb
	lzp-s	CSL16	10D2	12q15	AF432398	mCP23874	enzyme	9kb
	Gpx5	CSL221	13A2	6p21.2	AF517719	mCP22123	enzyme	15k
	L21	CSL22	4A5	17q12-q21	AF517700	mCP53488	ribosomal protein	5kb
	Rps15	CSL31	5E5	19p13.3	AF517701	mCP19940	ribosomal protein	5kb
	ribosomal protein L11	CSL71	12A1.1	1p36.1	AF517707	mCP87152	ribosomal protein	30kb
	ribosomal protein P0	CSL203	2H1	18	AF517733	mCP30352	ribosomal protein	7kb
	Elastase	CSL13	15F1	12q12	AF432397	mCP9570	protein metabolism	6kb
	Slc7a1	CSL212	5G3	13q12	AF517738	mCP18766	transporter	24kb
	phospholipid-transporting ATPase related	NFS61	3A3	3	AF517749	mCP3736	transporter	3kb
	putative protein	CSL16	2C1.1	2	AF517698	mCP49486	DNA repair	26kb
Casvis 38 *(3)*[a]	Mcp	CSL33	1H5/H6	1q32	AF432386	mCP15315	complement	40kb

							activation	
Casvis 39 *(3)*[a]	Kir5.1	NFS61	11E1/E2	17q23.1-q24.2	AF432395	mCP33805	ion channel	134kb
	Actg1	NFS107	11E2	17q25	AF432411	mCP18943	cytoskeleton	10kb
Casvis 40 *(2)*[a]	VSP41	DA2	13A1	7p14-p13	AF517754	mCP3593	unknown	6kb
Casvis 41 *(2)*[a]	putative protein	NFS58	18C	5q21	AF432385	mCP77581	unknown	6kb
	putative NAS protein	CSL212	9A2	19p13.2	AF517721	mCP55555	unknown	5kb
	putative protein	NFS58	12A1.2	2p25	AF517746	mCP78746	unknown	14kb
	putative protein	CSL203	16B5	3q13.1	AF517723	mCP87422	unknown	11kb
	putative protein	NFS124	6F1	9p13.3	AF517728	mCP62222	unknown	25kb
	putative protein	CSL201	15F1	12q12-q13	AF517742	mCP83351	unknown	13kb
	putative protein	NFS107	18D1/D2	unknown	AF517750	mCP77834	unknown	10kb
	putative protein	CSL65	12A1.1	2p24	AF517706	mCP86238	unknown	1kb
	putative protein	CSL78	3H2	1q31-32.1	AF517708	mCP89319	unknown	16kb
	putative protein	NFS61	3A3	3q26	AF517752	mCP25237	unknown	22kb

Proviral integration occurred within a certain distance of a gene. Distances between proviral insertions and potential target genes are presented in the last column. [a]Integrations in *Casvis 38-41* were found in more than one distinct leukemia. The number of independent leukemias in which the integrations were detected is between brackets in the first column. For further explanation, see Table 2.

Discussion

The identification of cVISs has previously become less labour-intensive through the development of various PCR procedures. In the present study we used two distinct complementary PCR procedures and applied an additional PCR/Southern strategy (Figure 1) to further increase the speed of identifying novel cVISs. Using these techniques we identified 126 virus integration sites in Cas-Br-M MuLV induced myeloid leukemias and determined that 39 of 41 insertions analysed represented a common VIS. Based on these findings, it may be deduced that of the other virus integration sites a comparable high percentage may in fact represent cVISs as well. PCR/Southern blotting analysis in the panel of leukemias using locus- and LTR-specific primers should assure whether this is indeed the case. The detection of previously cloned cVISs and their corresponding leukemia genes (*Evi1*, *Hoxa7*, *c-Myb*, *Cb2/Evi11*, *His1* and *Evi12*) indicates that the PCR/Southern based technique that we applied is reliable. It also emphasises the significance of the novel cVISs found, and warrants further exploration of the strategy to clone the large number of cVISs and disease genes that remain to be identified in leukemia and lymphoma.
The frequency of proviral insertions is sometimes exceptionally high, e.g. 58% for *Hoxa7* and *Transcription elongation factor SII* or 55% for *Midline-1*. Many other insertions were found at frequencies between 10 and 30%. In fact only a minority of common integrations were found at a frequency lower than 10%. The most likely explanation for these high frequencies is that these retrovirally-induced leukemias are oligoclonal [23, 32]. In fact, proviral insertion in certain genes (e.g. *Nm23-M2* or *Mid1*) identified by the PCR/Southern strategy, were undetectable by applying classical Southern blotting (data not shown), suggesting amplification of proviral integrations present in a minor clone. Since leukemia development is believed to be a multigenetic process it will be important to dissect which disease genes frequently cooperate in transformation. A thorough analysis of the large series of common integrations found in this cohort of leukemias should reveal which disease loci frequently coincide. However, oligoclonality does not allow a reliable analysis to identify cooperation between disease genes in these virus-induced tumours, a caveat also being recognised by other investigators [23, 32].
One group of genes frequently targeted by retrovirus comprises the ones that encode for nuclear proteins involved in transcriptional control or other nuclear functions. The fact that this group of genes is so often subject of mutations in mouse leukemias as well as in human disease underlines the critical role these proteins may have in development and indicates that mutations in those genes are key-events in leukemic transformation. Among integrations in previously reported DNA-binding protein genes *Evi1, Hoxa7* and *c-Myb*, we also found frequent proviral insertions in a region in the vicinity of *Erg*. The latter gene is the murine homologue of human *ERG*, which has been shown to be expressed as *TLS/ERG* fusion gene in human AML with translocation t(16;21)(p11;q22) [19].
The high-throughput search for novel cVIS provides a unique opportunity not only to clone novel transforming genes but may also lead to the identification of new disease pathways that have not previously been implicated in leukemia. Several novel potential disease genes have been identified as frequent proviral targets in our screen, which may relate with novel pathways. Among these are the *Mid-1* gene encoding the Midline-1 ringfinger protein, which is mutated in patients with Opitz-syndrome [33] and Nm23-M2 a gene of which a human homologue has been identified as a critical gene in lymphoma development. A large set of common virus insertion sites were

identified harbouring novel genes, encoding for putative proteins, with unknown function. Each of these novel disease loci may become of interest following further cloning, characterisation and functional screening using *in vitro* and *in vivo* models. Obviously, the PCR based strategies used are highly sensitive and almost every insertion that was found represented a common VIS. It is possible that certain VISs, particularly the ones found less frequently (<5%) have been pulled out due to the high sensitivity of our approach, and may not resemble disease loci. However, additional approaches are required anyway to further select for the critical genes that play a major role in leukemic transformation. An important question to be answered in the near future is whether the genes identified in our *in vivo* functional screens are of importance for the development of human disease. As a final filter, high-throughput nucleotide sequencing, real-time PCR and gene-array analysis should prove the role of these genes in the development of human AML.

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Chapter 5

Nm23-M1, *-M2*, and *–M3*, members of the nucleoside diphosphate kinase family of genes, are frequent proviral targets in Cas-Br-M MuLV induced leukemias in mice

Marieke Joosten, Fokke Lindeboom, Antoinette van Hoven-Beijen, Yolanda Vankan-Berkhoudt, Bob Löwenberg, Marieke von Lindern, and Ruud Delwel

Abstract

Nm23-M2 was recently identified as a common virus integration site in Cas-Br-M MuLV induced mouse leukemias. *Nm23-M2* is one of the eight members of the highly homologous family of *nm23*-genes, which can be divided into two groups. One group contains the genes *nm23-M1* to *M4*, which are 44% to 88% similar in amino acid sequence. The second group, consisting of *nm23-M5* to *M8*, shares far less homology with the first group or with each other (3% to 30%). *Nm23-M1* and *nm23-M2*, which share the highest homology, are located in tandem on mouse chromosome 11. Here we demonstrate by PCR/Southern blotting that *nm23-M1* and *nm23-M3* were frequent targets of proviral insertion as well. The other five members of this family appeared not to be located in a common virus insertion site. This indicates that multiple but not all members of the *nm23*-family may be involved in leukemic transformation. Since all members of the *nm23* family of genes contain nucleoside diphosphate kinase (NDPK) activity, and only three of the members may act as a disease gene, it is possible that another function these three genes may have in common is responsible for their transforming activity. Our findings that *nm23-M1*, *-M2*, and *M3* are frequent proviral targets in mouse leukemia and the fact that the human equivalents of nm23-M1 and –M2 are reported to be abnormally expressed in certain cases of leukemia or lymphoma, predicts a role for these genes in leukemia development. Experiments using the *in vitro* 32D system revealed that aberrant expression of nm23-M2 did not affect neutrophilic differentiation, a major characteristic of myeloid leukemia. Studies using alternative models should demonstrate how abnormal expression of the nm23 genes may affect myeloid development.

Introduction

Nm23-M2, also indicated as NDPK-B, is a member of a gene-family containing a **n**ucleoside **di**phosphate **k**inase domain capable to convert GDP to GTP. The nm23 family is highly conserved throughout species: *nm23* genes are not only found in mammals, but also in Zebrafish [1], Drosophila melanogaster (*awd*, abnormal wing discs) [2] and Dictyostelium discoideum [3]. The nm23 family consists of 8 members that are all found in human and mouse [4]. This high conservation suggests an important role for the *nm23*-genes in basic cellular processes. In addition to the kinase domain, nm23-M2 possesses a DNA binding domain by means of which it is able to regulate *c-Myc* expression in a positive way [5]. This transactivation of *c-Myc* has been shown to be independent of the NDPkinase activity [6].
Ninety-eight percent of the nm23-M2 amino acid sequence corresponds to the composition of the human homologue *nm23-H2*, located on chromosome 17q21.3 in tandem with the homologous isoform *nm23-H1* [7]. Nm23-H1 and nm23-H2 are designated differentiation inhibitory factors, a function that is independent of their NDPkinase activity [8]. Enhanced expression of nm23-H1 was reported to be associated with hematopoietic malignancies in man [9, 10]. Interestingly, high expression levels of the human homologue *nm23-H2* are associated with a poor prognosis in AML-M2, –M4, and –M5 [11].
Recently, the gene *nm23-M2* was found to be a common virus integration site (VIS) in primary mouse leukemias. Genes identified through retroviral insertional mutagenesis have been reported to play an important role in human leukemia as well. Examples are *Evi1* [12, 13], *Erg* [14, 15], *NF1* [16-18], *N-Myc* [19, 20], or *Bcl1* (*Cyclin D1*) [21, 22]. Proviral integration in a common virus insertion site may result in abnormal expression of the target gene. Insertion of the provirus 5' as well as 3' of a particular gene usually results in enhanced gene expression, while integration into the coding region may result in expression of aberrant (active or inactive) protein [23, 24]. Previous observations demonstrating that high Nm23 expression associates with aggressive leukemias and lymphomas [9-11] together with our finding that *nm23-M2* is a frequent target for proviral insertion in mouse disease, suggest a direct role for nm23 genes in malignant development of hematopoietic tissues.
To further investigate the importance of nm23-M2 and other family members in transformation of hematopoietic precursor cells we first studied the exact distribution of viral insertion in the nm23-M2 gene locus. We next investigated whether any of the other family members (nm23-M1 through -M8) are located in a common virus insertion site as well. To study the effect of aberrant expression of nm23-M2 on myeloid development, the gene was introduced into the 32D/G-CSF-R model. Our data demonstrate that of the eight different family members, nm23-M1, -M2 and -M3 are frequent targets for retroviral insertion, whereas in the loci containing the nm23-M4 through -M8 genes no viral insertions were detectable. None of the other family members have been shown to be involved in leukemia development. Using the 32D model a clear role for nm23-M2 in leukemia development was not identified yet.

Materials and Methods

PCR analysis and Southern blot hybridisation
The frequency of virus integrations was determined on primary leukemias (CSL 11-17, 19, 21-23, 25-27, 29, 31-33, 35, 36, 38, 39, 41, 43, 45-51, 53-61, 63-72, 74-83, 85, 87-109, 114, 117, and 123). To detect virus insertions in the 5'region of the gene, 1μg of genomic DNA from these leukemias was subjected to PCR with primer pLTR1 and a gene-specific primer A. 1μl of PCR product was transferred to a nested PCR reaction using primer pLTR2 and a nested gene-specific primer B. Cycle conditions for both reactions were 1 cycle 5' 94°C, 30 cycles 30"94°C, 1' Tm, 1'30"72°C, 1 cycle 5'72°C. Tm was specific for each primer combination and was between 48°C and 62°C. PCR was carried out using Taq polymerase (Amersham Pharmacia Biotech, Roosendaal, The Netherlands). PCR products were electrophoresed in a 1.5% agarose gel and transferred to Hybond-N^+ nylon membranes (Amersham Pharmacia Biotech) with 0.25 M NaOH/1.5 M NaCl. Membranes were hybridised with a ^{32}P-end-labelled locus specific primer C. Labelling was carried out using T_4kinase (USB, Cleveland OH, USA) according to the instructions of the provider. Subsequently, blots were stripped in 0.4M NaOH for 30 minutes at 45°C, neutralised using 0.2M Tris-HCl, 0.2% SDS, and 0.1xSSC for 15 minutes at 45°C and hybridised with ^{32}P-end-labelled Cas-Br-M MuLV specific primer pLTR3. Blots were exposed for autoradiography with a KODAK film and an intensifying screen. After 15 minutes of exposure films were developed and analysed. Nm23-M2 was analysed for the presence of proviral insertions in both 5'and 3'gene region, and in both orientations of the virus. For these experiments, primer combinations 1-A, 2-B, 3-C, 1-D, 2-E, 3-F, 4-A, 5-B, 6-C, 4-D, 5-E, 6-F were used (see also Figure 1). The sequences of the primers used for the detection of virus integrations in the different nm23 genes are present in Table 1. All primers were obtained from Gibco Life Technologies (Breda, The Netherlands).

Sequencing
Samples were prepared using the Bd-sequencing kit according to instructions from the provider (PE Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). Nucleotide sequencing was carried out on an ABI 310 automatic sequencer (PE Biosystems) using primers M13forward (5'-GTA AAA CGA CGG CCA GT-3') and M13reverse (5'-GGA AAC AGC TAT GAC CAT G-3').

Cells
32D/G-CSF-R-cells were cultured in RPMI 1640 medium (GibcoBRL., Life Technologies, Breda, The Netherlands) supplemented with 10% FCS and 10 ng/ml murine IL3 at a concentration of $2x10^5$ cells/ml. For experiments, cells were washed twice with HBBS (Gibco BRL.) and seeded in RPMI 1640 medium without any additives. Factor stimulation was with 100ng/ml IL3, 10ng/ml G-CSF.

Nm23-M2 expression constructs
cDNA of nm23-M2 (NCBI accession number X68193) was cloned in the pBluescript KS vector. A triple HA-tag was added 5' of the startcodon of the introduced sequence. Artificially introduced *NcoI* and *BspHI* restriction sites were used to isolate HA-nm23 and HA-LBD. Fragments were blunted by adding 1U of Alkaline Phosphatase (Roche, Mannheim, Germany) for 15' at 37°C. Subsequently the fragments were gel-

Table 1: Primer sequences used to detect integrations in *nm23* genes.

Gene	primer A	primer B	primer C	primer D	primer E	primer F
Nm23-M1	ATGATCTCGCCCA CCAGCC	CATCAGGCTTGAT GGCAATG	GTACGCTCACTGT TGGCC			
Nm23-M2	TCGCCCACCAGG CCGCGC	TGCACGCCATCTG GCTTG	CCAACCTCGAGCG TACCTTCATTG	GATGGGTTTCTGG ACAGAGCTC	CATCCCACTGAC AGGATGGATC	CAGTTCCAAAGTC TTTAT
Nm23-M3	CCCGCCCGGGTG CTCAC	GAGGTTAGCAAA GATGGT	AGCACCAGACAGA TCATG			
Nm23-M4	GCAGGCACTGAA AGCGTG	GCACAACAGCGC CCGCAG	GACGCGCCCGAAA AGGC			
Nm23-M5	CCTCTTCTTTGTC AACAAC	TGGCTTGATAAG GGCTAG	CTACATATATCTG AGGCAG			
Nm23-M6	GCATCAGGCTTG ATCAGG	GTGTGAGCTGGA GAGCTTG	GGACTTCGCAAGA TGGAG			
Nm23-M7	GACAGGCTCTCA TACTGC	CGAGGATGCAAC GCCGGC	GAAGAGAAGGCGT CAATAAC			
Nm23-M8	GTTCAGCAACAT CTCATC	CAAGTTCTGACTA TTGACG	CTGACTGTAGCTG GACTTC			

LTR	primer 1	primer 2	primer 3	primer 4	primer 5	primer 6
	CTTGCTGTTGCAT CCGACTGG	GTCTCGCTGTTCC TTGGGAGG	GGTCTCCTCAGAG TGATT	ACCACAGATATC CTGTTT	GTTCCTGACCTTG ATCGAAC	CCCTATTCTCAGT TCGGTA

Virus integrations in genes from the nm23-gene family were detected by a nested PCR-Southern blotting technique. For proviral insertions in the 5'region of the genes, primers A to C were used together with LTR primers 1, 2, and 3. For virus integrations in the nm23-M2 gene, both 3'and 5'regions were checked for integrations in two orientations (5'3'and 3'5'), using also primers D, E, and F, and LTR primers 4, 5, and 6. All primer sequences are given 5'to 3'.

purified and inserted into the blunted EcoRI site of the eukaryotic expression vector pLNCX.

Preparation of retrovirus and transduction of 32D cells
The retrovirus producer Phoenix-E cells were split to $2x10^6$/ml 24 hours prior to transfection. Chloroquine was added to each plate 5 minutes before transfection at a final concentration of 25μM. 10μg DNA was transfected in the cells by the calcium phosphate coprecipitation technique. Chloroquine was removed 9 hours after transfection and fresh medium was added to the cells supplemented with 10% foetal calf serum (FCS, Gibco Life Technologies) and 10ng/ml murine IL-3. The supernatant was collected from the cells 48 hours post-transfection. $2.5x10^5$ 32D/G-CSF-R cells were repeatedly transduced with 1.5 ml supernatant on retronectin coated plates. The cells were replaced from these plates 48 hours after transduction and the virus was removed by washing the cells three times with phosphate-buffered saline (PBS, Gibco Life Technologies). The transduced 32D cells were maintained in RPMI 1640 medium (Gibco Life Technologies) supplemented with 10% FCS and 10 ng/ml murine IL-3 at a concentration of $2x10^5$ cells/ml. Cells were subjected to limiting dilution to obtain monoclonal cell populations. The infected 32D clones were induced to terminally differentiate by shifting them from IL-3 supplemented medium to medium containing 10ng/ml G-CSF. Cytospins stained with May-Grünwald Giemsa and cell counting were done daily.

