Virology 268, 308–318 (2000)
doi:10.1006/viro.2000.0183, available online at http://www.idealibrary.com on IDEAL®

Phenotyping of *Evi1*, *Evi11/Cb2*, and *Evi12* Transformed Leukemias Isolated from a Novel Panel of Cas-Br-M Murine Leukemia Virus-Infected Mice

Marieke Joosten,* Peter J. M. Valk,* Yolanda Vankan,* Nico de Both,† Bob Löwenberg,* and Ruud Delwel*,[1]

*Institute of Hematology and †Department of Pathology, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands

Received July 13, 1999; returned to author for revision December 6, 1999; accepted January 4, 2000

Cas-Br-M murine leukemia virus (MuLV) is a slow-transforming retrovirus that potently induces leukemias in mice and therefore is 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 tumors found in leukemic animals were classified by gross pathology, morphology, and immunophenotype, as well as the incidence of known common virus integration sites (VISs) in MuLV-induced myeloid malignancies (i.e., *Evi1*, *Evi11/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 tumors 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 characterized by immunophenotyping with stem cell-, myeloid-, and erythroid-specific antibodies. The known Cas-Br-M MuLV common VISs (*Evi1*, *Evi11/Cb2*, and *Evi12*) were demonstrated in 19%, 12%, and 20% of the cases, respectively, whereas no *Fli1* and c-*Myb* rearrangements were found. Integrations into *Evi1* were restricted to myeloid leukemias, whereas those in *Evi11/Cb2* and *Evi12* were identified in myeloid as well as T-lymphoid leukemias. This panel of well characterized Cas-Br-M MuLV-induced hematopoietic tumors may be useful for the isolation and characterization of new proto-oncogenes involved in myeloid or T-cell leukemias.

Key Words: retrovirally induced leukemia; Cas-Br-M murine leukemia virus; common virus integration site.

INTRODUCTION

Slow-transforming retroviruses that do not contain oncogenes in their genome induce tumors by means of insertional mutagenesis (Ihle *et al.,* 1990; Jonkers and Berns, 1996; Wolff, 1997). The identification of common virus integration sites in retrovirally induced tumors provides a powerful strategy to isolate novel transforming genes from leukemias and lymphomas (Ihle *et al.,* 1990; Jonkers and Berns, 1996; Wolff, 1997). 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* (Morishita *et al.,* 1990, 1988), *Evi2 (NF1)* (Buchberg *et al.,* 1990; Shannon *et al.,* 1994; Side *et al.,* 1998), *Evi6 (Hoxa9)* (Nakamura *et al.,* 1996a,b), *Bcl*1 (cyclin D1) (de Boer *et al.,* 1997; Silver and Buckler, 1986), N-*Myc* (Hirvonen *et al.,* 1993; Setoguchi *et al.,* 1989), and *Erg* (Shimizu *et al.,* 1993; Valk *et al.,* 1997b)]. The molecular basis of retroviral insertional mutagenesis in the development of leukemias is the activation of proto-oncogenes or inactivation of tumor 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 (Jonkers and Berns, 1996; Wolff, 1997).

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 hematological diseases in NFS mice (Holmes *et al.,* 1986) and non-B-, non-T-cell leukemias in NIH/Swiss mice (Bergeron *et al.,* 1991). A number of recurrent targets have been observed: *Evi1* (Bergeron *et al.,* 1993; Morishita *et al.,* 1988; Mucenski *et al.,* 1988), *Evi11/Cb2* (Valk *et al.,* 1997a), *Evi12* (Valk *et al.,* 1999), and *Fli1* (Bergeron *et al.,* 1991) or, for Moloney and Friend MuLV [i.e., c-*Myb* (Nason-Burchenal and Wolff, 1993; Nazarov and Wolff, 1995; Shen-Ong and Wolff, 1987)]. *Evi1* encodes a zinc-finger transcription factor (Morishita *et al.,* 1988), and overexpression of *Evi1* has been shown to interfere in granulocytic (Morishita *et al.,* 1992) as well as erythroid differentiation (Kreider *et al.,* 1993). *Evi11* and *Evi12* are two relatively novel common VISs that we recently identified in the Cas-Br-M MuLV-induced interleukin-3-dependent myeloid cell line NFS107 and the Cas-Br-M MuLV-induced primary tumors described in this report (Valk *et al.,* 1997a, 1999). *Evi11* is located on mouse chromosome 4, and the

[1] To whom reprint requests should be addressed. Fax: 31104089470. E-mail: Delwel@hema.fgg.eur.nl.

0042-6822/00 $35.00

TABLE 1

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

target proto-oncogene in this locus is the gene encoding the hematopoietic peripheral cannabinoid receptor Cb2 (Valk *et al.,* 1997a). The candidate proto-oncogene in *Evi12*, which was mapped onto mouse chromosome 10 (Valk *et al.,* 1999), is currently unknown. The ets-transcription factor *Fli1* was originally identified by cloning proviral insertions from Friend MuLV-induced erythroid leukemias (Ben-David *et al.,* 1990), but proviruses in *Fli1* were later also shown in non-B-, non-T-cell leukemias (Bergeron *et al.,* 1991). The transcription factor c-*Myb* is one of the best-studied proto-oncogenes in myeloid disease (Introna *et al.,* 1994) and has been shown to be a target for proviral insertions in myeloid leukemias (Wolff, 1997)

To isolate novel common VISs and identify new target proto-oncogenes and cooperating proto-oncogenes (Valk *et al.,* 1999) involved in myeloid leukemia development, we generated a novel panel of Cas-Br-M MuLV-induced leukemias in NIH/Swiss mice. We characterized the hematological malignancies by gross pathology, cytology, and immunophenotyping using a panel of monoclonal antibodies (MAbs) 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. Although 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 characterized 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.

RESULTS

Leukemia development and gross pathology

Tumor 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 that were inoculated developed hematological malignancies, with 74 of these becoming severely sick with signs of splenomegaly and/or a thymoma (Table 1). The other 17 mice developed a mild disease, with a moderate increase (twofold) 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.

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 hematological malignancies. Interestingly, only 33 of 62 (53%) female mice developed severe leukemia, whereas tumor formation in male mice was evident in 41 of 54 (76%) cases. Furthermore, male mice developed leukemia more rapidly than female mice (Fig. 1). 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 2). Thirty-one of 41 male mice (71%) and 24 of 33 females (73%) manifested leukemia with a major spleen enlargement, whereas in 10 of 41 males (24%) and 7 of 33 females (21%), the mice also developed a thymoma. Two females developed thymoma without splenomegaly.

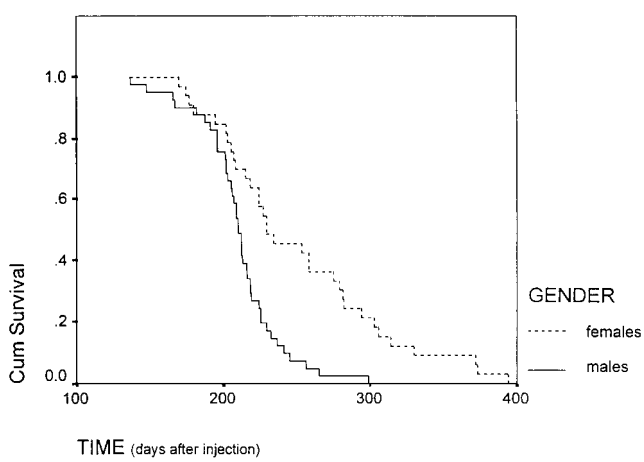

FIG. 1. Survival curves of male (—) and female (- - -) NIH/Swiss mice developing leukemia after inoculation with Cas-Br-M MuLV. A log-rank test was performed, based on Kaplan-Meier analysis. Males died significantly earlier than females ($P < 0.001$). The *x*-axis represents the time at which the mice developed leukemia after injection with the Cas-Br-M MuLV.

TABLE 2

Gross Pathology of Cas-Br-M MuLV-Induced Leukemias

Sex	Gross pathology	No. of mice	%	Latency period (days)	Mean
Males	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
Females	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 MAbs (Table 3).

T-cell leukemias. Thirteen cases displayed a solely T-cell phenotype [i.e., $CD3^{++}/Thy1^{++}$ (11 cases), $CD3^{++}/Thy1^{-}$ (1 case) or $CD3^{-}/Thy1^{++}$ (1 case)], without expression of myeloid markers (Fig. 2 and Table 4). In 10 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 8 of these displaying a thymus enlargement as well.

Myeloid leukemias. A $CD3^{-}/Thy1^{-}$ or $CD3^{-}/Thy1^{+}$ non-T-cell phenotype (Fig. 2 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 nonlymphoid compartment of the 13 mixed leukemias, containing both $CD3^{++}/Thy1^{++}$ and $CD3^{-}$ cells, were further immunophenotyped using the complete panel of MAbs (Table 3), which revealed heterogeneity among the cases. Six cases (5 non-T-cell and 1 mixed leukemia) showed an immature myeloid phenotype, that is, 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, that is, Imm^{+}, Gr^{+} ($Mac1^{+}$), but $F4/80^{-}$. Myeloid leukemias expressing monocyte/macrophage differentiation markers, that is, 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 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* (Bergeron *et al.,* 1993, 1992; Morishita *et al.,* 1988; Mu-

TABLE 3

Monoclonal Antibodies

Antibody	Antigen	Specificity	Reference
ER-MP54	ER-MP54 Ag	Myeloid cells	Leenen *et al.,* 1990b
ER-MP58	ER-MP58 Ag	Myeloid cells	Leenen *et al.,* 1990b
M1/70	Mac1	Nonfixed macrophages granulocytes, natural killer cells	Springer *et al.,* 1979
F4/80	F4/80 Ag	Macrophages	Austyn and Gordon, 1981
RB68C5	Gr1	Granulocytes	Hestdal *et al.,* 1991
ER-MP21	Transferrin receptor	Cells in cycle, erythroid cells	Leenen *et al.,* 1990a
TER119	Glycophorin A	Mature erythroid cells	Ikuta *et al.,* 1990
59-AD2.2	Thy1	T-cells, hematopoietic stem cells, myeloid cells	Ledbetter and Herzenberg, 1979
KT3	CD3	Mature functional T-cells	Tomonari, 1988
RA3 6B2	B220	B-cells, myeloid cells	Coffman, 1982
E13 161-7	Sca1	Hematopoietic precursors, T-cells	Aihara *et al.,* 1986