Western blotting
Samples of 32D/G-CSF-R-cells were taken at time points 0, 2, 4, 6, 7, 8, and 9 days during differentiation. Cells were washed twice with ice-cold phosphate-buffered saline with 10mM $NaVO_4$. Subsequently, cells were spun down and lysed by incubation for 10 minutes at 4°C in Lysis buffer (20mM Tris pH7.4, 150 mM NaCl, 1% NP40, 0.1% SDS, 0.5% Sodium deoxycholate, 5 mM EDTA). Insoluble material was removed by centrifugation for 15 minutes at 10000xg at 4°C. Following separation on 12.5% PAGE and Western blotting, filters were incubated with the nm23-M2 antibody (Seikagaku, Falmouth, MA, USA). Immune complexes were detected with horseradish peroxidase-conjugated goat-anti-rat IgG serum (Santa Cruz, Santa Cruz, CA, USA, catalogue #32006), followed by an enhanced chemoluminiscence reaction (NEN® Life Science Products, Zaventem, Belgium).

Results

Proviral insertions 5' and 3' of the nm23-M2 gene

We previously identified by inverse PCR a proviral insertion in the promoter sequence of nm23-M2. Directed PCR further demonstrated that the 5' region of nm23-M2 is a common site of proviral insertion. To study the frequency of proviral integrations in the nm23-M2 locus we designed a directed nested-PCR protocol applying LTR specific and locus specific primers followed by a Southern blot approach using additional LTR and locus specific primers (Figure 1). Proviral insertions were found in the 5' as well as 3' area of nm23-M2 (Figure 1). The insertions of the provirus occurred in the 5'3' as well as in the 3'5' orientation and were detected in 56% of the primary leukemias studied.

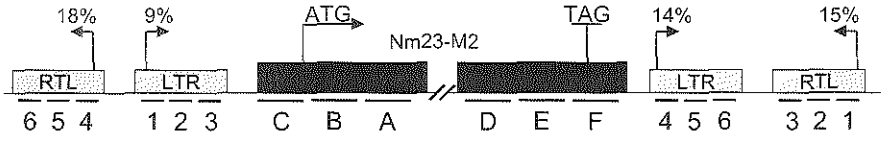

Figure 1. Schematic representation of the *nm23-M2* locus.
A PCR-Southern blotting approach was used to detect proviral insertions in the 5' and 3' regions of *nm23-M2*. Primers are depicted. For analysis of the 5' region of *nm23-M2* LTR primers 1, 2, and 3 and nm23-M2 primers A, B, and C were used to identify proviral insertions in the 5'3' orientation, and LTR primers 4, 5, and 6 in combination with the before mentioned nm23-M2 primers were used to detect integrations in the 3'5' orientation. For proviral insertions in the 3' region of the gene primers D, E, and F together with LTR primers 4, 5, and 6 resulted in identification of insertions in the 5'3' orientation, whereas with LTR primers 1, 2, and 3 integrations in the opposite direction were demonstrated. Proviral insertions were found in the 5' as well as 3' area of nm23-M2. The insertions of the provirus occurred in the 5'3' as well as in the 3'5' orientation and were detected in 56% of the primary leukemias studied. In the figure the percentage of occurrence of each type of integration is annotated.

Proviral insertions are present 5' of nm23-M1, M2 and M3

Nm23-M2 is part of a highly homologous family of nm23 genes. The amino acid comparison between the different nm23 homologues is shown in Figure 2A. The nm23-family can be divided into two groups. One group containing the first four members (nm23-M1, -M2, -M3 and -M4) between which homologies vary from 44% to 88% in mice (Figure 2B) and from 58 to 88% in human (data not shown). The expression of these genes is ubiquitous although variable in place and time during development [4]. The proteins from the second group containing the other members (nm23-M5, -M6, -M7 and -M8) share far less homology with the first group or with each other i.e. 3% to 30% in mice (Figure 2B) and 25% to 45% in human (data not shown). Nm23-M5, M7, and -M8 are mainly expressed in the testis, nm23-M6 is ubiquitously expressed [4].

Nm23-M2 shares the highest homology (88%) with the nm23-M1 family member. Interestingly, nm23-M1 and nm23-M2 are located in tandem on mouse chromosome 11 with a separation of approximately 3.8kb between the 3' end of nm23-M2 and the 5' mRNA start of nm23-M1 (Figure 3). We investigated whether nm23-M1 was also a

Figure 2A

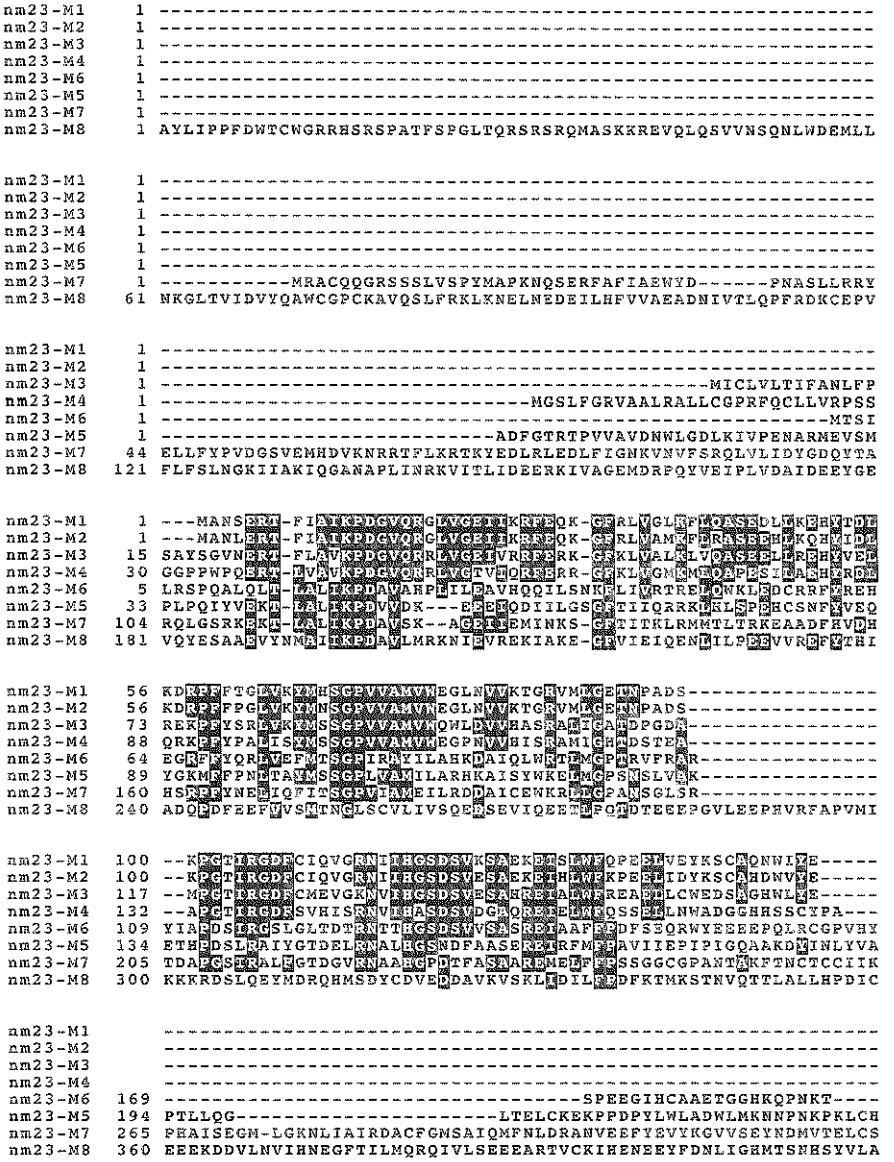

Figure 2.

B.

	nm23-M1	nm23-M2	nm23-M3	nm23-M4	nm23-M5	nm23-M6	nm23-M7
nm23-M2	88%						
nm23-M3	56%	55%					
nm23-M4	44%	44%	53%				
nm23-M5	20%	22%	19%	17%			
nm23-M6	25%	24%	27%	22%	30%		
nm23-M7	12%	11%	12%	13%	18%	14%	
nm23-M8	13%	11%	15%	14%	11%	14%	3%

Figure 2. Amino acid comparison between the 8 murine nm23 family members.
A. Visual comparison of the 8 nm23 genes. Identities are on a black background and similarities are on a gray background.
B. Homologies (% identity) between all 8 murine nm23 genes.

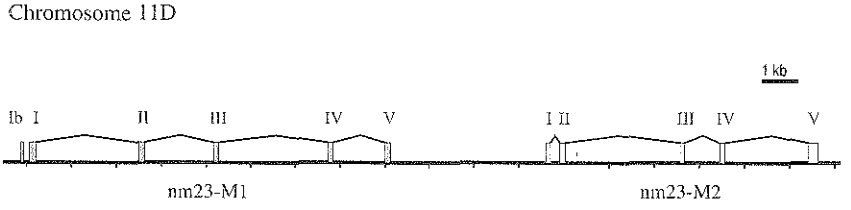

Figure 3. *Nm23-M1* and *nm23-M2* localisation in the mouse genome.
Nm23-M1 and *nm23-M2* are located in tandem on mouse chromosome 11 with a separation of approximately 3.8kb between the 3' end of nm23M2 and the 5' mRNA start of nm23-M1.

frequent target of proviral insertion. PCR/Southern blotting analysis using specific LTR primers and primers in exon 1 from nm23-M1 revealed that 48% of the primary CSL leukemias contained a VIS in the 5' area of the *nm23-M1* gene.
Since both nm23-M2 and M1 were frequent targets of proviral insertion, it was worthy to investigate which of the remaining *nm23*-family members should also be considered as potential leukemia disease genes. Therefore we designed primers in all other known nm23-genes to search for integrations in the 5' regions (sequences annotated in Table1). In 20% of the primary CSL leukemias proviral insertions had taken place 5' of the *nm23-M3* or *DR-nm23* gene. None of the other *nm23*-genes appeared to be located in a common virus integration site.

Overexpression of nm23-M2 in 32D/G-CSF-R cells
The 32D/G-CSF-R system has been shown to be a valuable model to study the effect of aberrantly expressed disease genes on neutrophilic differentiation. We generated nm23-M2/pLNCX expression constructs to which a HA-tag was cloned in frame 5'of the murine nm23-M2 cDNA. HA-nm23-M2 was introduced into 32D/G-CSF-R and Western blotting analysis demonstrated the presence of the ectopic protein (Figure 4A). In vitro cultures using IL3 or G-CSF were carried out using five HA/nm23-M2

expressing as well as five vector control 32D clones. All ten clones were fully dependent on growth factors and no differences in proliferative response were observed in response to either IL3 (data not shown) or G-CSF (Figure 4B). HA-nm23-M2 expressing as well as vector control 32D/G-CSF-R lines fully differentiated towards neutrophils in the presence of G-CSF (data not shown). So far, no differences were observed between the two distinct groups of clones.

Expression of nm23-M2 in 32D/G-CSF-R-cells during differentiation

Expression of endogenous *nm23-M2* is downregulated during differentiation of the erythroid progenitor cell line I/11 (Chapter 6). To investigate whether nm23-M2 expression decreases during granulocytic differentiation of the murine myeloid 32D/G-CSF-R-cell line, the cells were induced to terminal differentiation using G-CSF. Total protein was isolated from cells in different stages of differentiation and analysed by western blotting. Nm23-M2 protein levels did not decrease significantly following 9 days of culture of 32D/G-CSF-R with G-CSF (Figure 5).

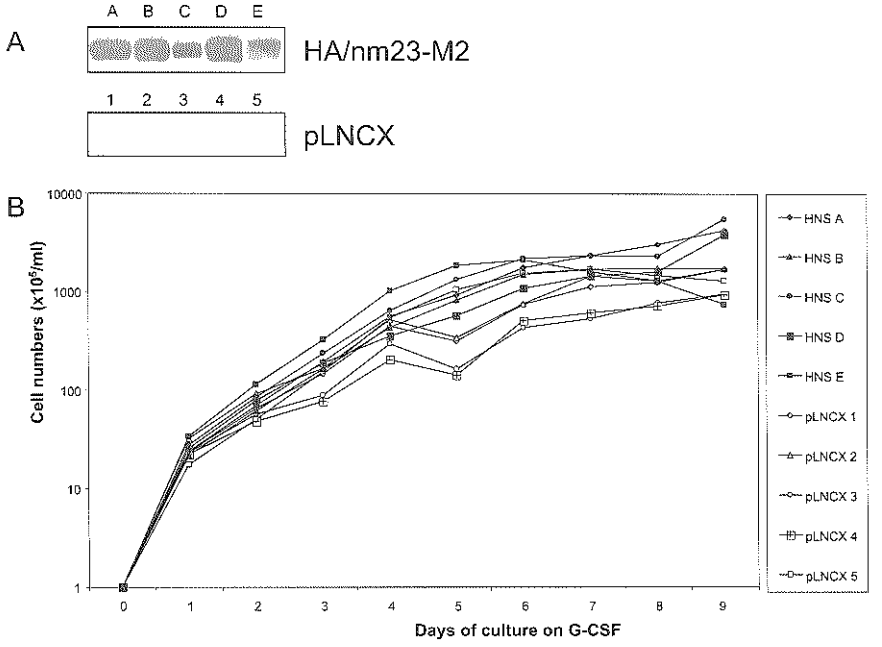

Figure 4.

A. Western blot showing expression of the HA tagged nm23-M2/pLNCX expression constructs in 32D/G-CSF-R (A to E). No nm23-M2 message was present in the pLNCX control transfectants (1 to 5).

B. No differences in proliferation were observed in 32D[G-CSF-R/HA-nm23-M2] cells compared to control clones in response to G-CSF.

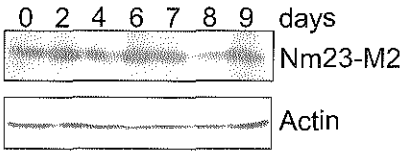

Figure 5. Normal expression of *nm23-M2* during differentiation of 32D/G-CSF-R cells.
Western blot demonstrating that nm23-M2 protein level did not decrease significantly following 9 days of culture of 32D/G-CSF-R with G-CSF. Staining for actin was used as a control for equal loading.

Discussion

The gene *nm23-M2* was identified as a common VIS in murine primary leukemias. Integrations had taken place both 5' and 3' of the coding sequence of the gene in different retrovirally induced leukemias in mice. In addition we showed that the two most closely related homologues of nm23-M2, i.e. nm23-M1 and nm23-M3 were also frequent targets for proviral insertion, the other five nm23 family members (M4 through M8) were not. Although these data clearly suggest a role of nm23-M1, M2 or M3 in leukemic transformation, aberrant expression of nm23-M2 into 32D/G-CSF-R-cells did not have an effect on the proliferative response or neutrophilic differentiation of these cells.

Proviral integrations 5' or 3' of a gene usually result in an overexpression, suggesting that high uncontrolled expression of the gene may be a critical event in leukemic transformation. High expression of the nm23 genes or their products has not been demonstrated in our retrovirally derived leukemias (unpublished data). The main reason for this cavity is that the tumours that are obtained from these mice are oligoclonal [25, 26]. The proviral insertions are therefore most likely present in only a certain percentage of leukemia cells in a particular mouse and high gene expression is most likely masked. The idea that high expression of nm23 genes may indeed be critical in leukemia progression comes from human studies. High expression of NM23-H2, the human homologue of nm23-M2, has been shown to be associated with a certain group of poor-risk myeloid leukemias [11, 27]. Furthermore, high expression of NM23-H1 has convincingly been shown to be associated with poor-risk lymphomas [9, 10]. *In vitro* or *in vivo* models should further elucidate how aberrant expression of nm23-M1, M2 or M3 may play a role in leukemia development.

One such model is the 32D *in vitro* system These 32D cells expressing the G-CSF-R can be maintained *in vitro* using IL3, but differentiate towards mature neutrophils in the presence of G-CSF. The model has been successfully used to demonstrate the transforming ability of e.g. Evi1, Myb, Cb2 or a mutated form of the G-CSF-R [28-31]. Introduction of the *nm23-M2* gene into the 32D-wt cells however, did not at all affect the proliferative capacity of the cells nor did it interfere with neutrophilic differentiation. Several possible explanations may explain this particular finding. It is possible that nm23-M2, like many other transforming genes, is oncogenic only in conjunction with other proto-oncogenes. This particular gene should then be introduced in 32D/G-CSF-R cells as well. It could also be that in the 32D cells the signalling pathway involved in the regulation of nm23-M2 expression is already

defective. This could explain our finding in Figure 5 showing that nm23-M2 protein levels are not downregulated during granulocytic differentiation of 32D cells. Other studies have nicely demonstrated that nm23 protein levels are normally downregulated during development [32]. Another possibility is that nm23 genes do not disturb granulocytic development but that they interfere with development of other lineages. AML is not only characterised by a block of neutrophilic differentiation, but also by a lack of mature cells of other differentiation lineages. Since the incapability of other hematologic precursors to develop may also be the result of genetic defects, we are currently investigating whether nm-23-M2 may interfere with erythroid development using an in vitro erythroid differentiation model. The *nm23*-genes are part of an evolutionary highly conserved gene family. Eight distinct members were identified of which the *nm23-M1* and *nm23-M3* members were originally identified in tumour tissue. *Nm23-M1* was identified in a murine melanoma cell line [33]. *Nm23-M3* was isolated from differential screening of a blast-crisis CML cDNA library [34]. The original identification of *nm23-H2* was based on sequence homology with *nm23-H1* [35]. All other nm23-genes have been found by screening of EST sequences in databases [36-38]. Since integrations were found in *nm23-M1*, *nm23-M2*, and *nm23-M3* genes and not in the other five family members, it is likely that the transforming capacity of these genes is not caused by an uncontrolled nucleoside diphosphate kinase activity. Studies on *nm23-M2* have demonstrated that besides a nucleotide diphosphate kinase activity the protein was also capable to act as a *c-Myc*-transactivator, indicating that these proteins may indeed possess other functions [5]. Since the Myc-transactivation capability was not ascribed to nm23-M1 or M3, we suggest that the transforming ability of these three nm23 homologues may be caused by yet another specific function that these three family members have in common.

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Chapter 6

Polysome association of nm23-M2 mRNA in erythroid cells is cytokine regulated, phosphoinositide-3-kinase dependent, and decreases with maturation

Marieke Joosten, Montserrat Blázquez Domingo, Florence Boulmé, Antoinette van Hoven-Beijen, Bianca Habermann, Ernst Müllner, Hartmut Beug, Bob Löwenberg, Ruud Delwel, and Marieke von Lindern

Abstract

Recently the gene nm23-M2 was found to be a common virus integration site, suggesting that this gene plays a role in leukemia development. Using a second approach to identify genes that may be involved in leukemogenesis, i.e. high throughput screening of cytokine induced genes by micro-array analysis, again nm23-M2 was identified. Northern blot analysis of nm23-M2 expression did not reveal alterations in mRNA levels between growth factor stimulated cells and controls. However, it was found that polysome binding of nm23-M2 mRNA is growth factor dependent, indicating a translational control of gene expression. Nm23-M2 polysome binding as well as protein expression declined during differentiation of the erythroid progenitor cell line I/11, indicating a change of translation of nm23-M2 during erythroid maturation. Regulation of nm23-M2 polysome binding was shown to be PI3K dependent, while inhibition of the MEK/ERK pathway did not result in a reduction in polysome association. Moreover, it was found that nm23-M2 transcript started with a Terminal Oligopyrimidine (TOP) tract, a stretch of pyrimidines that is present in many translationally controlled genes. Comparison of expression of nm23-M2 with other translationally regulated genes demonstrated that nm23-M2 behaves like other TOP-containing genes. These data together indicate that PI3K targets like nm23-M2 are likely involved in renewal induction, suggesting a mechanism by which nm23-M2 may be involved in leukemogenesis.

Introduction

Hemopoietic progenitors can be induced to undergo renewal divisions or they may differentiate into mature cells [1, 2]. This balance is controlled by proteins inherently expressed in the cells, including transcription factors, in concert with molecules that transmit signals in response to extracellular factors like growth factors, cytokines and hormones [3, 4]. Recently we described how human and murine erythroid progenitors can be induced to undergo renewal divisions in presence of erythropoietin (Epo), stem cell factor (SCF) and dexamethasone (dex), whereas they undergo terminal differentiation upon stimulation by Epo in absence of SCF [5, 6]. Leukemic alterations disturb the balance between expansion and differentiation as they constitutively mimic processes involved in renewal. For instance, the v-ErbB oncogene, encoded by the avian erythroleukemia virus (AEV) appeared to mimic the synergistic effect of Epo plus SCF induced signal transduction [6], which induces factor independent renewal divisions. Genes normally expressed under conditions of cell renewal, i.e. induced in presence of Epo plus SCF, and downmodulated during terminal differentiation are candidate genes to be upregulated in leukemia.

A direct approach to identify disease genes in hematopoietic malignancies is the characterisation of virus insertion sites in retrovirally induced leukemia. Genes recurrently targeted by retroviruses in experimental mouse leukemia models often appeared to be involved in human leukemia, either activated by translocations (e.g. *Evi* [7, 8], *Erg* [9, 10], *NF1* [11-13] and *N-Myc* [14, 15]) or by point mutations (e.g. *Bcll* (*Cyclin D1*) [16, 17]).

We have started to make an inventory of genes that may have a role in leukemogenesis, employing two different approaches, i.e. 1) high throughput screening of cytokine induced genes by means of micro-array analysis [18-20] and 2) identification of leukemia promoting genes by retroviral insertional mutagenesis.

In a recent screen of retroviral integration sites, 9 % of the samples in a panel of primary CSL leukemias harboured integrations in the *nm23-M2* gene [21]. Nm23-M2, or NDPK-B, is a member of a gene-family containing a **n**ucleoside **di**p**h**osphate **k**inase domain able to convert G-protein bound GDP to GTP [22-24]. Nm23-M2 also possesses a DNA binding domain by means of which it is able to regulate c-myc expression [25]. *Nm23-M2* has 98% amino acid identity with the human homologue *nm23-H2*, located on chromosome 17q21.3 in tandem with the isoform *nm23-H1* [26]. Nm23-H1 and nm23-H2 are considered differentiation inhibitory factors, a function that is independent of its NDPkinase activity [27]. Enhanced expression of nm23-H1 was reported to be associated with hematopoietic malignancies in man [28, 29].

Gene expression may be controlled at the transcriptional as well as at the translational level. Nuclear transcribed mRNAs contain a cap-structure (m^7GpppN) at their 5'end. Eukaryote initiation factor 4E (eIF4E) binds the cap structure and forms a complex with other translation initiation factors [30]. This complex scans the mRNA until it encounters an ATG-startcodon in a favourable sequence environment [31, 32]. Subsequently the initiation factors dissociate and a 60S ribosomal subunit associates, and protein synthesis starts. Structural elements in the UTRs can render translation dependent on cell type, developmental stage or environmental conditions. Such structures may consist of a terminal **o**ligo**p**yrimidine-(TOP)-tract, repeats forming dsRNA stretches that require RNA helicase activity, structures that are stabilised by conditional binding of proteins, or upstream ATGs and ORFs that require recruitment of the translation machinery to an **i**nternal **r**ibosomal **e**ntry **s**ite (IRES). In such

situations, specific cellular signals, e.g. phosphorylation of proteins involved in recruitment may be required for mRNA translation. Signalling pathways known to regulate translation following growth factor stimulation include the MEK/ERK pathway, resulting in activation of $p90^{RSK}$ and MNK-1,-2, and PI3K/PKB which activates mTOR and S6kinase [33].

Genes upregulated upon growth factor stimulation may be actively involved in preserving cell growth, while inhibiting differentiation. In the present study, we carried out micro-array analysis, using cDNA derived from growth factor stimulated (Epo plus SCF; representing renewal conditions) versus factor deprived erythroid precursor cells. In order to identify genes that are differentially expressed either at the transcriptional or at the translational level, cDNA prepared from polysomal mRNA was used. Here we demonstrate that expression of the nm23-M2 gene, previously identified as a common virus integration site, was differentially expressed in factor stimulated cells. Furthermore, nm23-M2 protein expression was shown to be down regulated during erythroid differentiation. To study whether nm23-M2 expression was regulated at the transcriptional or at the translational level, nm23-M2 Northern analysis was performed using mRNA fractions blotted after sucrose gradient isolation, separating polysomal from non-polysomal mRNA. We report that although mRNA transcripts from the *nm23-M2* gene in erythroid progenitors are constitutively high, polysome binding of nm23-M2 mRNA, suggesting protein expression control, is regulated at the level of translation initiation. Polysome binding of *nm23-M2* in erythroblasts is strictly dependent on Epo/SCF signalling to PI3K. Cloning of the full 5'UTR of *nm23-M2* revealed that it contains both a TOP-sequence and an inverted repeat which may both be crucial for translation regulation.

Materials and Methods

Cells
I/11 cells were cultured in StemPro medium supplemented with 0.5U/ml Epo, (Ortho-Biotech, Tilburg, The Netherlands), 100 ng/ml SCF (Amgen, Breda, The Netherlands) and 10^{-6}M dexamethasone (Sigma-Aldrich, Zwijndrecht, The Netherlands) as described [6]. To analyse cellular signalling mechanisms, cells were washed twice with HBBS (Gibco BRL., Life Technologies, Breda, The Netherlands) and seeded in Iscove's medium without any additives. Factor stimulation was with 5U/ml Epo, 200ng/ml SCF.

RNA isolation and Northern blot analysis
Isolation of total RNA and Northern blot analyses was performed as described by Chomczynski et al. [34] with minor modifications [35]. Isolation of polysomal RNA by sucrose gradient fractionation was performed essentially as described [36]. Cell extracts were prepared by lysis at 4°C in extraction buffer (10 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1.5 mM MgCl2, 0.5% Nonidet-P40, 20 mM dithiothreitol, 150 µg/ml cycloheximide, 1 mM phenylmethylsulfonyl fluoride and 500 U/ml RNAsin), and nuclei were removed by centrifugation (12000 g, 10 s, 4°C). The supernatant was supplemented with 665 µg/ml heparin and and centrifuged (12000 g, 5 min, 4°C) to eliminate mitochondria. The supernatant was layered onto a 10 ml (ATLAS hybridisation and Northerns) or 4 ml (Taqman analysis) linear sucrose gradient (15–40% sucrose [w/v] supplemented with 10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 1.5 mM MgCl2, 10 mM dithiothreitol, 100 µg/ml cycloheximide, and 0.5 mg/ml heparin) and centrifuged in a SW41Ti rotor (Beckman, Palo Alto, CA, USA.) 38000 rpm, 120 min, 4°C, or in a SW50Ti rotor 42000 rpm, 60 min, 4°C without brake. Fractions (550 µl) were collected and digested with 100 µg proteinase K in 1% sodium dodecylsulfate (SDS) and 10 mM EDTA (30 min, 37°C). RNAs were then recovered by phenol-chloroform-isoamyl alcohol extraction, followed by ethanol precipitation. RNAs were analysed by electrophoresis on denaturing 1.2% formaldehyde agarose gels and subsequent Northern blotting (using Hybond-N^+ membranes; Amersham Pharmacia Biotech, Roosendaal, The Netherlands). These gels indicated that fractions 1-11 (10 ml gradient) and 1-4 (4 ml gradient) contain nonpolysomal and subpolysomal mRNA, while fractions 13-20 (10 ml gradient) and 5-8 (4 ml gradient) consisted of polysomal RNA. The latter fractions were pooled for array hybridisation and Taqman analysis respectively. As probes we used a 612 bp *nm23-M2* cDNA fragment, or cDNAs containing the entire ORF of *pim1* or *c-jun*. After hybridisation, filters were scanned on a phosphor-imager and signals were quantified by PhosphorImaging (Molecular Dynamics, Sunnyvale, CA, USA.)

Poly(A)$^+$ mRNA isolation and cDNA synthesis
Total RNA from sucrose gradients was pooled into two major fractions: free RNA and polysome-bound RNA. RNA was quantified by UV-absorbance. Poly(A)$^+$ mRNA was purified from isolated total RNA (25-30 µg) with the oligotex mRNA minikit (Qiagen Gmbh, Hilden, Germany) according to the manufacturer's recommendations. Equivalent amounts of polyA$^+$ RNA (1-2 µg) were reverse transcribed into cDNA using 2 µl CDS primers provided by the ATLAS kit. RNA was denatured (7', 65°C; 7' on ice), RNAsin (10U), DTT (10mM), nucleotides (100nM each) and buffer supplied with the enzyme plus 100 U SSII* Reverse Transcriptase (Gibco) were

added and the reaction was put at 42°C for 1.5 hours. Subsequently RNA was degraded in 0.3M NaOH at 65°C for an hour, which was stopped by adding Tris pH7.5 to 20mM and HCl to neutralise the NaOH. CDNA was precipitated in the presence of glycogen (Boehringer) and dissolved in 15 µl H_2O. 1 µl was used in a random labelling reaction (Random primed labelling kit, Roche) using ^{32}P-dATP. After removal of free nucleotides the ^{32}P-incorporation was measured in a Cherenkov-counter and the same amount of counts was used in hybridisations of a single experiment [18, 37].

Microarray hybridisations
Hybridization of Atlas™ cDNA arrays (588 mouse cDNA probes; Clontech laboratories, Palo Alto, CA) was performed basically as recommended by the manufacturer, with the modifications described below. Filters were prehybridised for 8h at 68°C in 10 ml of prewarmed ExpressHyb plus denatured sheared salmon sperm DNA, both provided by the kit. Subsequently, 6 x 10^7 dpm of denatured radioactive cDNA (kept 5 min at 95°C without denaturing solution and thereafter chilled on ice) were added and hybridised for 20 hours. Filters were washed three times at 68°C in 200 ml of 2 x SSC / 1%SDS for 40 min. Thereafter the filters were washed again three times in 0.2 x SSC / 0.5% SDS for 20 min and subjected to phosphor-imaging (Molecular Dynamics).

Western blotting
I/11 cells were factor deprived and stimulated as described above. Samples were taken at regular intervals and processed as described before [38]. Following separation of proteins on 10% PAGE and Western blotting, filters were incubated with the nm23-M2 antibody (Seikagaku, Falmouth, MA, USA). Immune complexes were detected with horseradish peroxidase-conjugated goat anti rat IgG antiserum (Santa Cruz, Santa Cruz, CA, USA, catalogue no #2006), followed by an enhanced chemoluminescence reaction (NEN® Life Science Products, Zaventem, Belgium).

Isolation and cloning of the 5'untranslated region of nm23-M2
A nested PCR was performed on an oligo-d(T)-primed cDNA library of primary erythroblasts (a gift from Walbert Bakker, Institute of Hematology, Erasmus University, Rotterdam, The Netherlands). The library was ligated into the lambda ZAP express® vector (Stratagene, Amsterdam, The Netherlands). The first PCR (45" at 94°C, 1' at 57°C, and 1' at 72°C; 25 cycles) was performed using the M13 reverse primer (5'ACA GGA AAC AGC TAT GAC CTT G3') in the vector in combination with pN6 (5'TCG CCC ACC AGG CCG CGC3') in *nm23-M2* on 50ng of cDNA. 1µl of PCR product was transferred to the nested PCR (45" at 94°C, 1' at 56°C, and 2' at 72°C (30 cycles)). The T3 primer (5'AAT TAA CCC TCA CTA AAG GG3') in the vector and pN8 (5'TGC ACG CCA TCT GGC TTG3') in *nm23-M2* were used for this reaction. Subsequently, the final products were cloned directly into pCR2.1 (Invitrogen, Breda, The Netherlands) according to the instructions of the manufacturer. Nucleotide sequencing was carried out using the Bd-sequencing kit according to instructions from the provider (PE Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) Sequencing was carried out on an ABI 310 automatic sequencer (PE Biosystems) using the M13forward primer (5'GTA AAA CGA CGG CCA GT3'). All primers were obtained from Life Technologies (Breda, The Netherlands).

Primer pairs

Gene-specific primers corresponding to *nm23-M2* (NCBI accession number X68193), YB-1 (X57621), rpS4 (M73436), eEF-1β2 (BC023139), Fli-1 (X59421) and αTUB4 (M13444) were obtained from Life Technologies. The sequence of the primers used for the amplification of nm23-M2 were: forward 5'TGG CCA ACC TCG AGC GTA C3', reverse 5'TTG AGC CCC TCC CAG ACC A3'; YB-1, forward 5'TGC AGG AGA GCA AGG TAG AC3', reverse 5'TGG TGG ATC GGC TGC TTT TG3'; rpS4, forward 5'TAG CGC AGC CAT GGC TCG TG3', reverse 5'TCA TCT CCA GTC AGG GCA TAC3'; eEF-1β2, forward 5'ATG GGA TTC GGA GAC CTG AA3', reverse 5'TCA GCA GGT GGT GGA CCA GA3'; Fli-1, forward 5'TGC AGC CAC ATC CAA CAG AG3', reverse 5'TGA AGG CAC GTG GGT GTT AG3'; and αTUB4, forward 5'TGC AGC GTG CTG TGT GCA TG3', reverse 5'TCC TCT CGA GCC TCA GAG AA3'.

Real-time PCR

The real-time PCR assay involves TaqMan technology (PE Applied Biosystems Model 7700 sequence detector), which combines rapid thermocycling with on-line fluorescence detection of the PCR products. The reactions were performed in a volume of 25 μl of a mixture containing 4 μl of the respective cDNA dilution, primers at 5 μM and 12.5 μl of 2X SYBR green PCR Master mix (PE Biosystems) containing Amplitaq Gold® DNA polymerase, reaction buffer, dNTP mix with dUTP, passive reference and the double stranded DNA (dsDNA)-specific fluorescence dye SYBR green I. Samples were placed into a 96-well plate, capped and placed into the TaqMan sequence detector. The amplification program consisted of 1 cycle of 50°C with 2-minutes hold (AmpErase UNG incubation), 1 cycle of 95°C with 10-minutes hold (AmpliTaq Gold Activation), followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 62°C for 30 s and extension at 62°C for 30 s. All the different primer pairs have the identical optimal PCR annealing temperature. Acquisition of the fluorescence signal from the samples was carried out at the end of the elongation step. To confirm amplification specificity the PCR products form each primer pair were subjected to agarose gel electrophoresis.

Results

Nm23-M2 expression is regulated by control of translation

Similar to primary erythroid progenitors, the erythroid cell line I/11 proliferates in presence of erythropoietin (Epo), Stem cell factor (SCF) and dexamethasone (Dex), while the cells undergo terminal differentiation into enucleated erythrocytes in the presence of Epo and insulin [6, 18]. We are interested in genes whose expression is induced by Epo plus SCF as these genes may be actively involved in maintaining cell expansion while inhibiting differentiation. As signal transduction may control activation of both transcription and translation, we used polysome bound mRNA, derived from I/11 erythroid cells factor deprived and stimulated with Epo, SCF or Epo plus SCF, to screen ATLAS filters containing 588 cDNA probes (see material and methods, [18]). In this screen we found *nm23-M2* to be upregulated by Epo or SCF (Figure 1A). The highest expression was observed when cells were stimulated with Epo plus SCF. To validate the results, we tested total RNA derived from cells that were factor deprived and similarly stimulated by Epo, SCF or Epo plus SCF for *nm23-M2* expression on a Northern blot containing total mRNA. Surprisingly, we did not detect altered expression, although the Epo-target gene *Pim1* was upregulated by Epo and the SCF-target gene c-Jun was upregulated by SCF (Figure 1B). Subsequently we hybridised Northern blots containing fractions of subpolysomal and polysome bound RNA with the *nm23-M2* probe. Quantitative analysis showed that in absence of factor, less than 20% of all *nm23-M2* mRNA is present in the polysome bound fractions. Upon stimulation with Epo 53% of nm23-M2 mRNA shifts into the polysome bound fractions. In the presence of SCF 64% and in the presence of Epo plus SCF 78% of the nm23-M2 mRNA was found in the polysome bound fractions (Figure 1C). These data suggest that expression of *nm23-M2* is controlled at the level of translation initiation rather than by control of transcriptional activation. The highest percentage of polysome binding by *nm23-M2* transcripts occurs following stimulation by the combination of Epo and SCF.

Loss of polysomal association of nm23-M2 mRNA and decrease of protein levels during erythroid differentiation

Previous studies reported nm23-M2 protein expression to be downregulated in myeloid [39] and in erythroid differentiation [40]. Our finding that the highest percentage of polysome-bound nm23-M2 mRNA was observed when stimulated with Epo plus SCF is in agreement with these findings. To investigate whether nm23-M2 downregulation was due to a change in translation, we studied nm23-M2 mRNA polysome binding in I/11 cells during terminal differentiation. I/11 cells were shifted from medium supplemented with Epo, SCF, and dexamethasone to medium supplemented with Epo and Insulin. Western blotting shows that nm23-M2 protein is downmodulated between 36 and 48 hours upon differentiation induction (Figure 2A). This corresponds to the differentiation stage in which I/11 cells stop to divide and start to accumulate hemoglobin [6, 18]. On Northern blots containing fractions of subpolysomal and polysomal mRNA derived from cells at different stages of differentiation, the nm23-M2 mRNA shifted significantly to the subpolysomal between 20 and 48 hours after differentiation induction (Figure 2B).