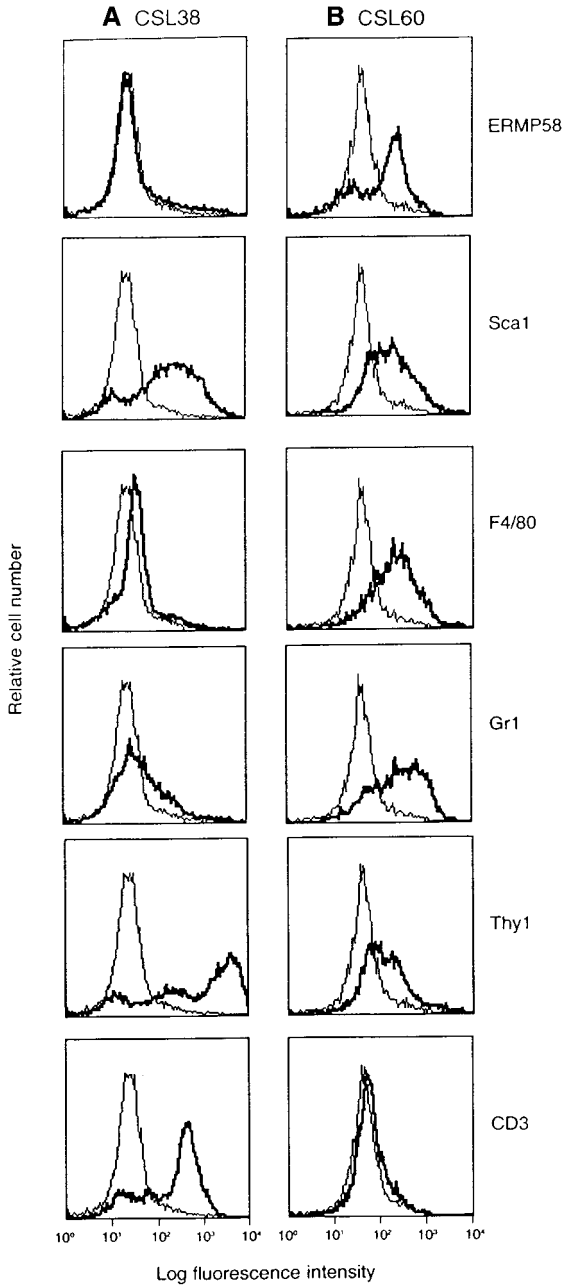

FIG. 2. Immunophenotyping of Cas-Br-M MuLV-induced primary CSL tumors. Representatives of leukemias with a T-cell phenotype ($Thy1^{++}$ and $CD3^{++}$) (A, CSL38) and with a non-T-cell phenotype ($CD3^{-}$ and $Thy1^{+\ or\ -}$) (B, CSL60). CSL60 is a typical example of a leukemia type IV (see Table 5) (i.e., blasts with granulocytic and monocytic differentiation).

censki *et al.,* 1988), *Evi11/Cb2* (Valk *et al.,* 1997a), *Evi12* (Valk *et al.,* 1999), and *Fli1* (Bergeron *et al.,* 1991), whereas Moloney and Friend MuLV also target c-*Myb* (Nason-Burchenal and Wolff, 1993, 1995; Shen-Ong and Wolff, 1987). 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 (Fig. 3). No abnormalities in *Fli1* or c-*Myb* were observed. In 48 of 74 cases, no retroviral insertions in any of these regions could be identified.

Evi1. Rearrangements in the *Evi1* gene were found in 14 of 74 cases (Figs. 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 (Fig. 4B) (Bartholomew *et al.,* 1989; Bergeron *et al.,* 1993; Valk *et al.,* 1997b). *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, five leukemias showed an erythroid phenotype (i.e., the cells were ER-MP21 and TER119 positive; Table 5). Three mice, from which the tumors were later shown to have *Evi1* rearrangements, died before any viable cell could be harvested and immunophenotyped.

Evi11/Cb2. The target gene in *Evi11* has been shown to be *Cb2*. Retroviral insertions in *Cb2* occur either 5′ or 3′ of the protein-coding region (Valk *et al.,* 1997a, 1999). Rearrangements in *Evi11/Cb2* were found in 9 of the 74 cases studied (Fig. 3). The exact positions of these *Evi11* retroviral insertion sites have been documented recently (Valk *et al.,* 1999). *Evi11* rearrangements were found in T-cell, myeloid, or mixed leukemias (Table 6). Three *Evi11* rearrangements were found in leukemic spleens obtained from mice that died before immunological analysis could be carried out. Interestingly, in addition to the nine leukemic cases, Southern blot analysis revealed rearrangements in the *Evi11/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 (Fig. 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 (Valk *et al.,* 1999). The target gene in *Evi12* is currently unknown. As with *Evi11/Cb2* rearrangements, retroviral insertions in *Evi12* have been found in T-cell (three cases), non-T-cell (two cases), and mixed leukemias (four cases) (Table 6). Of the 74 mice that developed a severe leukemia, five died before any viable cell could be harvested. Interestingly, all five mice carried a retroviral insertion in *Evi12*.

Tumors with multiple rearrangements. Coincidence of *Evi1* with other integrations appeared to be rare: *Evi1* and *Evi11/Cb2* in one case and *Evi1* and *Evi12* in two cases, whereas *Evi1, Evi11/Cb2*, and *Evi12* were also observed in one case (Fig. 3). Interestingly, six of the *Evi11/Cb2* rearranged cases also harbored *Evi12* virus

TABLE 4

Immunophenotyping of Cas-Br-M MuLV-Induced Leukemias: T-Cell versus Non-T-Cell Leukemias

	Immunophenotype	Splenomegaly	Thymoma[a]	Total
T-cell leukemias	$CD3^{++}/Thy1^{++}$	3[b]	10[c]	13
Non-T-cell leukemias[d]	$CD3^{-}/Thy1^{-\text{ or }+}$	36	1	37
Mixed leukemias[e]	$CD3^{++}/Thy1^{++}$ and $CD3^{-}/Thy1^{-\text{ or }+}$	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.

integrations (Fig. 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 analyzed phenotypically. One leukemia had a T-cell phenotype (CSL14), and two mixed leukemias expressed T-cell as well as myeloid markers (CSL12 and CSL16).

DISCUSSION

Cas-Br-M MuLV-injected newborn NIH/Swiss mice developed detectable leukemias by approximately 140–400 days postinoculation. 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 (Holmes *et al.,* 1986, 1985). Bergeron *et al.* (1993, 1992, 1991) 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 *et al.* used a molecular clone NE-8, described by Jolicoeur *et al.* (1983), whereas we inoculated a biologically cloned Cas-Br-M MuLV stock obtained by limiting dilution (Hartley and Rowe, 1976) in the same mouse strain. In contrast to our studies, Bergeron *et al.* (1993, 1992, 1991) observed frequent insertions of Cas-Br-E MuLV in *Fli1*, whereas these investigators observed no Cas-Br-E provirus insertions in either *Evi11/Cb2* or *Evi12* loci (E. Rassart, personal communication). In fact, only mink cell focus forming retroviral integrations were identified in *Evi11* and *Evi12 (Valk et al.,* 1999), suggesting the presence of amphotropic virus contaminant in the Cas-Br-M stock, which is absent in Cas-Br-E (Ott *et al.,* 1992; Rasheed *et al.,* 1982) 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 (Nason-Burchenal and Wolff, 1993; Nazarov and Wolff, 1995; Shen-Ong and Wolff, 1987) but not for Cas-

TABLE 5

Immunophenotyping of Non-T-Cell Leukemias and Number of *Evi1*-Positive Tumors among Them

	Leukemia type	Immunophenotype[a]	Non-T-cell leukemias	Mixed leukemias[b]	*Evi1*-positive 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	$ER\text{-}MP21^{+}$, $TER119^{+}$, Imm^{+}, $(Mac1^{+})$, $(F4/80^{+})$, and $(Gr1^{+})$	6	4	5
VI:	B-lymphocytic	$B220^{+}$ and $other^{-}$	2	0	0
Total			37	13	11

Note. Leukemias were immunophenotyped using the monoclonal antibodies indicated in Table 3.
[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 analyzed.
[c] Expression markers between parentheses may be positive or negative.