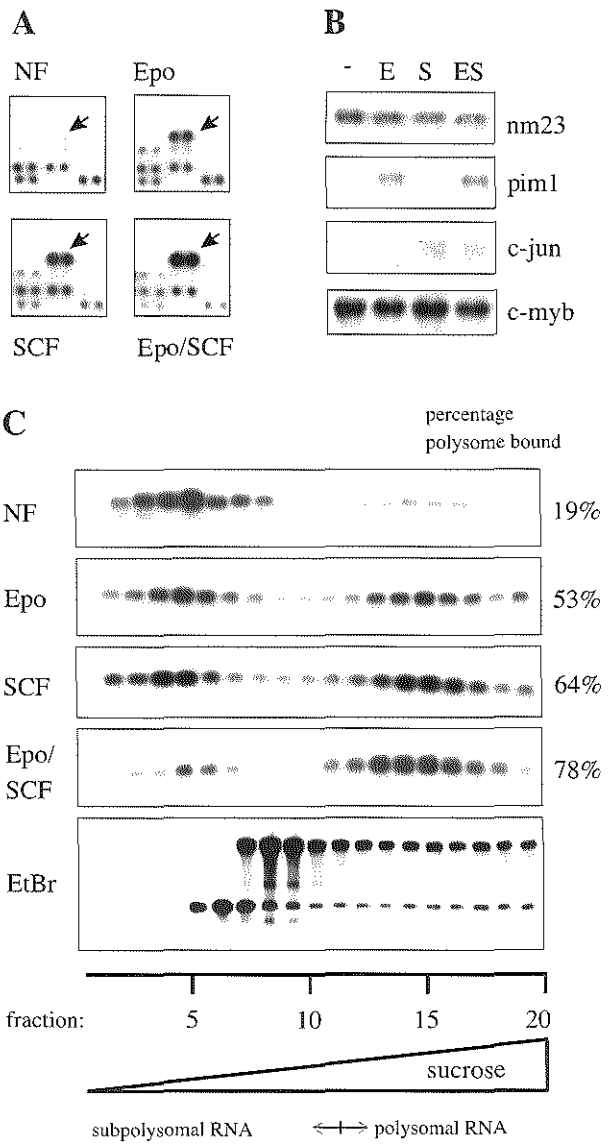


Figure 1. Nm23-M2 polysome association is induced by mitogenic signals.
Clone I/11 erythroblasts were factor deprived and subsequently left unstimulated (NF) or stimulated with Epo (5U/ml), SCF (100 ng/ml) or both (Epo/SCF) for two hours. **A**, polysomal mRNA was hybridised to ATLAS filters (see materials and methods). Arrows indicate spot C4c, *nm23-M2*, to be induced by Epo and SCF. **B**, Total RNA of cells similarly treated was used to generate a Northern blot, which was hybridised to probes representing *nm23-M2*, pim1, c-jun and c-myb (in all cases probes comprised full ORF). Surprisingly, *nm23-M2* expression was not induced, though *pim1* expression was induced by Epo (***E***) and Epo plus SCF (***ES***) and *c-jun* was expressed by SCF (***S***) and Epo plus SCF (***ES***). **C**, Separate fractions of RNA isolated from the sucrose gradient (concentration gradient indicated below) were tested for expression of *nm23-M2*. The first fractions contain subpolysomal RNA, the latter fractions contain RNA recovered from polysomes. The percentages indicated at the right represent the percentage of *nm23-M2* mRNA present in polysomes (fraction 11 and above) under the respective conditions.

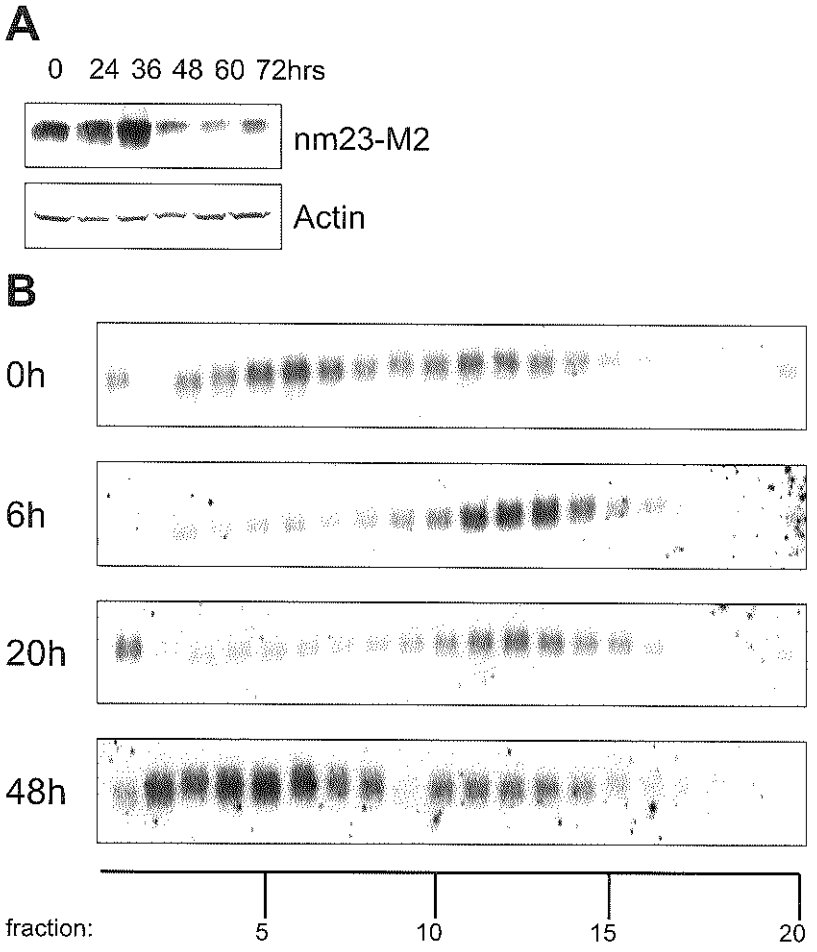


Figure 2. Nm23-M2 translation is downregulated upon differentiation induction.
I/11 cells were induced to differentiation. **A**, cells were harvested at times indicated and derived protein samples were analysed for *nm23-M2* expression on Western blots. Expression of Actin served as a loading control. In erythroid cells, the anti-nm23-M2 antibodies we used recognise two proteins with almost similar electrophoretic mobility. Nm23-M2 is the faster migrating protein. **B**, Polysome-bound mRNA was isolated at various time points after differentiation induction. RNA isolated from 20 distinct fractions of a sucrose gradient was tested for *nm23-M2*. *Nm23-M2* message in proliferating cells is present in the polysome-bound and unbound RNA fractions. After 6 hours a slight shift of nm23-M2 to polysome bound mRNA is present. Twenty hours after differentiation induction the signal starts to move to the non-polysome bound fraction. After 48 hours the differentiating cultures have become stationary and the *nm23-M2* signal is present in the subpolysomal fractions.

Cytokine regulated polysome binding of nm23-M2 mRNA is PI3K dependent
The signalling routes that may be involved in growth factor stimulated translational control are the MEK/ERK pathway resulting in activation of p90RSK and MNK-1,-2, and the PI3K/PKB pathway activating mTOR and S6kinase [33]. To test whether these pathways are involved in growth factor induced polysome association of nm23-M2 mRNA, we factor-depleted and subsequently SCF-stimulated I/11cells in absence and presence of the PI3K-inhibitor LY294002, the mTOR inhibitor rapamycin and the MEK1 inhibitor PD98059. In presence of the PI3K inhibitor LY294002 polysome association of nm23-M2 mRNA was completely abrogated, whereas in the presence of the MEK inhibitor PD98059 the distribution of the nm23-M2 transcripts was identical to the untreated SCF-control samples (Figure 3). Rapamycin only weakly reduced polysome association of nm23-M2 mRNA following SCF treatment.

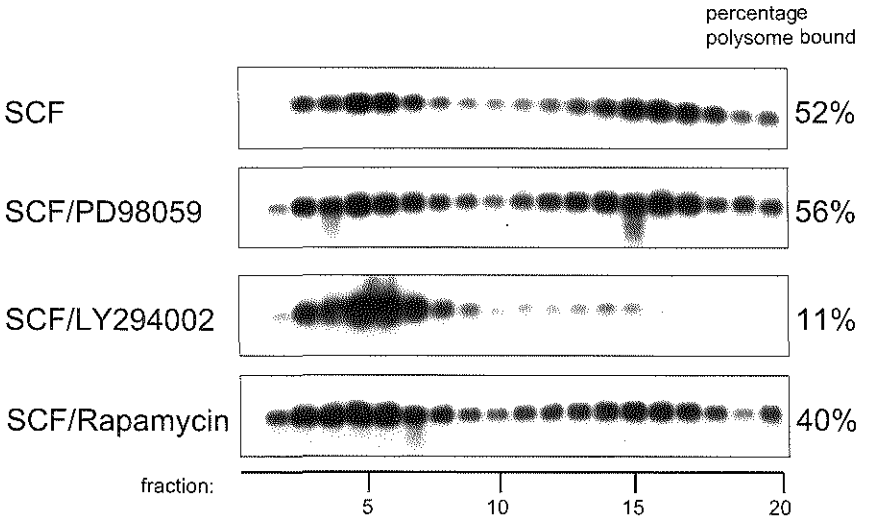


Figure 3. Translation of nm23-M2 occurs in a PI3K dependent way.
A culture of I/11 cells was factor deprived for 4 hours and subsequently stimulated with SCF (100 ng/ml) in absence of low molecular weight inhibitors, or in combination with the PI3K inhibitor LY294002 (15μM), MEK inhibitor PD98059 (25μM), or the mTOR inhibitor Rapamycin (20ng/ml) for two hours. Separate fractions of RNA isolated from the sucrose gradient (concentration gradient indicated below) were tested for the presence of *nm23-M2*. *Nm23-M2* message in the SCF stimulated cells is mainly present in the polysome associated fractions. Incubation with PD98059 does not have an effect on the polysome association of *nm23-M2* mRNA. Incubation with LY294002 causes a major shift of the *nm23-M2* signal to the subpolysomal fractions. Rapamycin has a similar, though less evident effect on the *nm23-M2* mRNA.

Nm23-M2 mRNA starts with a Terminal Oligopyrimidine (TOP) tract and contains a inverted repeat

Translation regulation may occur through specific sequences and structures present in the 5'- and/or 3'-untranslated region (UTR) of the mRNA. The 5'UTR sequence of nm23-M2 available in the public databases did not contain any sequence known to be involved in translational control. We obtained 5' fragments of nm23-M2 cDNA from a cDNA library by PCR. Fragments of approximately 170 bp, primed from the ATG in *nm23-M2* to the 5'cloning site of the vector were isolated and cloned. Three independent clones were sequenced, which all started at their 5' site with a pyrimidine stretch of 14 nucleotides respectively. Terminal oligopyrimidine (TOP) tracts are structures known to be essential in the control of translation of several genes [41]. In addition, we observed a 9bp inverted repeat (indicated as a stem-structure in Figure 4). Modelling of the 5'UTR, using the digital program RNA structure 3.6 [42], indicated that this inverted repeat was able to form a structure with a free energy of approximately –15.4 kcal/mole (Figure 4). Mapping of the novel 5'cDNA sequence to genomic DNA revealed that the extra 90 nucleotides in the mRNA are located on a separate exon which precedes the second exon from which the *nm23-M2* open reading frame starts.

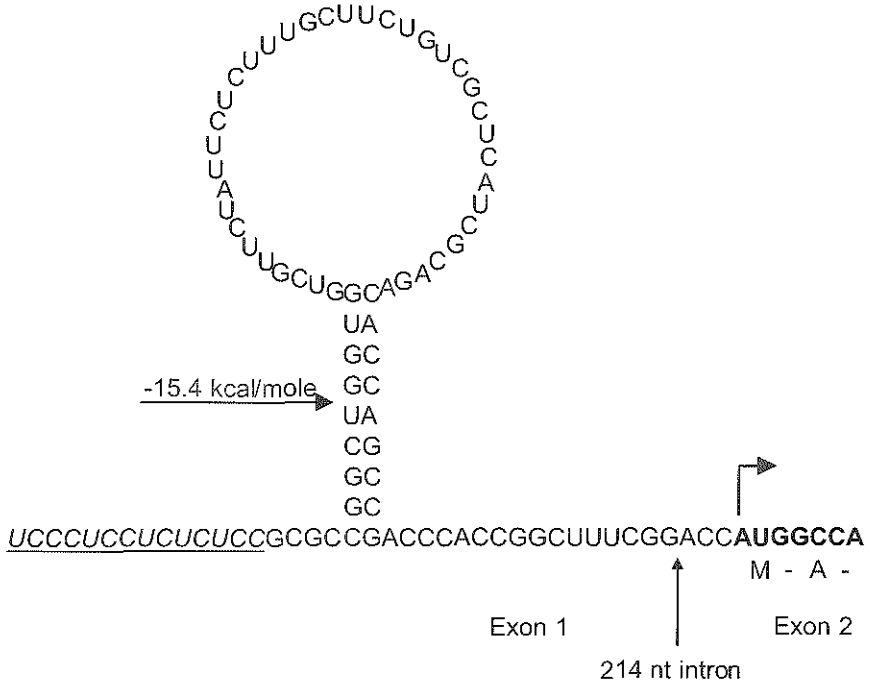


Figure 4. The 5'untranslated region of nm23-M2 contains a TOP-tract and an inverted repeat.
The 5'UTR of *nm23-M2* was cloned by RT-PCR from a cDNA library of I/11 cells. Three independent clones were sequenced. The cDNA of *nm23-M2* started with a stretch of pyrimidines (underlined italics). The 5'UTR contains a 9 nucleotides inverted repeat, creating a potential stem-loop structure as indicated with a predicted free energy of –15.4 kcal/mole. Within the gene, the TOP and potential stem-loop sequence are separated from the translation start site by an intron of 214 nt (indicated by an arrow below the sequence). The start of the protein coding region is marked in bold and the encoded amino-acids are written below in the 1-letter code.

SCF/Epo and PI3K dependent polysomal association of RpS4, EF1β2, YB1 and nm23-M2 mRNA

To relate the observed inhibition of translation initiation by LY294002 to the TOP-tract and/or the inverted repeats, we compared regulation of nm23-M2 polysome association with several other mRNAs wich appeared to be upregulated as determined by our Atlas screen, i.e. (*i*) ribosomal protein S4 (rpS4) [20] and elongation factor 1B2 (EF1β2) [20], two mRNAs with an exemplary TOP-tract but no other 5'structure, (*ii*) YB1 a mRNA which also harboured an inverted repeat in its 5'UTR but does not contain a TOP-tract, (*iii*) FLI-1 a mRNA subject to translational regulation but via distinct mechanisms, and (*iv*) alpha-tubulin as control mRNA. The cells were treated as described above, subpolysomal and polysomal fractions were isolated and pooled into two fractions and transcribed to cDNA. Real-time PCR (Taqman, PE) was performed to quantitatively determine mRNA expression (Figure 5). *RpS4, EF1β2, YB1* and *nm23-M2* showed significant polysome association following Epo/SCF stimulation (more than 8-fold). Furthermore, *nm23-M2*, *rpS4, EF1β2* and *YB1* showed very similar behaviour in that polysome association was almost completely abrogated by LY294002 and largely reduced by rapamycin. In contrast, Epo/SCF induced a minor increase in polysome association of *Fli-1* or *α-tubulin* (less than 2-fold) and the presence of LY294002 or rapamycin hardly affected polysome association by these particular transcripts. Apparently, the TOP-sequence (rpS4, EF1β2 and nm23-M2) and inverted repeats (YB1 and nm23-M2) similarly render translation dependent on PI3K activity.

Figure 5. Real Time PCR.

A culture of 1/11 cells was factor deprived for 4 hours and subsequently left unstimulated (-) or stimulated with Epo (5U/ml) and SCF (100 ng/ml) for two hours. Real-time PCR was performed on cDNA isolated from polysome bound and non-polysome bound RNA from these cells. For six translationally regulated genes the response to growth factor stimulation and to the PI3K inhibitor LY294002 or the mTOR inhibitor Rapamycin was measured and compared to the effect on the unstimulated cells.

A. Nm23-M2 mRNA, containing a TOP-tract and an inverted repeat in the 5'UTR, showed a large increase of ribosome binding upon growth factor stimulation. This increase was almost completely vanished when incubated with LY. Rapamycin did inhibit this binding, but not as much as LY.

B. The TOP-containing rpS4 mRNA showed a less big increase than nm23-M2 and YB-1 upon growth factor stimulation. LY and Rapamycin were both able to reduce this effect, though LY had a larger impact than Rapamycin.

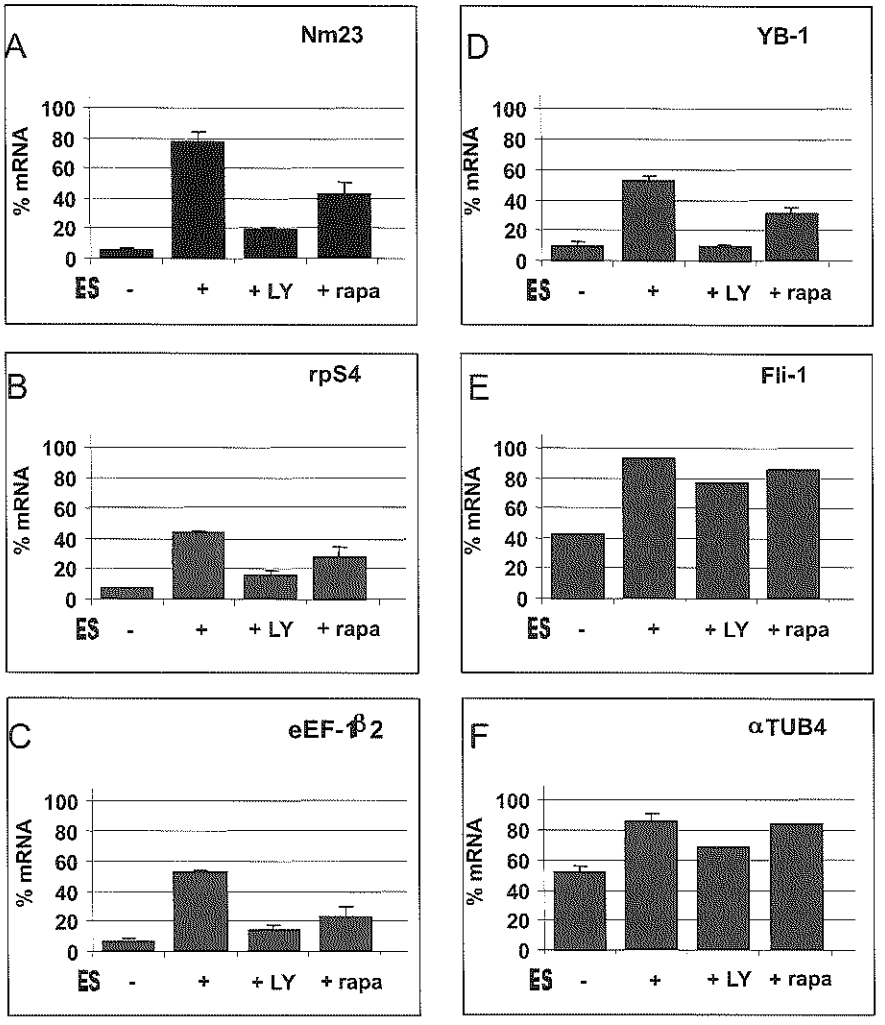
C. The translation elongation factor eEF-1β2 that only holds a TOP tract shows a similar response to growth factor stimulation and inhibition of PI3K and mTOR as rpS4.

D. YB-1, containing an inverted repeat in its 5'UTR, responded in the same fashion to growth factor stimulation as *nm23-M2*. Blocking of the signalling intermediates demonstrated that there was no increase of polysome binding after incubation with LY, and only a minor increase upon mTOR inhibition.

E. Fli-1, that is translationally regulated through an IRES, demonstrated an increase of mRNA-polysome binding when stimulated with Epo and SCF, but no clear effect of both LY and Rapamycin was detected.

F. Approximately 50% of the mRNA of the control mRNA alpha-Tubulin is already bound to polysomes in the unstimulated cells. Stimulation with growth factors resulted in an increase of this fraction. No obvious inhibition resulting from the presence of LY or Rapamycin was found.

Figure 5. Real Time PCR.



Discussion

Expression of the putative oncogene *nm23-M2* in erythroid progenitors was found to be controlled by the mitogenic factors Epo and SCF. *Nm23-M2* gene transcription is constitutively high, while mRNA translation appeared strictly controlled by pathways dependent on PI3K and mTOR. The nm23-M2 starts with an oligopyrimidine tract and contains an inverted repeat, structures known to invoke control of translation by growth factors [41, 43].

To stimulate cell proliferation growth factors need to activate multiple processes, required for cell cycle progression. To allow cell growth, regulation of translation is as important as transcriptional control. Growth factors enhance expression of translation factors like initiation- and elongation factors and ribosomal proteins [44, 45]. They do so by inducing mRNA specific translation rather than gene transcription, which ensures a very rapid response. Many translation factors are encoded by mRNAs containing a TOP-sequence [41], which causes inhibition of translation in absence of growth factors and nutrients and enhances translation in presence of growth factors. It is crucial that the very first nucleotide following the cap structure is a pyrimidine and a stretch of 5 pyrimidines is sufficient to execute its function [41]. A TOP-sequence generally contains a similar proportion of C and U residues. Furthermore, a TOP-tract is followed by a GC-rich sequence and no upstream AUGs should be present. The *nm23-M2* TOP-sequence consisting of 5 U and 9 C residues meets these requirements. The mechanism by which a TOP-sequence inhibits translation is not known. Overexpression of the cap-binding protein eIF4E in general overcomes the requirement for growth factors to translate TOP-mRNAs, which suggested that activation of eIF4E by phosphorylation and/or release of eIF4E from 4E-BPs may be required. However, a recent report suggests the existence of a factor that actively inhibits translation by binding to TOP-sequences [46].

Other structural motives present in UTRs may also be critical regulators of growth factor-dependent translational control of gene-expression. *Nm23-M2* contains an inverted repeat able to form a stem-loop structure. However, the free energy of this structure (–15.4 kcal/mole energy as calculated by RNA structure 3.6 [42]) is too small to impair scanning by the initiation complex and can easily be resolved by eIF4A. The structure may, however, be stabilised by an interaction with RNA-binding proteins. The stem-loop structure in ferritine, the iron response element, is stabilised by a protein whose association is controlled by iron binding [43]. Similarly, the potential stem-loop structure in *nm23-M2* may be stabilised by a protein whose association is dependent on its phosphorylation status. On the basis of the presented data it can not be decided whether both the TOP-sequence and the inverted repeat contribute to translation control. Comparison with known TOP-mRNAs that contain no other regulatory domains show that growth factor-dependence of *nm23-M2* mRNA translation is even more strict than that of ribosomal protein S4 and elongation factor-1β. This suggests that both potential control mechanisms in the *nm23-M2* 5'UTR may contribute to its translational control.

PI3K controls nm23-M2 mRNA polysome association

Translation of most TOP-mRNAs, is under the control of S6kinase. Activation of S6kinase occurs through phosphorylation by mTOR and PI3K-dependent pathways, which target separate domains of S6kinase. The translation of *nm23-M2* is fully repressed upon inhibition of PI3K. Inhibition of mTOR by rapamycin appears less

complete. However, translation of EF1β and S4 is also less efficiently repressed by rapamycin. Therefore, the effectiveness of LY294002 compared to rapamycin may be specific for the erythroid progenitors and the conditions used, rather than for the *nm23-M2* mRNA. In erythroid progenitors, the cooperation of Epo and SCF induces renewal divisions and delays differentiation [47]. Inhibition of PI3K abrogates renewal and induces differentiation [6], while inhibition of the MEK/ERK pathway does not affect the balance between expansion and differentiation. This indicates that PI3K-targets like *nm23-M2* may be involved in renewal induction. Enhanced activity of PI3K and its downstream targets is also crucial in tumours induced by v-p3k, the oncogene present in avian sarcoma virus 16 (ASV16) [48] and in Friend spleen focus-forming virus induced erythroleukemia [49]. We showed that *nm23-M2* mRNA translation is controlled by Epo/SCF-induced PI3K-activity in erythroid progenitors, but we do not know whether the same mechanisms are used to control mRNA translation during differentiation.

The nm23 family and malignant transformation

Enhanced expression of the *nm23* family of genes has been detected in a variety of malignancies [50-54] and frequently correlated with a poor differentiation stage of certain tumours [50]. Some studies examined nm23 mRNA levels, others investigated protein levels but seldom the relation between mRNA and protein levels was studied. Therefore, it is not clear whether abberrant control of *nm23-M2* mRNA translation is involved in enhanced protein expression in malignancy. Most likely transcriptional as well as translational control mechanisms regulate *nm23-M2* expression. In the retrovirally induced mouse leukemias the large majority of the integrations occur in, or just upstream of the 5'UTR. The retroviral LTR may act as a promoter enhancer element. However, in the leukemias tested, virus integration often resulted in a fusion transcript in which the translational control elements of *nm23-M2* (TOP-sequence and repeat) are replaced with viral sequences that ensure rapid scanning of the translation initiation complex (data not shown). As a result, the viral integrations may render nm23-M2 expression independent of growth factor availibility. Unfortunately, it appeared impossible to isolate polysome-associated mRNA from primary leukemias. Therefore the effect of virus integration on translational regulation of nm23-M2 in the leukemias containing LTR-*nm23-M2* fusion transcripts could not be determined. Moreover, the oligoclonal nature of the leukemias renders protein expression assays difficult to interpret.

Oncogenesis and translational control

Control of translation initiation via mRNA-specific mechanisms is increasingly recognised as a potentially important control level in lineage determination, cell survival, proliferation and differentiation. Overexpression of eIF4E, the limiting factor in translation initiation, is in its own tumourigenic [55-57]. In addition, the expression of many potential oncogenes appears to be controlled at the level of translation since it allows rapid changes in protein levels. Expression of AML1 is regulated through usage of alternative promoters coupled with IRES-mediated translation control [58]. An IRES is also present in the 5'UTR of c-Myc. In several bone marrow samples from patients with multiple myeloma there is aberrant translational regulation of c-myc and this correlates with a C-T mutation in the c-myc-IRES [59]. FLI-1 is expressed as two protein isoforms generated by alternative translation initiation from two highly conserved in-frame initiation codons [60].

Overexpression of the proto-oncogene Fli-1 is associated with multiple virally induced leukemias in mouse, and the human counterpart is translocated in Ewing tumours [61].
Several mechanisms for retrovirally induced malignant transformation have so far been identified, ranging from activation of transcription to mRNA stabilisation or gene inactivation. Data presented in this paper suggest that an important mechanism may so far have been underestimated: the replacement of untranslated sequences in the mRNA encoding a potential oncogene, such that translation is no longer controlled by e.g. growth factors.

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Chapter 7

Leukemic predisposition of *pSca-1/Cb2* transgenic mice

Marieke Joosten, Peter J.M. Valk, Meritxell Alberich Jordà, Yolanda Vankan-Berkhoudt, Sandra Verbakel, Marion van den Broek, Antoinette Beijen, Bob Löwenberg, and Ruud Delwel

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Abstract

Objective. The gene encoding the peripheral cannabinoid receptor, *Cb2* is located in the common virus integration site *Evi11* and is associated with hematopoietic malignancies in mice. To determine the effect of Cb2 overexpression on hematopoietic development *in vivo*, *Cb2* transgenic mice were generated.
Materials and Methods. A *Cb2* expression vector was constructed containing a *Cb2* cDNA fragment cloned into the 14kb *Sca-1 (Ly-6E.1)* gene. Two transgenic lines in which Cb2 expression is controlled by the *Sca-1* promoter, were generated and the effect on hematopoietic development was studied. The expression of *Cb2* mRNA or protein was studied by means of RNase protection analysis and ligand binding assays, respectively. Leukemic predisposition was investigated by injecting newborn transgenic as well as control animals with a retrovirus, i.e. Cas-Br-M Murine Leukemia Virus (Cas-Br-M MuLV).
Results. Although increased expression of the *Cb2* gene was observed in hematopoietic tissues, a follow-up of more than one year did not reveal any hematological defect. Interestingly, infection of newborn *pSca-1/Cb2* transgenic mice with Cas-Br-M MuLV revealed that significantly more transgenic mice developed leukemia than virus treated control littermates. Since these studies provide evidence for the cooperative potential of Cb2 in leukemia progression, we wished to identify genes that may collaborate with *Cb2* in leukemic transformation. Our study suggests that *Evi1*, another common target for proviral integration in mouse leukemias may be overexpressed in virus-induced leukemias in *pSca-1/Cb2* transgenic mice.
Conclusions. These data indicate that hematopoietic precursor cells that express high levels of Cb2 possess increased susceptibility for leukemia development and that *Cb2* and *Evi1* might collaborate in leukemogenesis.

Introduction

Cb2, the gene encoding the peripheral cannabinoid receptor has been shown to be a frequent target for proviral integration in retrovirally induced leukemias [1, 2]. In mice, the protein-coding region of *Cb2* is located on a single exon of approximately 4 kb (exon-2). Two non-protein coding first exons have been identified, i.e. exons-1A or 1B, previously designated exon-1 and exon-1', respectively [1]. In cells expressing exon-1A/exon-2 mRNA, e.g. in thymocytes or in the majority of myeloid cell lines, no or only low levels of the peripheral cannabinoid receptors are detectable [3]. In contrast, in spleen cells expressing both transcripts high receptor numbers are present, suggesting that protein expression is the result of translation from the exon-1B/exon-2 *Cb2* mRNA transcript. Interestingly, retroviral insertion in *Cb2* in mouse leukemia may cause expression of exon-1B/exon-2 transcripts and consequently lead to high receptor numbers [3]. These results indicate that Cb2 receptor overexpression may be a critical step in tumour progression in retrovirally-induced leukemias.

To study the effect of *Cb2* overexpression on hematopoiesis *in vivo* and investigate its role in hematopoietic malignancies, *Cb2* transgenic mice were generated in a FVB/n background. A construct was made with the *Cb2* gene, i.e. exon-1B/exon-2 under the control of the *Sca-1* (Ly-6E.1) promoter [4]. Genes controlled by regulatory elements of the *Sca-1* gene will be expressed in primitive hematopoietic cells, as has previously been shown for *LacZ* [5] as well as for *Evi1* [6].

Retroviral insertions in *Cb2* occur in mice that develop leukemia with a long latency, after Cas-Br-M Murine Leukemia Virus (Cas-Br-M MuLV) infection, suggesting that a *Cb2* defect is one of a series of mutations, leading to full transformation. To study the transforming potential of *Cb2* and investigate whether aberrant expression in marrow precursor cells may be part of the multigenetic process of leukemia progression, *pSca-1/Cb2* transgenic mice were treated with retrovirus in analogy with other transgenic mouse models [7, 8]. Transgenic mice as well as FVB/n background control animals were inoculated with Cas-Br-M MuLV. This combination was chosen since Cas-Br-M MuLV, which is a N-tropic retrovirus, only rarely causes development of leukemias in non-susceptible FVB/n control mice [9]. On the other hand, we recently reported that transgenic animals. e.g. Evi1 transgenics, are susceptible for leukemia induction using this virus [6]. Thus, inoculating Cas-Br-M MuLV into FVB/n mice may particularly result in the development of leukemia in case other defects have been introduced. Our data demonstrate enhanced susceptibility to Cas-Br-M MuLV-induced tumour formation of *pSca-1/Cb2* transgenic mice as compared to retrovirus-treated control littermates as well as non-treated *Cb2*-transgenics. To investigate in those leukemias whether Cb2 would cooperate with other established transforming genes, we studied the abnormal expression of *Evi1*, another common VIS targeted by Cas-Br-M MuLV in mouse leukemias. The data presented suggest cooperation between Cb2 and Evi1 in leukemia progression.

Materials and Methods

Generation and propagation of pSca-1/Cb2-transgenic mice
A *Cb2* expression vector was constructed containing *Cb2* exon-1B/exon-2 cDNA under the control of regulatory elements of the murine *Sca-1 (Ly6E.1)* gene (Figure1). The *Eco*RI/*Bam*HI cDNA fragment encompassing the complete coding region was isolated and filled in with the Klenow fragment of DNA polymerase I. The resulting fragment was cloned into the *Cla*I site of pL6Cla (a gift from Dr. E. Dzierzak, Dept of Cell Biology and Genetics, Erasmus University, Rotterdam, The Netherlands) to yield *pSca-1/Cb2*. The plasmid pL6ClaI contains the full-length 14-kb *Sca-1* (Ly-6E.1) gene [5]. The proper orientation of the *Cb2* cDNA insert was verified by restriction enzyme analysis. Transgenic mice were engineered according to standard techniques [10]. Briefly, a linearised DNA fragment of 15.5 kb in length was derived by *Not*I digestion of *pSca-1/Cb2* (see above) and purified by means of 0.8 % agarose gel electrophoresis and electro-elution. This DNA fragment was microinjected into the most accessible pronucleus of (FVBxFVB) fertilised mouse eggs. These eggs were re-implanted into pseudo-pregnant BCBA female foster mice. Transgenic mice were identified by Southern blot analysis of tail-derived DNA according to standard procedures [11]. In short, DNA was isolated, digested with *Bgl*II, electrophoresed in a 0.6% agarose gel and transferred to Hybond-N$^+$ nylon membranes (Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands) with 0.25 M NaOH/1.5 M NaCl. Membranes were hybridised with a random-primed ^{32}P-labelled 1.2-kb *Cb2* cDNA probe [1]. Transgenic founder mice were bred with normal FVB mice to establish transgenic lines. All mice were maintained under standard conditions and treated according to institutional animal health care and use guidelines.

RNase protection analysis
Total cellular RNA was prepared from spleen and thymus by homogenising tissue cells in 4M guanidium thiocyanate, followed by phenol-chloroform extraction and isopropanol precipitation [11]. The RNA samples were subjected to an RNase protection assay, essentially as described by the supplier (Promega, Leiden, The Netherlands). For this purpose a 249 bp *Cb2* cDNA fragment (See Figure 1) was cloned into a pBluescript II SK+ vector and linearised by using *Hind*III. To study *Evi1* mRNA expression a 350 bp antisense riboprobe was used as described previously [6]. For comparison of the signals a radiolabelled *GAPDH* riboprobe was synthesised [12]. RNA probes were synthesised using T_3 or T_7 polymerase. For each incubation, 10 µg mRNA was suspended with radiolabelled RNA probe (15.000 cpm) in 30µl of hybridisation buffer. The samples were heated to 85 °C for 5 minutes and after hybridisation of overnight at 50°C digested with RNase One (Promega, Leiden, The Netherlands) and electrophoresed in a 6% polyacrylamide-8 M urea sequencing gel. After running, the gels were dried and exposed for autoradiography with a KODAK film and an intensifying screen.

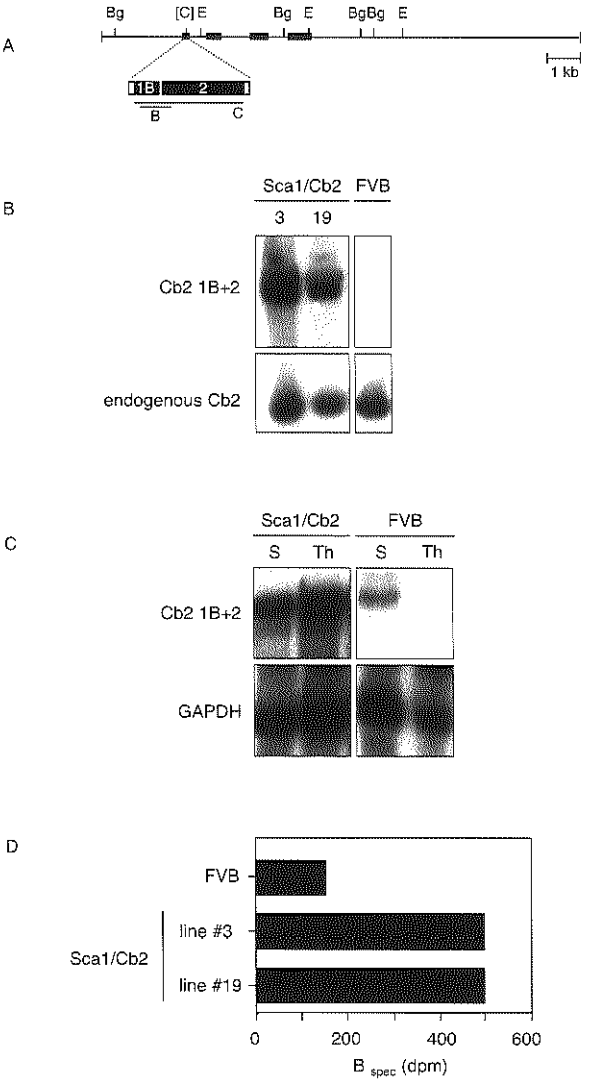

Figure 1. Generation of *pSca-1/Cb2* transgenic mice.

A. Schematic representation of the 14 kb *Sca-1* transgenic construct. A 1.2 kb *Cb2* cDNA was cloned into the genomic construct giving *pSca-1/Cb2* (See Materials and Methods). Abbreviations used: Bg, *Bgl*II; E, *Eco*RI; [C], *Cla*I lost upon subcloning. Locations of probes B and C are indicated.

B. Southern blot analysis of *Bgl*II-digested tail DNA from *pSca-1/Cb2* transgenic lines #3 and 19 and non-transgenic FVB control animals. Blots were hybridised with a exon-1B/exon-2 total cDNA probe C.

C. RNase protection using probe B (See Figure 1A and 4A) on 10 µg total mRNA from spleen (S) or thymus (Th) isolated from *pSca-1/Cb2* transgenic (line # 3) and control FVB mouse.

D. Expression of Cb2 ligand binding sites using [^{3}H]-CP55,940 on spleen cells isolated from two *pSca-1/Cb2* transgenic mice and one FVB control mouse.

Membrane preparation and [^{3}H]-CP55,940 binding assays
Frozen cell and tissue pellets were kept at –80°C until use. For binding assays, pellets were thawed and suspended in assay buffer (50 mM Tris-HCl pH 7.0, 1 mM EDTA, 3 mM MgCl2, containing 0.1% BSA (Serva, Heidelberg, Germany). Membrane suspensions were homogenised and centrifuged at 10,000xg_{av} for 10 min (4°C). Pellets were then resuspended in 5 ml in assay buffer and homogenised using a Potter-Elvejhem homogeniser. Suspensions were finally resuspended in assay buffer at a final membrane concentration equivalent to 10^6 cells/ml.
For binding experiments 160 µl membrane suspension (10^6 cells/ml) was incubated in 96-well plates (flat bottom plates, Greiner, Frickenhausen, Germany) with 20 µl [^{3}H]-CP55,940 (165µCi/nmol) (DuPont-New England) in a saturable concentration of 1 nM and 20 µl assay buffer for total binding or assay buffer containing 10^{-6}M non-labelled CP 55,940 to assess non-specific binding. Mixtures (200 µl final volume) were incubated at 30°C for 50 min after which suspensions were filtrated over Unifilter GF/B plates using a Filtermate-196 Harvester (Packard, Meriden, CT, USA) and washed twice for 5 sec with 200 µl ice-cold washing buffer (50 mM Tris-HCl pH 7.0, containing 0.25% BSA). Subsequently, filtration plates were sealed at the bottom, after which 25 µl scintillation fluid (Microscint-O; Packard) was added per well, radioactivity was counted in a TopCount scintillation counter (Canberra Packard Central Europe GmbH, Schwadorf, Austria) and the $B_{specific}$ was determined.