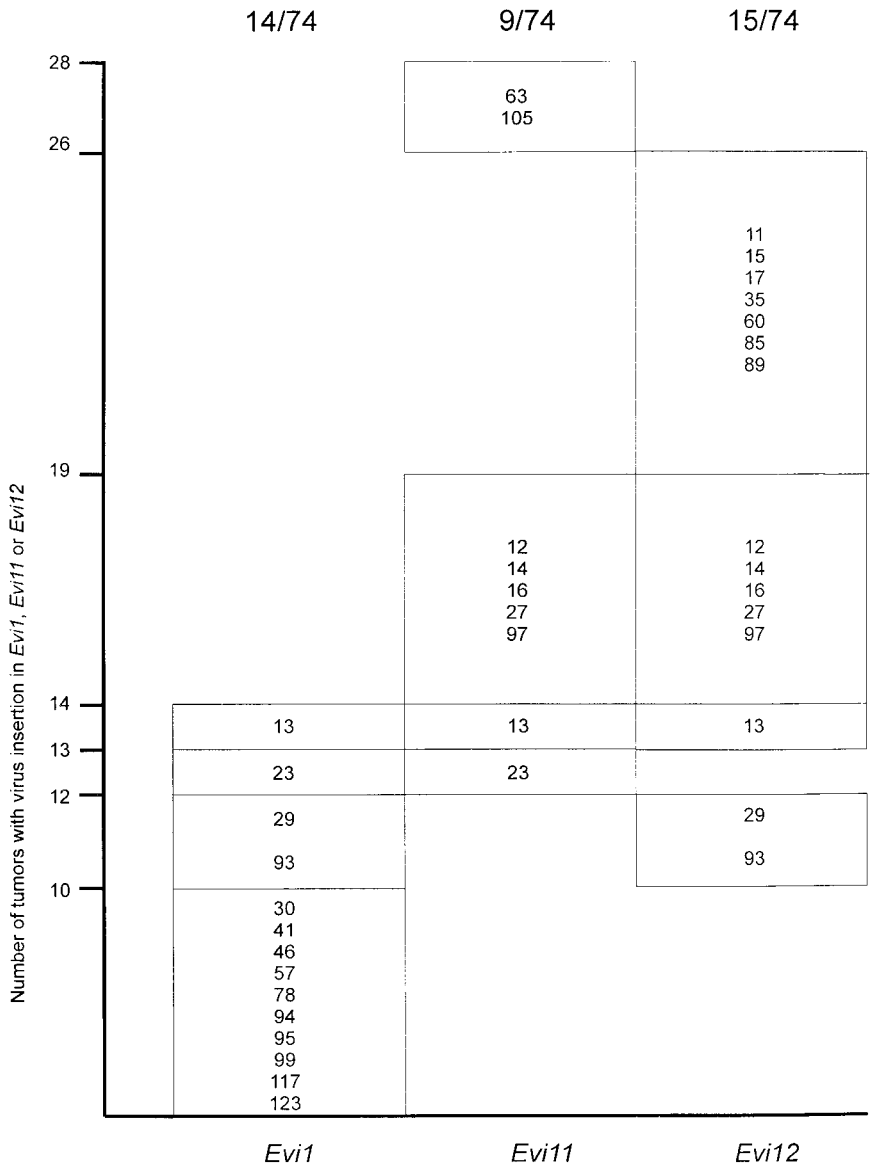

FIG. 3. Common virus integration sites *Evi1*, *Evi11/Cb2*, and *Evi12* in primary CSL tumors. Diagram represents the different provirus insertions in *Evi1*, *Evi11/Cb2*, and *Evi12* in the primary CSL tumors. Overlapping boxes symbolize coincidence of provirus insertions in two or three common VISs (i.e., *Evi1*, *Evi11/Cb2*, and/or *Evi12*). In each box, the CSL leukemia numbers are depicted. The *y*-axis represents the sum of the leukemias.

Br-type viruses. The results from these studies together reemphasize that infection with 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 (Jonkers and Berns, 1996; Wolff, 1997).

More males then females developed leukemia after Cas-Br-M MuLV-injection. In fact, seven female mice did not develop any hematological malignancy at all, even at 13 months after inoculation. Moreover, male mice developed leukemia more rapidly than did females. Gender-related sensitivity to tumor-inducing agents in rodents has been documented in several tumorigenic conditions previously. For instance, female animals have been reported to be more sensitive to certain tumor-inducing agents or viruses (Siglin *et al.,* 1995; Storch and Chused, 1984), whereas other studies have demonstrated a higher tumor incidence in males after exposure to radiation (Di Majo *et al.,* 1996; Yoshida *et al.,* 1993). As an interesting parallel to our study, the radiation-induced tumors, showing greater susceptibility in male mice, represent cases of acute myeloid leukemia (Di Majo *et al.,* 1996; Yoshida *et al.,* 1993). Explanations to clarify these gender-specific differences are mainly based on speculation, including possible differences in sensitivity of certain tumor cells to gender-specific hormones (Siglin *et al.,* 1995; Yoshida *et al.,* 1993). 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-in-