In vitro colony formation
Bone marrow cells were harvested by crunching femurs and tibia of *Cb2* transgenics and non-transgenic FVB control mice. Single cell suspensions were washed twice in HBSS (HANKS'; GIBCO Life Technologies Inc., Gent, Belgium) and resuspended in α-minimum essential medium (_-MEM; GIBCO Life Technologies Inc., Gent, Belgium). Percoll fractionated bone marrow cells [13] were plated at 1 x 10^4 cells/ml in 35-mm dishes (Falcon® 3001; Becton Dickinson, Plymouth, England) in methylcellulose media (Methocult GF M3434; Stem Cell Technology, Vancouver, BC, Canada) supplemented by 15% FCS (GIBCO Life Technologies Inc., Gent, Belgium), 2 mM L-glutamine, with G-CSF (10U/ml), IL3 (10U/ml), Epo+SCF (3U/ml, 100ng/ml resp), or without additional growth factors. For erythroid colony development 10^{-4} mol/L hemin was added to the cultures. Dishes were incubated at 37°C in 5% CO_2. Assays were performed in triplicate. Colonies were counted at day 7 (G-CSF-derived colonies), and day 14 (BFU-E-derived and IL3-derived colonies) using an inverted microscope.

Peripheral blood cell counts and bone marrow analysis
Peripheral blood (PB) was collected in EDTA-coated micro-tubes (SARSTEDT, Etten-Leur, The Netherlands) by heart or tail puncture. Peripheral blood counts and hematological parameters were determined using an automated blood cell counter (Cobas, ABX La France, Montpellier, France). Differential counts of peripheral blood and bone marrow cells were performed microscopically on May-Grunwald-Giemsa stained smears and cytospins, respectively. Numbers of neutrophils, lymphocytes and platelets are expressed as 10^9cells/l whereas the number of erythrocytes is indicated as 10^{12}cells/ l.

Tumour induction and DNA analysis
For tumour induction Cas-Br-M MuLV was used to infect 19 *Cb2* transgenic mice

and 39 non-transgenic littermates. Cas-Br-M MuLV producing NIH 3T3 cells (a gift from Dr. J. Hartley, National Institutes of Health, Bethesda, MD, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO Life Technologies Inc., Gent, Belgium) containing 10% Fetal Calf Serum (FCS). A subconfluent culture of Cas-Br-M MuLV-producing NIH 3T3 cells was incubated in 5 ml of culture medium for 18 hours at 37°C, in a 75 cm^2 culture flask. Newborn mice were injected subcutaneously with 100 _l filtered (pore size, 0.22 _m; Nucleopore Corp., Pleasanton, CA, USA) tissue culture medium. Mice were sacrificed when moribund. Tumour tissues, i.e. bone marrow, spleen and thymus were isolated and cryopreserved and stored in liquid nitrogen. For Southern blot analysis tumour spleen DNA was digested with *Bam*HI and analysed as described above. A 570 nt *Evi1* specific cDNA fragment [14] was used as a probe for hybridisation.

Immunological staining and flowcytometric analysis

Single-cell suspensions of normal bone marrow cells and spleen tumour cells were analyzed by flow cytometry using a FACScan flow cytometer (Becton Dickinson and Co, Mountain View, CA, USA). Tumour cells were analysed by single staining using rat monoclonal antibodies. The following antibodies were used: ER-MP58 (anti-ER-MP58); M1/70 (anti-Mac-1) (CD11b); F4/80 (anti-F4/80); 59-AD2.2 (anti-Thy1)(CD90); KT-3 (CD3); E13161-7 (anti-Sca-1); or MEC14.7 (CD34) [15]. As a second step reagent GαRa (Goat-anti-Rat)-FITC (Nordic, Tilburg, The Netherlands) was used. Immunophenotyping of percoll separated normal bone marrow cells from transgenic mice and control littermates was performed by flowcytometric analysis using double-staining. FITC-conjugated E13161-7 (anti-Sca-1, BD Pharmingen, San Diego, CA, USA), was combined with PE-conjugated MEC14.7 (CD34, Beckman Coulter, Fullerton, CA, USA), 2B8 (anti-c-Kit, Beckman Coulter), 59-AD2.2 (anti-Thy1, BD Pharmingen)(CD90), or M1/70 (anti-Mac-1, Beckman Coulter)(CD11b). Labelling with FITC-conjugated RB68C5 (anti-Gr1, BD Pharmingen) was carried out in combination with PE-conjugated E13161-7 (anti-Sca-1, BD Pharmingen).

Results

Generation of pSca-1/Cb2 transgenic mice and analysis of transgene expression
To study the effect of *Cb2* over-expression in primitive hematopoietic cells *in vivo*, transgenic animals were generated. Exon1-B/exon-2 *Cb2* cDNA was cloned into the 14 kb genomic DNA fragment containing the *Sca-1* promoter (Ly-6E.1) (Figure 1A). The complete construct was injected into pronuclei of fertilised oocytes. By Southern blot analysis two transgenic founder animals carrying the *Cb2* exon-1B/exon-2 gene (lines #3 and #19) were obtained (Figure 1B). RNase protection using the *Cb2* cDNA probe B (Figure 1A) revealed excess of *Cb2* splice-variant exon-1B/exon-2 mRNA in spleen and thymus as compared to control FVB animals (Figure 1C). Using [^{3}H]-CP55,940 the presence of the cannabinoid receptors in spleen was studied (Figure 1D). Ligand-binding to spleen cells from *pSca-1/Cb2* transgenic mice of both lines was higher than to spleen cells from control FVB/n mice.

Analysis of blood and bone marrow cells
To study the effect of *Cb2* receptor overexpression on hematopoiesis we carried out standard blood cell analysis as well as bone marrow, spleen and thymus histology. Those analyses did not reveal any abnormality in transgenic animals (data not shown). No differences between respectively *pSca-1/Cb2* transgenic animals (n=10) and control mice (n=10) were observed in platelet (1228 ± 257 versus 1329 ± 153), neutrophil (1.4 ± 0.8 versus 1.9 ± 1.1), lymphocyte counts (9.7 ± 3.4 versus 9.4 ± 2.1), expressed as 10^9cells/Liter or erythrocyte counts (10.8 ± 3.3 versus 11.4 ± 1.9), expressed as 10^{12}/liter. The values are given as mean ± standard deviation.
To investigate the characteristics of blood progenitor cells of *pSca-1/Cb2* transgenic mice more specifically, percoll separated bone marrow cells from two transgenics and two control mice were analysed by flow cytometry. Cells were labelled with anti-Sca-1 (E13161-7) in combination with different membrane markers, i.e. anti-CD34 (MEC14.7), anti-c-kit (2B8), anti-Thy1 (59-AD2.2), anti-Mac1 (M1/70), anti-Gr1 (RB68C5). The representative experiment shown in Figure 2 demonstrates that no differences were observed between the phenotype of transgenics and control litter mates in the distribution of these surface markers.
To investigate whether the expression of the Cb2 transgene in hematopoietic precursor cells had affected the behaviour of immature colony forming cells, colony culture were carried out. No clear differences were observed between transgenic mice and normal control mice in the numbers of IL3 stimulated colonies, G-CSF induced granulocytic colonies or EPO/SCF induced BFUe (Table 1).

Table 1. In vitro colony formation of bone marrow precursors cells.

Stimulus	pSca/Cb2	FVB/n
No factor (n = 5)	0	0
IL3 (n = 11)	30 ± 8	36 ± 17
G-CSF (n = 5)	34 ± 15	67 ± 21
Epo/SCF (n = 9)	109 ± 44	97 ± 37

Numbers represent mean colony numbers ± SD per 10^4 percoll-separated bone marrow cells.

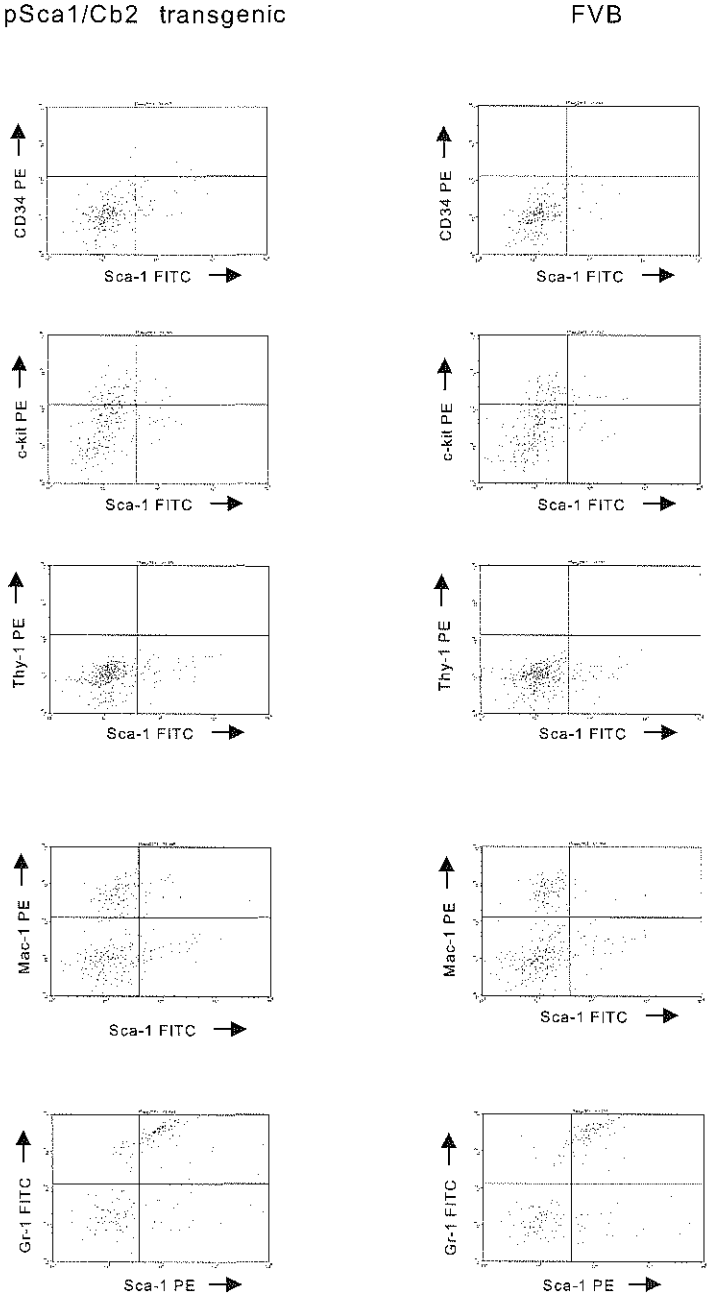

Figure 2. Immunological analysis of transgenic and control bone marrow cells.
Percoll separated bone marrow cells were double labelled using the indicated membrane markers and analysed by flow cytometry.

Increased tumour incidence by Cas-Br-M MuLV in pSca-1/Cb2 transgenic mice

To further investigate the role of *Cb2* in leukemogenesis and study its potency as a collaborating proto-oncogene in tumour progression, newborn transgenic mice and non-transgenic littermates were infected with Cas-Br-M MuLV. Animals were monitored for tumour development. Twenty-two months after retroviral infection 11 of 19 (58%) *pSca-1/Cb2* transgenic mice developed hematological malignancies, whereas leukemias were observed in only 4 out of 39 (10%) virus-infected control animals (Figure 3). Autopsy of moribund animals revealed an enlarged spleen (5-10-fold enlargement; [6]) in almost all of the cases (Table 2). In one case (CFL 7) a thymoma was observed. In cases with a splenomegaly, morphological analysis demonstrated high blast percentages in five cases and accumulation of granulocytic cells at all stages of differentiation in the other five. Morphologic analysis of bone marrow cells showed accumulation of blasts or granulocytic cells as well (Table 2). Immunological analysis of the leukemic spleen cells demonstrated the presence of primitive membrane markers (*Sca-1*; CD34 and/or ER-MP58) in all cases examined (Table 2). In 6 cases monocytic markers were observed and in 3 positivity for granulocytic markers (Gr1 and Mac-1 positivity, but negative for F4/80) was present. In one case (CFL 7) the T-cell markers Thy1 and CD3 were present. The hematopoietic profile as determined by blood smears revealed that most mice were severely anemic. Furthermore, in the majority of cases there was a strong reduction in white blood cell counts (Table 2).

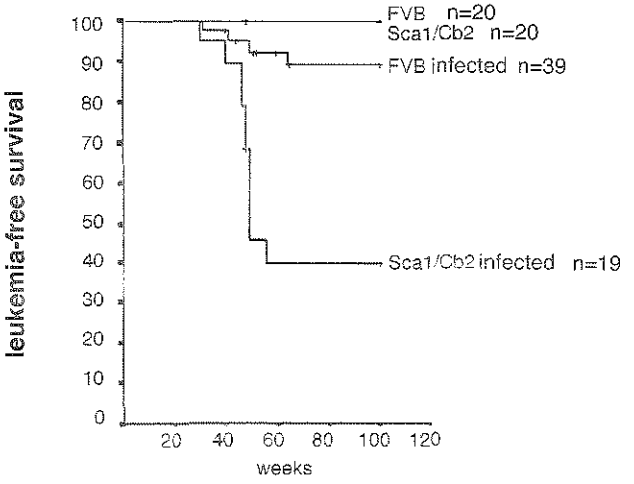

Figure 3. Increased incidence of leukemia in *pSca-1/Cb2* transgenic mice.
Cas-Br-M MuLV infected *pSca-1/Cb2* (n=19) and FVB (n=39) as well as non-infected *pSca-1/Cb2* (n=20) and FVB (n=20) were monitored for tumour development. The graph indicates the percentage of mice remaining tumour-free (vertical axis) at the indicated ages (horizontal axis).

Overexpression of Cb2 and the Evi1 proto-oncogene in pSca-1/Cb2 transgenic leukemias

To investigate whether leukemic organs in transgenic animals expressed high Cb2 mRNA levels, an RNase protection analysis was performed using probe B, representing exon 1B + 2. As expected leukemic cells of *pSca-1/Cb2* transgenic mice demonstrated high Cb2 mRNA expression (Figure 4A).

Leukemic transformation is a multistep process involving activation of multiple proto-oncogenes and/or inactivation of tumour suppressor genes. One of the most commonly found retroviral insertions in Cas-Br-M MuLV-induced leukemias is *Evi1*. Moreover, in NFS78, a leukemia expressing high Cb2 protein levels as the result of retroviral insertion in the gene, *Evi1* mRNA and protein overexpression has been demonstrated. RNase protection revealed high *Evi1* mRNA levels in spleen cells from 6 out of 8 *pSca1/Cb2* derived leukemias studied (Figure 4B). Southern blot analysis using an *Evi1* specific probe [14], on *Sst*I-digested genomic DNA was carried out on 5 of those cases (Figure 4C). This analysis demonstrated rearrangements as the result of proviral insertion in the *Evi1*-locus in 2 of those cases. These data suggest that aberrantly expressed Cb2 cooperates with elevated levels of Evi1 in the transformation of myeloid precursor cells.

Table 2. Characterisation of the leukemic *pSca1/Cb2* transgenic mice.

Tumour[1]	Gross pathology[2]	Blood counts		Blast percentage		Immunology			
		White[3]	Red[4]	Marrow	Spleen	Mono[5]	Gran[6]	T[7]	Pre[8]
CFL7	T	ND	ND	ND	ND	+	-	+	+
CFL13	S	ND	ND	ND	ND	-	-	-	+
CFL14	S	3.3	7.8	ND	ND	-	-	-	+
CFL23	S	ND	ND	18*	2*	ND	ND	ND	ND
CFL25	S	2.7	1.1	77	53	ND	ND	ND	ND
CFL28	S	7.3	5.5	ND	ND	+	+	-	+
CFL29	S	0.6	2.4	82	77	+	-	-	+
CFL32	S	3.4	5.2	ND	4*	+	+	-	+
CFL34	S	ND	ND	16*	0*	+	+	-	+
CFL37	S	41	11.3	2*	23	ND	ND	ND	ND
CFL44	S	42.6	3.4	46	12	+	-	-	+

[1]The tumors are designated CFL (Cas-Br-MuLV; FVB background; Leukemia).
[2]The majority of the mice presented with a splenomegaly (S). One mouse had a thymoma (T).
[3]White blood cell counts represent numbers X 10^9 per liter.
[4]Red blood cell counts represent numbers X 10^{12} per liter.
[5]Monocytic marker positivity (Mono), i.e. M1/70 (Mac1) or/and F4/80.
[6]Granulocytic marker positivity (Gran), i.e. RB68C5 (Gr1) and M1/70 (Mac-1), without positivity for F4/80.
[7]T-lymphocytic marker positivity (T), i.e. KT-3 (CD3) and 59-AD2.2(Thy1)
[8]Precursor marker positivity (Pre), i.e. ER-MP58, E13 161-7 (Sca1)or MEC 14.7 (anti-CD34).
*In the cases with low blast percentages differential counts revealed the presence of myelo/granulocytic cells from all stages of differentiation.
A sample was considered positive for a particular marker when 30% of the cells or more showed positive fluorescence [15].

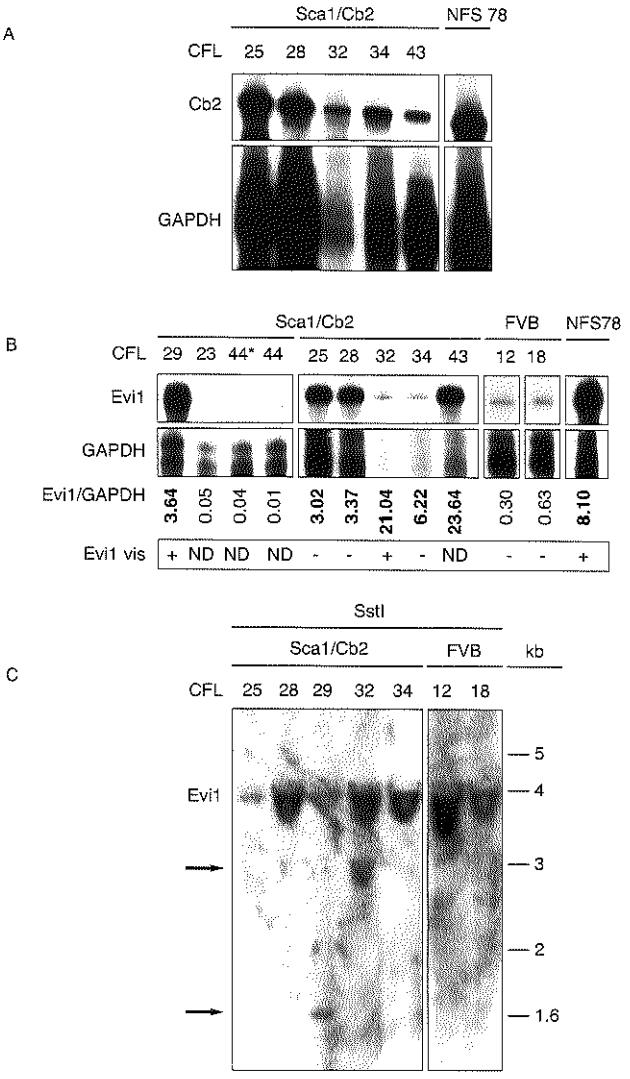

Figure 4. *Evi1* mRNA expression in leukemic spleen and marrow isolated from Cas-Br-M MuLV treated *pSca-1/Cb2*transgenic and FVB mice.