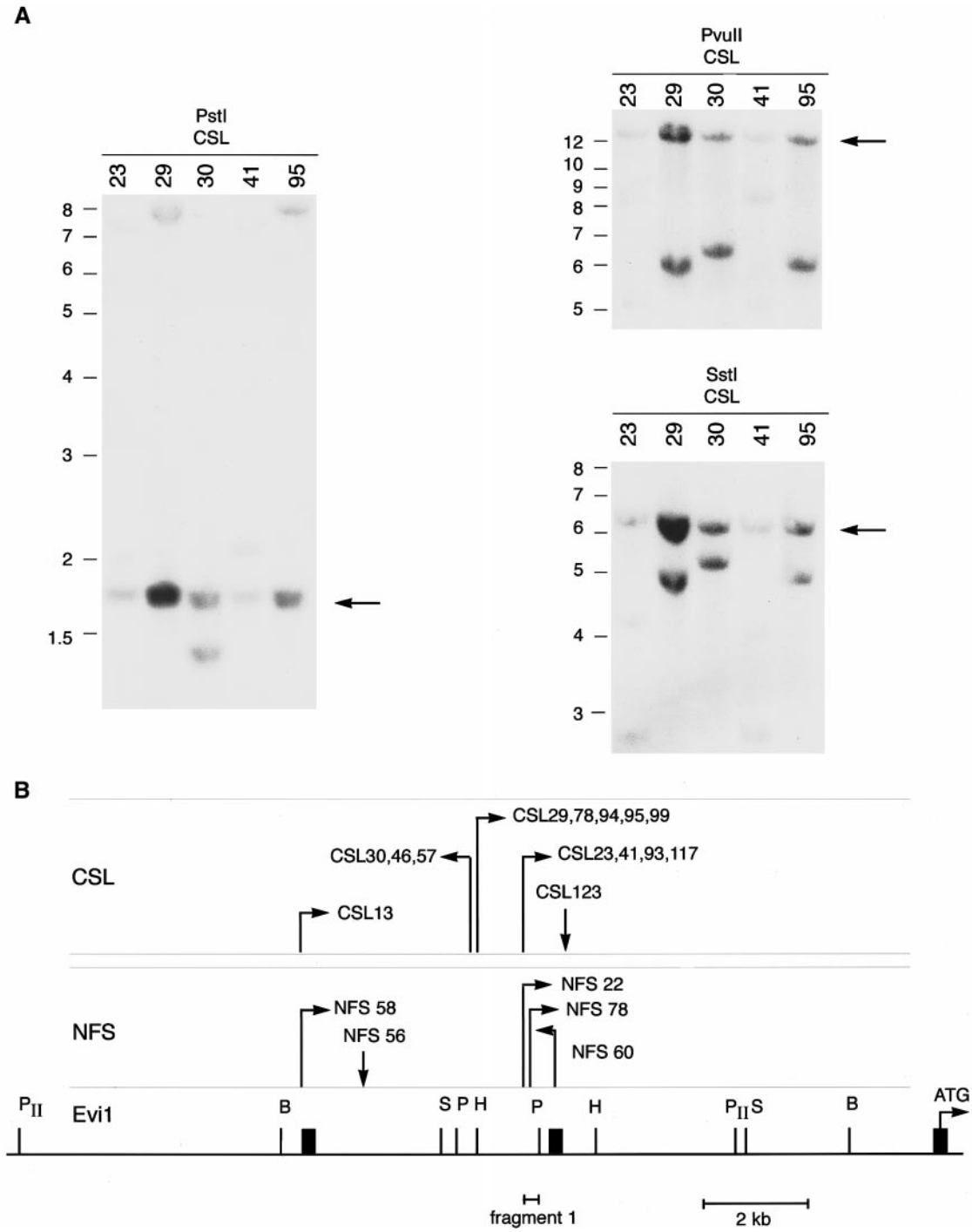

FIG. 4. Provirus insertion in the common VIS *Evi1*. (A) Southern blot analysis of *Pst*I-, *Pvu*II-, and *Sst*I-digested genomic DNA of primary CSL tumors. Filters were hybridized with fragment 1 (B), a cDNA specific for the *Evi1* locus (Valk *et al.,* 1997b). The size marker is depicted in kilobases, and an arrow indicates the size of the normal allele. All tumors (i.e., CSL23, CSL29, CSL30, CSL41, and CSL95) 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 tumors and NFS cell lines (Holmes *et al.,* 1985) are depicted by arrows.

duced 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 (Bartholomew *et al.,* 1989; Bergeron *et al.,* 1993, 1992; Morishita *et al.,* 1988). In humans, *EVI1* has shown to be mutated in acute myeloid leukemias with chromosome 3q26 abnormalities (Mor-

TABLE 6

Common Virus Integration Sites in Cas-Br-M MuLV-Induced Tumors: Immunologic Characterization

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.

ishita *et al.,* 1990) and in certain cases of myelodysplastic syndrome (Dreyfus *et al.,* 1995), a preleukemic disease characterized by a severe anemia. Overexpression of *Evi1* in 32Dcl3 cells has been shown to interfere with granulocytic differentiation of these cells when stimulated with granulocyte colony-stimulating factor (Morishita *et al.,* 1992). In addition, Kreider *et al.* (1993) demonstrated a block of erythroid differentiation by *Evi1 in vitro*. Possibly, Evi1 interferes with GATA1, a transcription factor that is indispensable for erythropoiesis (Pevny *et al.,* 1995, 1991). Recent studies with *Evi1* transgenic mice also support this hypothesis (i.e., a defective erythropoiesis as a result of *Evi1* overexpression) (Louz *et al.,* manuscript in preparation). 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, such as *Evi1* retroviral insertions, may determine whether myeloid or lymphoid leukemias evolve.

Leukemia initiation and progression involve aberrant expression of multiple genes (Adams and Cory, 1992). Two or more VISs have frequently been shown to be present in one particular tumor or tumor cell line. For instance, *Hoxa-* and *Pbx1*-related genes in myeloid leukemias (Nakamura *et al.,* 1996b), c-*Myc* and *M-CSF* in a monocyte tumor (Baumbach *et al.,* 1988), *IL-3*, *SCL*, and *Hoxb8* in myeloid WEHI-3B(D^-) cells (Tanigawa *et al.,* 1994), *IL-3* or *GM-CSF* and c-*Myb* in WEHI-274 cells (Leslie *et al.,* 1991), *IL-3* and *GM-CSF* in *in vivo*-passaged FDC-P1 cells (Duhrsen *et al.,* 1990), *p53* and *PU.1* in erythroleukemias (Moreau-Gachelin *et al.,* 1988), and *Fli1* and *p53* in non-T-, non-B-cell leukemias (Bergeron *et al.,* 1993). These data indicate that leukemogenesis, like oncogenesis in general (Hunter, 1991; Vogelstein and Kinzler, 1993), is indeed a multistep process involving mutation of multiple oncogenes (Adams and Cory, 1992). 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, CSL13 and CSL23. In fact, as a result of retroviral insertions in both loci, aberrant expression of *Evi1* and *Evi11/Cb2* has been demonstrated in the myeloid cell line NFS78 (Valk *et al.,* 1997a). 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 recently generated (unpublished results). *Evi1* also appears to collaborate with an *Evi12*-related proto-oncogene because three leukemias contain insertions in both of these loci (i.e., CSL13, CSL29, and CSL93). Two common VISs that frequently coincide are *Evi11/Cb2* and *Evi12*, that is, rearrangements were shown in both common VISs in CSL12, CSL13, CSL16, CSL27, and CSL97. 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*, harbor Cas-Br-M provirus, which may imply that indeed three or maybe more genetic defects are required for full leukemic transformation (Vogelstein and Kinzler, 1993).

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 signaling pathways leading to full malignant transformation of hematopoietic cells.

MATERIALS AND METHODS

Mice, viruses, and pathology of tumors

NIH/Swiss mice were obtained from Harlan (Horst, The Netherlands). Cas-Br-M MuLV-producing NIH 3T3 cells (donated by Dr. Janet W. Hartley, National Institutes of Health, Bethesda, MA) (Hartley and Rowe, 1976) were cultured in Dulbecco's modified Eagle's medium (GIBCO, Gent, Belgium) containing 10% FCS. To obtain virus, a 70% subconfluent culture of Cas-Br-M MuLV-producing NIH 3T3 cells was incubated in 5 ml of culture medium for 18 h at 37°C in a 75-cm^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 5–13 months after inoculation, with mice sacrificed when moribunded. Leukemic mice had enlarged spleens, frequently accompanied by gross thymus enlargements. Diagnosis was based on gross pathology, cytological staining with May-Grünwald/Giemsa, and flow-cytometric analysis using MAbs directed to membrane surface antigens (Table 3). Leukemias were also characterized for DNA rearrangements within immunoglobulin heavy chain (IgH) genes. Cells were cryopreserved in aliquots of 50×10^6 cells in 7.5% DMSO and 20% FCS using a controlled freezing apparatus and stored in liquid nitrogen.

Labeling of cells with MAbs and flow cytometry

Cells were labeled sequentially with rat MAbs followed by GARa (goat anti-rat)–FITC (Nordic, Tilburg, The Netherlands). All incubations were carried out for 30 min on ice in PBS supplemented with 1% BSA. The specifications of the MAbs used in this study are described in Table 1. Cell surface fluorescence was analyzed 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 (Fig. 2). In the case of Thy1 expression, a discrimination was made 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) (Fig. 2).

DNA isolation, Southern blot analysis, and probes

Isolation of genomic DNA and Southern blot analysis was carried out exactly as described previously (Valk *et al.,* 1997a). The IgH region was analyzed with an *Eco*RI–*Bgl*II (2.5-kb) J_h probe from clone H24 (Honjo *et al.,* 1981) (a gift from Dr. T. Honjo, Kyoto University, Japan). The T-cell receptor β-chain gene was analyzed with a 4-kb *Hin*dIII $J_{\beta 1}$ probe (Duby *et al.,* 1985). Rearrangements in the *Evi1* locus were detected using the 535-bp cDNA fragment that we recently described (Valk *et al.,* 1997b), whereas those in the *Evi11/Cb2* locus were studied with a 1.2-kb *Eco*RI–*Bam*HI *Cb2* cDNA fragment (Valk *et al.,* 1997a). *Evi12* rearrangements were studied with the 503 probe obtained with a RT-PCR-based strategy to isolate cDNA fragments flanking retrovirus integration sites (Valk *et al.,* 1997b). Rearrangements in the c-*Myb* gene were determined with a 450-bp *Nco*I–*Eco*RI and a 500-bp *Pst*I–*Pst*I cDNA probe spanning exons 3–6 of the gene (Shen-Ong *et al.,* 1986). *Fli1* insertions were studied using a 1.7-kb *Eco*RI cDNA probe (Ben-David *et al.,* 1991).

ACKNOWLEDGMENTS

We thank Karola van Rooyen for preparation of the figures and Dr. Alister C. Ward for critical reading of the manuscript. This work was supported by the Dutch Cancer Foundation Koningin Wilhelmina Fonds and the Netherlands Organisation for Scientific Research NWO. R.D. is a Fellow of the Royal Dutch Academy of Sciences KNAW.