A. RNase protection using probe B on 10 µg total mRNA isolated from leukemic spleens. The protected *Cb2* bands are indicated. NFS 78 mRNA has been taken along as a positive control and GAPDH was used as an internal control

B. RNase protection using an *Evi1* specific RNA probe [6] on 10 µg mRNA isolated from leukemic spleens or bone marrow*. The protected *Evi1* bands are shown. NFS 78 mRNA has been taken along as a positive control and GAPDH was used as an internal control. *Evi1* mRNA expression levels were quantified relative to the GAPDH levels, using GeneTools (version 2.10.03 ©1998 Synoptics Ltd, Cambridge, UK).

C. Southern blot analysis has been carried out to investigate the presence of a retroviral insertion into the *Evi1* locus (See Materials and Methods). Southern blot analysis demonstrated the presence of a proviral insertion in the *Evi1* locus (arrows). Evi1 rearrangements (Evi vis) have also been indicated in figure 3B (+/-). ND = not done.

Discussion

Previously, *Cb2* has been identified as a target for retroviral insertion and it may therefore be a proto-oncogene involved in the development of certain mouse leukemias. *Cb2* retroviral integrations were found in the myeloid cell lines NFS 78 and NFS107, and in a large panel of primary leukemias that were generated by injecting newborn NIH-Swiss mice with Cas-Br-M MuLV [1]. Phenotyping of the tumours revealed that virus integrations in *Cb2* may occur in myeloid as well as in T-lymphoid leukemias [15]. To study the effect of enforced expression of Cb2 in primitive hematopoietic cells transgenic mice were generated using a *Sca-1 (Ly-6E.1)* promoter construct. The Sca-1 protein is a membrane surface marker expressed on hematopoietic precursors as well as on a subset of T-lymphocytes [16-18]. *pSca-1/LacZ* transgenic mice have previously been generated to study primitive hematopoiesis [5]. Recently, we successfully expressed the *Evi1* proto-oncogene in the hematopoietic system using the *Sca-1* promoter construct [6]. In this report we demonstrate that expression of *Cb2* mRNA (RNase protection) and protein (ligand binding assay) is high in spleens of *pSca-1/Cb2* transgenic animals.

Although examples exist, showing that a single defect may result in a severe hematological abnormality [19-21], other transgenic models reveal that additional genetic lesions may be required for progression towards leukemia or lymphoma [22-24]. During follow-up of more than one year *Cb2* transgenic mice did not develop leukemia or another apparent hematological defect. The lack of significant hematological effects caused by Cb2, as determined by hematological and immunological analysis and in vitro colony cultures (Figure 2 and Table 1), may be due to the choice of the *Sca-1* promoter, which functions in a small population of primitive hematological precursors [5]. In addition, it is possible that enforced expression of Cb2 alone is not sufficient to cause hematological defects. Previous studies have demonstrated that *Cb2* is a frequent target of retroviral insertion. Leukemias with *Cb2* abnormalities arise with long latency, suggesting that multiple 'hits' in time are required for full transformation of hematopoietic precursors [15]. In order to investigate the role of *Cb2* as a collaborating oncogene, *Cb2* transgenics were infected with Cas-Br-M MuLV. Significantly more *pSca-1/Cb2* mice developed leukemia than non-transgenic control mice indicating that *Cb2* transgenic mice indeed carry a higher susceptibility for Cas-Br-M MuLV-induced tumour formation. Determination of the phenotype of these leukemias revealed a large variation present between the distinct tumours. Since in the FVB control population only 4 mice developed leukemia, a comparison between phenotypes in this setting is not reliable. However, primary leukemias that were generated previously by infecting newborn NIH-Swiss mice with Cas-Br-M MuLV demonstrated a similar spectrum of phenotypes [15].

We decided to investigate which other transforming genes may collaborate with Cb2 in leukemic transformation. Overexpression of the peripheral cannabinoid receptor as the result of virus insertion in *Cb2* has been demonstrated in the myeloid precursor cell line NFS 78 ([1, 3]. Interestingly, in this cell line the *Evi1* gene is also highly transcribed as the result of proviral integration [25] (Figure 4). RNase protection using RNA from leukemias derived from *pSca-1/Cb2* transgenics revealed high *Evi1* mRNA levels in 6 out of 8 cases studied. In 2 out of 5 *Evi1* mRNA expressing leukemias a virus insertion was observed by means of Southern blot analysis. In three cases high *Evi1* mRNA levels were found whereas no retroviral insertion in the *Evi1*

locus was observed. It is possible that in these leukemias retroviral insertion took place in the *Fim-3* locus, which is located approximately 90 kb upstream of *Evi1*[26]. In the murine leukemic cell lines DA3 and DA34 virus integrations in the *Fim-3* region result in over-expression of Evi1 [26]. On the other hand, it is also possible that Evi1 is overexpressed by other mechanisms. Interestingly, preliminary results obtained from real-time RT-PCR studies in human AML, demonstrate that *EVI1* is expressed in at least 5% of de novo AML cases without a 3q26 abnormality [27]. No matter the mechanism, the observed association of increased *Cb2* and *Evi1* gene expression may be suggestive for a cooperative action between these two genes in leukemic transformation. Breeding of *Cb2* transgenic animals with *Evi1*-mutant mice possibly followed by retroviral infection should provide further documentation of the cooperative action of these two genes in leukemia progression.

Primary leukemias containing an integration in *Cb2* displayed a myeloid or a T-lymphoid phenotype [15]. In contrast, the leukemias from the *pSca-1/Cb2* mice mainly exhibit a myeloid phenotype. It is unlikely that the *Sca-1* promoter construct employed for ectopic expression of Cb2 selects for myeloid malignancies rather than for a T-lymphoid disease. The discrepancies could be the result of the genetic differences of the mouse strains used in these different studies. Animals with NIH-Swiss background were previously injected with Cas-Br-M MuLV for the identification of novel virus integration sites in leukemia [15], whereas FVB mice were used in the study presented here.

It is currently unclear by which mechanism Cb2 may be involved in leukemic transformation. *Cb2* encodes a $G_{\alpha i}$-coupled peripheral cannabinoid receptor. This receptor, like other $G_{\alpha i}$-coupled seven transmembrane receptors, may be involved in chemoattraction. Myeloid precursor cells overexpressing Cb2 migrate in a trans-well assay, when stimulated with the endogenous ligand 2-arachidonoylglycerol (2-AG) [3]. Altered migration *in vivo* as the result of stimulation of the Cb2 receptor may cause differences in homing of the cells. Changes in homing of myeloid precursors may result in altered regulation of growth and development. Infection of normal bone marrow precursors with Cb2/EGFP using retroviral vectors may be carried out to study the effects of peripheral cannabinoid receptor expression on homing of myeloid precursors *in vivo*. The Cb2 specific antagonist SR144528, when included in these experiments, should interfere with altered homing of myeloid precursors *in vivo*. The availability of the Cb2 specific antagonists may also be used to investigate whether blocking of the Cb2 receptor on leukemia cells, interferes with leukemic progression *in vivo*. Leukemias generated in the *pSca-1/Cb2* transgenic mice presented in this study may be of value for these investigations.

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Chapter 8

Summary and Discussion
&
Samenvatting en Discussie (in Dutch)

Summary and Discussion

Leukemia is characterised by an accumulation in the bone marrow of non-functional blood cells arrested at a particular stage of differentiation. In the process of normal hematopoiesis, errors may occur as the result of mutations in the DNA of hematopoietic precursor cells. These genetic lesions may lead to activation of proto-oncogenes, inactivation of tumour suppressor genes or expression of aberrant gene products. The combination of different genetic lesions in a hematopoietic progenitor cell may ultimately result in the development of leukemia. An approach to find leukemia disease genes is the identification and cloning of common virus integration sites in murine leukemias. This approach has proven to be a sensitive tool to identify novel proto-oncogenes as well as tumour suppressor genes.

We used the slow transforming retrovirus Cas-Br-M murine leukemia virus (Cas-Br-M MuLV) in NIH/Swiss mice to establish a panel of leukemias (Chapter 2). All tumours found in leukemic animals were classified by gross pathology, morphology, and immunophenotype as well as the incidence of known common virus integration sites in murine leukemia virus-induced myeloid malignancies, i.e., *Evi1* [1], *Evi1/Cb2* [2], *Evi12* [3], *Fli1* [4] and c-*Myb* [5]. While most of the immunophenotyped Cas-Br-M MuLV induced tumours were of myeloid origin (58%), also numerous T-cell leukemias (21%) and mixed myeloid/T-cell leukemias (21%) were found. The myeloid leukemias and myeloid compartment of the mixed leukemias were further characterised by immunophenotyping with stem cell-, myeloid- and erythroid-specific antibodies. The known Cas-Br-M MuLV common virus integration sites *Evi1*, *Evi1/Cb2* and *Evi12* were demonstrated in 19%, 12% and 20% of the cases, respectively, whereas no *Fli1* or c-*Myb* rearrangements were found. Integrations into *Evi1* were restricted to myeloid leukemias, whereas those in *Evi1/Cb2* and *Evi12* were identified in myeloid as well as T-lymphoid leukemias. This panel of well-characterised Cas-Br-M MuLV-induced hematopoietic tumours may be useful for the isolation and characterisation of new proto-oncogenes involved in murine myeloid or T-cell leukemias.

The identification of common virus integration sites and isolation of potential disease genes in those loci used to be a laborious and time-consuming procedure. We established a rapid and simple RT-PCR based method, which facilitates the identification of virus integration sites and novel "leukemia" genes (Chapter 3). In addition to the RT-PCR technique, we used an inverse-PCR procedure combined with automated sequencing on the Cas-Br-M MuLV-induced primary mouse leukemias to isolate novel virus integration sites (Chapter 4). As a result, 126 different virus integration sites were cloned. Subsequently, a nested-PCR/Southern-blotting procedure was developed on genomic DNA from a large panel of MuLV-induced leukemias, to investigate whether a particular virus insertion represented a common virus integration site. This analysis was carried out for approximately one third of the virus integrations. The majority of the virus integration sites analysed appeared to represent a common virus integration site, suggesting that the other virus insertions represented common virus integration sites as well and may also harbour novel disease genes. Among the novel virus integration sites we found insertions in or near genes encoding nuclear proteins, novel putative signalling or transporter proteins, a ringfinger-protein gene and a panel of genes encoding novel proteins with unknown function. The specificity of the two methods described in Chapters 3 and 4 was confirmed by the detection of previously identified common virus integration sites

and their corresponding leukemia genes, e.g. *Evi1* [1, 6], *Evi2* [7], *Hoxa7* [8], *c-Myb*, *Cb2/Evi11* [2], *His1* [9], and *Evi12* [3]. These findings emphasise the significance of the novel common virus integration sites found in our studies.

Large scale cloning of common virus integration sites and identification of the human homologues of the affected genes will serve a clinical interest. Retrospective studies in large cohorts of patient samples may demonstrate a correlation between expression or mutation of a (novel) transforming gene and prognosis of disease development. A representative example of a gene identified as a common virus insertion which may also be critical in human AML development is Evi1. Murine *Evi1* was identified as a common virus integration site in myeloid leukemia [1] (Chapters 2, 3 and 4). *EVI1* encodes a DNA binding protein and is located on human chromosome 3q26 [6]. Normally the gene is expressed at low levels in hematopoietic tissues, whereas high expression was observed in AML and MDS cases carrying a 3q26 abnormality [10, 11]. In a retrospective study, analysing 319 de novo AML patients, Barjesteh Van Waalwijk Van Doorn-Khosravani et al. demonstrated applying real-time PCR analysis that high EVI1 expression occurred in approximately 10% of de novo AML cases. EVI1 expression appeared independent of the presence of 3q26 abnormalities and was highly predictive for unfavourable outcome of treatment [12]. High throughput analysis of gene expression through microarray technology allows the simultaneous analysis of thousands of parameters within a single experiment. Considering technical possibilities this is an even more rapid and pluriform technique than Real-time PCR to analyse aberrant expression of genes. Changes in expression patterns resulting from transcriptional, posttranscriptional or posttranslational control, and differences in subcellular localisation of proteins may be observed using this approach [13, 14].

Mutation analysis should provide additional information concerning the role of novel leukemia genes in human AML. High throughput sequencing protocols are now available to identify mutations in disease genes in AML on a large-scale. It has for instance been demonstrated by investigators in our institute that C-terminal mutations in the C/EBPalpha gene in AML with intermediate-risk karyotype predict a good prognosis [15]. Patients with C-terminal C/EBPalpha mutations had remission and relapse rates comparable to AML patients carrying a good-risk karyotype (i.e. t(15;17), t(8;21), or inv(16)/del(16) [16-18]). Although C/EBPalpha has not been identified as a common virus integration site, these data demonstrate the value of high-throughput sequencing of candidate genes in leukemia.

The group of novel common virus integration sites that were identified in Chapter 4, contained the *nm23-M2* gene. *Nm23-M2* is one of eight members of the highly homologous family of *nm23*-genes [19], which can be divided into two groups (Chapter 5). One group contains the genes *nm23-M1* to *-M4*, which are 44% to 88% similar in amino acid sequence. The second group, consisting of *nm23-M5* to *-M8*, share considerably less homology with the first group or among each other (3% to 30%). *Nm23-M1* and *nm23-M2*, which share the greater homology, are located in tandem on mouse chromosome 11. Moreover, the human equivalent of *nm23-M1* has been reported to be abnormally expressed in leukemias or lymphomas [20, 21]. High expression levels of *NM23-H2* are associated with a poor prognosis in AML-M2, -M4, and –M5 [22]. PCR/Southern blotting demonstrated that also *nm23-M1* and *nm23-M3* were frequent targets of proviral insertion. This indicates that multiple but not all members of the *nm23*-family may be involved in leukemic transformation.

Since gene expression may be controlled at multiple levels, a lack of mRNA expression regulation does not exclude the presence of altered protein levels. In

Chapter 6 it is demonstrated that expression of the gene *nm23-M2* may be influenced by the hematopoietic growth factors erythropoietin (EPO) and stem cell factor (SCF). However, we did not detect altered expression in nm23-M2 mRNA levels between growth factor stimulated cells and starved cells by Northern blot analysis. We demonstrate, however, that nm23-M2 polysome binding was growth factor dependent, indicating a translational control of gene expression by EPO and/or SCF.

To evaluate how a particular gene may be aberrantly expressed and what its role may be in transformation, insight into the mechanism of its normal control and function will be of great value. *In vivo* and *in vitro* models have been developed to evaluate the role of particular genes in development. The nm23-M2 gene may normally be expressed in hematopoietic precursors. Regulation of nm23-M2 expression in development was investigated in the erythroid progenitor cell line I/11(Chapter 6). This precursor cell is capable of differentiating towards mature erythrocytes in vitro when stimulated with EPO [23, 24]. We demonstrated in Chapter 6 that association of nm23-M2 mRNA with polysomes as well as protein expression decreased during EPO induced differentiation of I/11 cells, indicating a change of translation of *nm23-M2* with erythroid maturation. Regulation of nm23-M2 polysome binding also appeared dependent on PI3K. Moreover, it was found that nm23-M2 mRNA started with a TOP-tract, a stretch of pyrimidines that is often found in translationally controlled genes. The data indicate that control of nm23-M2 protein expression may occur at the level of translation and suggest that abberant gene expression in malignant disease may not only occur by aberrant control of transcription.

The growth factor dependent 32D myeloid cell line is capable of terminal differentiation towards neutrophils following stimulation with G-CSF [25]. In previous studies it has been demonstrated that this differentiation model may be of great value to study the role of potential disease gene in leukemic transformation. Introduction of *Evi1*, *Notch1*, or a truncated form of the G-CSF receptor resulted in a block of differentiation [26-28]. As described in Chapter 5 we made use of the 32D system to investigate the potential role of *nm23-M2* in myeloid development. In contrast to what has been demonstrated with other disease genes, aberrant expression of nm23-M2 did not affect neutrophilic differentiation. Adaptation of the cells might have caused unresponsiveness to the aberrant protein expression, or the gene may have been switched off during cell growth. Inducible systems have been designed to avoid such limitations and study the role of nm23-M2 in altered myelopoiesis. On the other hand it is also possible that alternative models are required to study the effect of nm23-M2 in transformation. As an alternative, it would be of interest to study the effect of uncontrolled expression of nm23-M2 in the erythroid cell line I/11.

To investigate the role of a potentially transforming gene in hematopoiesis *in vivo* transgenic mice may be generated as discussed for *Cb2* in Chapter 7. Although increased expression of the *Cb2* gene was observed in hematopoietic tissues, during a follow-up of more than one year no hematological defects became apparent. Expression of Cb2 alone may not be adequate to cause any hematological abnormalities. Taking into account that overexpression of Cb2 *in vitro* is sufficient for blocking the differentiation of myeloid precursor cells [29], it may be more plausible that the lack of a direct effect of Cb2 overexpression is due to the choice of the promoter used to express the transgene in the mice. The *Sca-1* promoter functions in a small population of primitive hematologic precursors [30]. To further investigate the role of Cb2 receptor *in vivo*, transgenics could be made under the control of another promoter. Alternatively it would be wise to generate inducible and/or conditional

constructs to overexpress a potential gene of interest. Cre-recombinase is becoming an important instrument for achieving precise genetic manipulation in mice. Using the recombinase activity as a genetic activation or inactivation switch, conditional transgenic or conditional knock-out mice are currently being created. The Cre protein catalyses the recombination between the two *loxP* recognition sites [31]. For conditional transgenesis, the promoter and the coding region of a gene of interest are separated by a *loxP* flanked STOP region, which does not allow any transcription initiated from the promoter to read through and include the gene. When this region is removed by Cre-mediation, the gene of interest may be expressed. In order to create an inducible system, the Cre-recombinase may be fused to the LBD of the mutated estrogen receptor. In the presence of tamoxifen the Cre-fusion protein translocates into the nucleus and will induce the Cre-mediated excision [32].
Since the Cb2 transgenic mice did not develop any hematological defects during a long-time follow up, the cooperative potential of Cb2 was investigated by infection of newborn *pSca-1/Cb2* transgenic mice with Cas-Br-M MuLV (Chapter 7). This revealed that significantly more virus treated transgenic mice developed leukemia than virus exposed control littermates, suggesting that Cb2 indeed plays a role in leukemia progression. Moreover, Evi1 overexpression was observed in several independent virus-induced leukemias in *pSca-1/Cb2* transgenic mice. These data would indicate that hematopoietic precursor cells that express high levels of Cb2 possess increased susceptibility for leukemia development. The linked overexpression of *Cb2* and *Evi1* would suggest collaboration in leukemogenesis.

This thesis describes the generation of a novel panel of primary mouse leukemias. By two newly developed and rapid techniques, common virus integrations sites could be identified. The isolation of known cVISs, such as Evi1, Nf1, Evi11/Cb2, and Evi12 confirmed the specificity of the methods. Two genes that were found to be a cVIS were analysed functionally for involvement in leukemogenesis; i.e. nm23-M2 and Evi11/Cb2.

Samenvatting en Discussie

Leukemie wordt gekenmerkt door een opeenhoping van niet-functionele cellen in het beenmerg die tijdens een bepaald stadium in de differentiatie tot staan zijn gebracht. In het proces van normale hematopoiese kunnen fouten optreden als resultaat van mutaties in het DNA van hematopoietische voorlopercellen. Deze genetische lesies kunnen leiden tot activatie van proto-oncogenen, inactivatie van tumorsuppressor genen of expressie van aberrante genprodukten. De combinatie van verschillende genetische defecten in een hematopoietische voorlopercel kan uiteindelijk leiden tot het ontstaan van leukemie. Een benadering om leukemie ziektegenen te vinden is het identificeren en kloneren van zogenaamde "common virus integratie sites" in leukemieën bij muizen. Het is bewezen dat deze benadering een gevoelige methode is die het identificeren van zowel nieuwe proto-oncogenen als tumorsuppressorgenen mogelijk maakt.
Wij hebben gebruik gemaakt van het langzaam transformerende retrovirus Cas-Br-M muizenleukemievirus (Cas-Br-M MuLV) om een groep leukemieën te verkrijgen bij NIH/Swiss muizen (Hoofdstuk 2). Alle tumoren die gevonden werden in leukemische dieren werden geclassificeerd op basis van globale pathologie, morfologie en immunofenotype, en ook op basis van bekende common virus integratie sites in muizenleukemievirus-geinduceerde maligniteiten zoals *Evi1* [1], *Evi11/Cb2* [2], *Evi12* [3], *Fli1* [4] en c-*Myb* [5]. Terwijl de meeste geïmmunofenotypeerde Cas-Br-M MuLV geïnduceerde tumoren van myeloide origine waren (58%), werden ook talrijke T-cel leukemieën (21%) en gemengd myeloide/T-cel leukemieën (21%) gevonden. De myeloide leukemieën en het myeloide compartiment van de gemengde leukemieën werden verder geanalyseerd met behulp van immunofenotypering met stamcel-, myeloid- en erythroid-specifieke antilichamen. De bekende Cas-Br-M MuLV common integratiesites *Evi1*, *Evi11/Cb2* en *Evi12* werden in respectievelijk 19%, 12% en 20% van de gevallen aangetoond, terwijl er geen rearrangeringen in *Fli1* en c-*Myb* werden gevonden. Integraties in *Evi1* waren beperkt tot myeloide leukemieën, terwijl die in *Evi11/Cb2* en *Evi12* gevonden werden in zowel myeloide als T-lymfoide leukemieën. Deze groep van goed beschreven Cas-Br-M MuLV-geïnduceerde hematopoietische tumoren kan bruikbaar zijn voor het isoleren en karakteriseren van nieuwe proto-oncogenen die betrokken zijn bij myeloide of T-cel muizenleukemieën.
De identificatie van common virus integratiesites en isolatie van mogelijke ziektegenen in deze loci is altijd een arbeidsintensieve en tijdverslindende procedure geweest. Wij hebben een snelle en simpele methode ontwikkeld, die gebaseerd is op RT-PCR, en die de identificatie van virus integratiesites en nieuwe "leukemiegenen" vergemakkelijkt (Hoofdstuk 3). Als aanvulling op de RT-PCR techniek hebben we een inverse-PCR procedure toegepast in combinatie met geautomatiseerde sequentiebepaling op de Cas-Br-M MuLV geïnduceerde primaire muizenleukemieën om nieuwe common virus integratiesites te isoleren (Hoofdstuk 4). Als resultaat werden 126 verschillende virus integratiesites gekloneerd. Vervolgens werd een interne PCR-Southern-blot procedure ontwikkeld op genomisch DNA van een grote groep muizenleukemievirus geïnduceerde leukemieën, om te onderzoeken of een bepaalde virus integratie ook een common integratiesite vertegenwoordigde. Deze analyse werd uitgevoerd op ongeveer een-derde van de virus integratie sites. De meerderheid van de virus integratiesites die geanalyseerd werden, bleek een common integratie site te vertegenwoordigen. Dit suggereert dat de andere virus integratiesites ook common zijn en nieuwe ziektegenen kunnen bevatten. Onder de nieuwe virus

integratiesites vonden wij inserties in of nabij genen die coderen voor kerneiwitten, nieuwe potentiele signaal- of transporteiwitten, een ringfinger-eiwitgen en een groep genen die voor nieuwe eiwitten met onbekende functies coderen. De specificiteit van de methodes die in Hoofdstuk 3 en 4 beschreven worden werd bevestigd door de detectie van eerder beschreven common virus integratiesites en hun correponderende leukemiegenen, zoals *Evi1* [1, 6], *Evi2* [7], *Hoxa7* [8], *c-Myb*, *Cb2/Evi11* [2], *His1* [9] en *Evi12* [3]. Deze bevindingen benadrukken de significantie van de nieuwe common virus integratiesites die gevonden zijn in onze studies.
Het op grote schaal kloneren van common virus integratiesites en identificeren van de humane homologen van de aangedane genen dient een klinisch belang. Retrospectieve studies in grote cohorten met patiëntenmateriaal kunnen een correlatie tussen expressie of mutatie van een (nieuw) transformerend gen en de prognose van de ziekteontwikkeling aantonen. Een representatief voorbeeld van een gen dat geïdentificeerd is als een common virus integratiesite die ook kritiek kan zijn in de ontwikkeling van humane AML is *EVI1*. Muizen-*Evi1* was geidentificeerd als een common virus integratiesite in myeloide leukemie [1] (Hoofdstukken 2, 3 en 4). *EVI1* codeert voor een DNA-bindend eiwit en is gelegen op humaan chromosoom 3q26 [6]. Normaalgesproken wordt dit gen op een laag niveau tot expressie gebracht in hematopoietische weefsels, terwijl hoge expressie gezien is in gevallen van AML en MDS die een 3q26 afwijking hadden [10, 11]. In een retrospectieve studie waarin 319 *de novo* AML patienten geanalyseerd werden, toonden Barjesteh Van Waalwijk Van Doorn-Khosravani et al. door middel van real-time PCR analyse aan dat hoge EVI1 expressie in ongeveer 10% van de *de novo* AML gevallen voorkwam. EVI1 expressie leek onafhankelijk te zijn van de aanwezigheid van 3q26 afwijkingen en was in hoge mate voorspellend voor een ongunstige uitkomst van de behandeling [12]. "High throughput" analyse van genexpressie via mico-array technologie maakt gelijktijdige analyse van duizenden parameters mogelijk binnen een enkel experiment. Rekening houdend met technische mogelijkheden is dit een nog snellere en meer pluriforme techniek dan Real-time PCR analyse om aberrante expressie van genen te analyseren. Veranderingen in patroon die het resultaat zijn van transcriptionele, posttranscriptionele of posttranslationele controle en verschillen in subcellulaire lokalisatie van eiwitten kan door middel van deze benadering bestudeerd worden [13, 14].
Mutatie analyse zou aanvullende informatie betreffende de rol van nieuwe leukemiegenen in AML moeten brengen. High-throughput sequentie protocollen zijn nu beschikbaar om op grote schaal mutaties in ziektegenen in AML te identificeren. Het is bijvoorbeeld aangetoond door onderzoekers van ons instituut dat C-terminale mutaties in het C/EBPalpha gen in AML met gemiddeld-risico karyotype een gunstige prognose voorspellen [15]. Patienten met C-terminale C/EBPalpha mutaties hadden remissie en relapse cijfers die vergelijkbaar waren met die van AML patienten met een gunstig-risico karyotype (bijv. t(15;17), t(8;21), of inv(16)/del(16) [16-18]). Hoewel C/EBPalpha nooit geidentificeerd is als een common virus integratie site, tonen deze data de waarde van high-throughput sequentiebepaling van kandidaat-leukemiegenen aan.
De groep van nieuwe common virus integratie sites die geidentificeerd werden in Hoofdstuk 4, bevatte het *nm23-M2* gen. *Nm23-M2* is een van de acht leden van de zeer homologe familie van nm23-genen [19], die onderverdeeld kan worden in twee groepen (Hoofdstuk 5). Eén groep bevat de genen *nm23-M1* tot en met *-M4*, die 44% tot 88% gelijk zijn in aminozuurvolgorde. De tweede groep, bestaand uit *nm23-M5* tot

en *met –M8*, delen aanzienlijk minder homologie met de eerste groep, maar ook met elkaar (3% tot 30%). *Nm23-M1* en *nm23-M2*, die het meest homoloog zijn, liggen in tandem op muizenchromosoom 11. Ook is gepubliceerd dat de humane equivalent van *nm23-M1* abnormaal tot expressie gebracht wordt in leukemieën en lymfomen [20, 21]. Hoge expressie van *NM23-H2* is geassocieerd met een slechte prognose in AML-M2, -M4 en –M5 [22]. PCR/Southern blotting heeft aangetoond dat ook in *nm23-M1* en *–M3* frequent provirale insertie optreedt. Dit geeft aan dat meerdere, maar niet alle leden van de nm23-familie betrokken zouden kunnen zijn in leukemische transformatie.

Aangezien genexpressie op meerdere niveaus gecontoleerd kan worden, sluit een tekort aan mRNA expressie regulatie niet uit dat er sprake kan zijn van een veranderde eiwitexpressie. In Hoofdstuk 6 wordt aangetoond dat expressie van het gen *nm23-M2* beïnvloed kan worden door de hematopoietische groeifactoren erythropoietine (EPO) en stam cel factor (SCF). Toch konden wij door middel van Northern blot analyse geen veranderde expressie van nm23-M2 mRNA aantonen tussen groeifactor gestimuleerde cellen en gestarveerde cellen. Wij tonen echter aan dat binding van nm23-M2 aan polysomen groeifactor afhankelijk is, wat aangeeft dat er sprake is van translationele controle van genexpressie door EPO en/of SCF.

Om te evalueren hoe een bepaald gen aberrant tot expressie gebracht kan worden en wat zijn rol in transformatie kan zijn, is inzicht in het mechanisme van normale controle en functie van grote waarde. Er zijn *in vivo* en *in vitro* modellen ontwikkeld om de rol van bepaalde genen in de ontwikkeling te onderzoeken. De expressie van nm23-M2 kan normaal zijn in hematopoietische voorlopercellen. Regulatie van nm23-M2 expressie gedurende ontwikkeling werd onderzocht in de erythroide voorlopercellijn I/11 (Hoofdstuk 6). Deze voorlopercel kan *in vitro* tot een volwassen erythrocyt differentiëren na stimulatie met EPO [23, 24]. In Hoofdstuk 6 tonen wij aan dat zowel de associatie van nm23-M2 mRNA met polysomen als de eiwitexpressie omlaag gaat gedurende EPO-geinduceerde differentiatie van I/11 cellen. Dit geeft aanwijzingen voor een verandering in *nm23-M2* translatie tijdens erythroide maturatie. Regulatie van nm23-M2 polysoombinding lijkt ook afhankelijk te zijn van PI3K. Bovendien is gevonden dat het nm23-M2 mRNA met een TOP-tract begint, een rij pyrimidines die vaak in translationeel gereguleerde genen gevonden wordt. De data duiden erop dat controle van nm23-M2 eiwitexpressie op het niveau van translatie kan gebeuren en suggereren dat aberrante genexpressie in maligne ziekten niet alleen kan optreden door aberrante controle van transcriptie.

De groeifactor afhankelijke myeloide cellijn 32D kan volledig uitrijpen tot een neutrofiele granulocyt volgend op stimulatie met G-CSF [25]. In voorheen uitgevoerde studies is aangetoond dat dit differentiatiemodel grote waarde kan hebben voor het bestuderen van de rol van mogelijke ziektegenen in leukemische transformatie. Introductie van *Evi1*, *Notch1* of een verkorte vorm van de G-CSF receptor resulteert in een blokkade van differentiatie [26-28]. Zoals beschreven in Hoofdstuk 5 hebben wij gebruik gemaakt van het 32D systeem om de mogelijke rol van *nm23-M2* in myeloide ontwikkeling te onderzoeken. In tegenstelling tot wat voor andere ziektegenen aangetoond is, heeft aberrante expressie van nm23-M2 geen invloed op neutrofiele differentiatie. Een mogelijke verklaring hiervoor is dat adaptatie van de cellen ervoor gezorgd kan hebben dat zij geen reactie vertonen op de aberrante eiwitexpressie. Een andere oorzaak zou kunnen zijn dat het gen uitgeschakeld is gedurende celgroei. Om dergelijke beperkingen te vermijden zijn induceerbare systemen ontworpen om zo de rol van nm23-M2 in veranderde

myelopoiese te bestuderen. Aan de andere kant is het ook mogelijk dat andere modellen nodig zijn om het effect van nm23-M2 op transformatie te bestuderen. Als alternatief zou het interessant zijn om het effect van ongecontroleerde nm23-M2 expressie in I/11 cellen te onderzoeken.
Om de rol van een potentieel transformerend gen in hematopoiese *in vivo* te onderzoeken kunnen transgene muizen gemaakt worden, zoals beschreven voor *Cb2* in Hoofdstuk 7. Alhoewel toegenomen expressie van het *Cb2* gen in hematopoietische cellen waargenomen werd, werden er geen hematologische defecten gezien gedurende een follow-up van meer dan een jaar. Expressie van Cb2 alleen zou niet adequaat kunnen zijn om hematologische afwijkingen te veroorzaken. Rekening houdend met het feit dat overexpressie van Cb2 *in vitro* voldoende is om de differentiatie van myeloide voorlopercellen te blokkeren [29], is het meer aannemelijk dat het gebrek van een direct effect van Cb2 overexpressie te wijten is aan de keuze van de gebruikte promoter om het transgen tot expressie te brengen in de muis. De *Sca1*-promoter functioneert in een kleine populatie van primitieve hematologische voorlopers [30]. Om de rol van de Cb2-receptor *in vivo* verder te onderzoeken zouden transgenen onder de controle van een andere promoter gemaakt moeten worden. Als alternatief zou het verstandig zijn om induceerbare en/of conditionele constructen te maken om een gen van interesse tot expressie te brengen. Cre-recombinase wordt meer en meer een manier om preciese genetische manipulatie in muizen te bewerkstelligen. Gebruik makend van de recombinase activiteit als genetische activatie of inactivatieswitch, worden op dit moment conditionele transgene of conditionele knock-out muizen gecreëerd. Het Cre-eiwit katalyseert de recombinatie tussen de twee *loxP* herkenningsplaatsen [31]. Voor conditionele transgenese worden de promoter en de coderende regio van een gen van interesse van elkaar gescheiden door een *loxP* geflankeerde STOP regio, zodat geen transcriptie toegestaan wordt vanaf de promoter waarbij het gen geïncludeerd wordt. Als deze regio verwijderd is door middel van Cre tussenkomst, kan het gen van interesse tot expressie worden gebracht. Om een induceerbaar systeem te creëren, kan de Cre-recombinase gefuseerd worden aan de LBD van de gemuteerde oestrogeen receptor. In aanwezigheid van tamoxifen verplaatst het Cre-fusie-eiwit zich naar de celkern en zal de Cre gemedieerde excisie induceren [32].
Aangezien de Cb2-transgene muis gedurende een lange follow-up tijd geen hematologische stoornis ontwikkelde, werd de samenwerkende potentie van Cb2 onderzocht door pasgeboren *pSca-1/Cb2* transgenen te infecteren met Cas-Br-M MuLV (Hoofdstuk 7). Dit liet zien dat significant meer transgenen die behandeld waren met het leukemievirus leukemie ontwikkelden dan controlemuizen, wat aanduidt dat Cb2 inderdaad een rol speelt in leukemievoortgang. Bovendien werd overexpressie van Evi1 gezien in verschillende onafhankelijke virus geïnduceerde leukemieën bij *pSca-1/Cb2* transgenen. Deze data zouden kunnen betekenen dat hematopoietische voorlopercellen die Cb2 hoog tot expressie brengen een verhoogde mogelijkheid bezitten voor ontwikkeling van leukemie. De samenhang tussen overexpressie van *Cb2* en *Evi1* zou een samenwerking in leukemogenese kunnen aanduiden.

Dit proefschrift beschrijft de ontwikkeling van een nieuwe groep primaire muizenleukemieën. Via twee nieuw ontwikkelde en snelle technieken konden common virus integratiesites geïdentificeerd worden. De isolatie van bekende common virus integratiesites, zoals Evi1, Nf1, Evi11/Cb2, en Evi12 bevestigt de specificiteit van de

methoden. Twee genen die gevonden werden als common virus integratiesite werden functioneel geanalyseerd voor betrokkenheid bij leukemogenese, i.e. nm23-M2 en Evi11/Cb2.

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List of abbreviations

ALL	acute lymphoblastic leukemia
AML	acute myeloid leukemia
bp	basepair
Casvis	Cas-Br-M virus integration site
CFL	Cas-Br-M MuLV FVB Leukemia
CLL	chronic lymphocytic leukemia
CML	chronic myeloid leukemia
CSL	Cas-Br-M MuLV Swiss Leukemia
cVIS	common virus integration site
DA	IL-3 dependent murine leukemia cell line (Moloney MuLV / Balb/c-mice)
eEF	eukaryote elongation factor
eIF	eukaryote initiation factor
EPO	erythropoietin
ERK	extracellular regulated kinase
Evi	ecotropic virus integration site
FACS	fluorescence-activated cell sorting
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte-macrophage-colony stimulating factor
HGF	hematopoietic growth factors
IL	interleukin
IPCR	inverse PCR
IRES	internal ribosomal entry site
LTR	long terminal repeat
MAPK	mitogen-activated protein kinase
M-CSF	macrophage-colony stimulating factor
MDS	myelodysplastic syndrome
mTOR	mammalian target of rapamycin
MuLV	murine leukemia virus
NCBI	National Center for Biotechnology Information
NFS	IL-3 dependent murine leukemia cell line (Cas-Br-E MuLV / NFS-mice)
nt	nucleotide
PCR	polymerase chain reaction
PI3K	phosphatidylinositol 3-kinase
RT	reverse transcriptase
SCF	stem cell factor
TIM	the incredible man
TOP	terminal oligopyrimidine
TPO	thrombopoietin
VIS	virus integration site

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Curriculum Vitae

The author of this thesis was born on 2 December 1974 in Emmeloord, Noordoostpolder, The Netherlands. She graduated gymnasium-β at Theresialyceum in Tilburg in 1992. After finishing the theoretical part of medical school performing a research project on novel common virus integration sites in murine leukemias, she started the studies described in this thesis at the institute of Hematology, Erasmus MC (late Erasmus University Rotterdam) under the supervision of Prof.Dr. B. Löwenberg and Dr. H.R. Delwel. From October 2001 she is completing the second phase of medical school at Erasmus MC, Rotterdam.

De schrijfster van dit proefschrift werd op 2 december 1974 geboren in Emmeloord, Noordoostpolder. Ze behaalde haar gymnasium-β-diploma in 1992 aan het Theresialyceum in Tilburg. Nadat zij haar doctoraalopleiding geneeskunde aan de Erasmus Universiteit Rotterdam in 1996 had afgerond met een onderzoek naar nieuwe common virus integratiesites in leukemie bij muizen, begon zij aansluitend aan het in dit proefschrift beschreven onderzoek bij het instituut Hematologie aan het Erasmus MC (voorheen Erasmus Universiteit Rotterdam) onder leiding van Prof.Dr. B. Löwenberg en Dr. H.R. Delwel. Sinds oktober 2001 is zij bezig met het voltooien van de tweede fase van haar studie aan het Erasmus MC in Rotterdam.

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