REFERENCES

Adams, J. M., and Cory, S. (1992). Oncogene co-operation in leukaemogenesis. *Cancer Surv.* **15,** 119–141.

Aihara, Y., Buhring, H. J., Aihara, M., and Klein, J. (1986). An attempt to produce "pre-T" cell hybridomas and to identify their antigens. *Eur. J. Immunol.* **16,** 1391–1399.

Austyn, J. M., and Gordon, S. (1981). F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *Eur. J. Immunol.* **11,** 805–815.

Bartholomew, C., Morishita, K., Askew, D., Buchberg, A., Jenkins, N. A., Copeland, N. G., and Ihle, J. N. (1989). Retroviral insertions in the CB-1/Fim-3 common site of integration activate expression of the Evi-1 gene. *Oncogene* **4,** 529–534.

Baumbach, W. R., Colston, E. M., and Cole, M. D. (1988). Integration of the BALB/c ecotropic provirus into the colony-stimulating factor-1 growth factor locus in a myc retrovirus-induced murine monocyte tumor. *J. Virol.* **62,** 3151–3155.

Ben-David, Y., Giddens, E. B., and Bernstein, A. (1990). Identification and mapping of a common proviral integration site Fli-1 in erythroleukemia cells induced by Friend murine leukemia virus. *Proc. Natl. Acad. Sci. USA* **87,** 1332–1336.

Ben-David, Y., Giddens, E. B., Letwin, K., and Bernstein, A. (1991). Erythroleukemia induction by Friend murine leukemia virus: Insertional activation of a new member of the ets gene family, Fli-1, closely linked to c-ets-1. *Genes Dev.* **5,** 908–918.

Bergeron, D., Houde, J., Poliquin, L., Barbeau, B., and Rassart, E. (1993). Expression and DNA rearrangement of proto-oncogenes in Cas-Br-E-induced non-T-, non-B-cell leukemias. *Leukemia* **7,** 954–962.

Bergeron, D., Poliquin, L., Houde, J., Barbeau, B., and Rassart, E. (1992). Analysis of proviruses integrated in Fli-1 and Evi-1 regions in Cas-Br-E MuLV-induced non-T-, non-B-cell leukemias. *Virology* **191,** 661–669.

Bergeron, D., Poliquin, L., Kozak, C. A., and Rassart, E. (1991). Identification of a common viral integration region in Cas-Br-E murine leukemia virus-induced non-T-, non-B-cell lymphomas. *J. Virol.* **65,** 7–15.

Buchberg, A. M., Bedigian, H. G., Jenkins, N. A., and Copeland, N. G. (1990). Evi-2, a common integration site involved in murine myeloid leukemogenesis. *Mol. Cell. Biol.* **10,** 4658–4666.

Coffman, R. L. (1982). Surface antigen expression and immunoglobulin gene rearrangement during mouse pre-B cell development. *Immunol. Rev.* **69,** 5–23.

de Boer, C. J., van Krieken, J. H., Schuuring, E., and Kluin, P. M. (1997). Bcl-1/cyclin D1 in malignant lymphoma. *Ann. Oncol.* **8** (suppl 2), 109–117.

Di Majo, V., Coppola, M., Rebessi, S., Saran, A., Pazzaglia, S., Pariset, L.,

and Covelli, V. (1996). The influence of sex on life shortening and tumor induction in CBA/Cne mice exposed to X rays or fission neutrons. *Radiat. Res.* **146,** 81–87.

Dreyfus, F., Bouscary, D., Melle, J., Ribrag, V., Guesnu, M., and Varet, B. (1995). Expression of the Evi-1 gene in myelodysplastic syndromes. *Leukemia* **9,** 203–205.

Duby, A. D., Klein, K. A., Murre, C., and Seidman, J. G. (1985). A novel mechanism of somatic rearrangement predicted by a human T-cell antigen receptor beta-chain complementary DNA. *Science* **228,** 1204–1206.

Duhrsen, U., Stahl, J., and Gough, N. M. (1990). In vivo transformation of factor-dependent hemopoietic cells: Role of intracisternal A-particle transposition for growth factor gene activation. *EMBO J.* **9,** 1087–1096.

Hartley, J. W., and Rowe, W. P. (1976). Naturally occurring murine leukemia viruses in wild mice: Characterization of a new "amphotropic" class. *J. Virol.* **19,** 19–25.

Hestdal, K., Ruscetti, F. W., Ihle, J. N., Jacobsen, S. E., Dubois, C. M., Kopp, W. C., Longo, D. L., and Keller, J. R. (1991). Characterization and regulation of RB6–8C5 antigen expression on murine bone marrow cells. *J. Immunol.* **147,** 22–28.

Hirvonen, H., Hukkanen, V., Salmi, T. T., Pelliniemi, T. T., and Alitalo, R. (1993). L-myc and N-myc in hematopoietic malignancies. *Leuk. Lymphoma* **11,** 197–205.

Holmes, K. L., Langdon, W. Y., Fredrickson, T. N., Coffman, R. L., Hoffman, P. M., Hartley, J. W., and Morse, H. C. d. (1986). Analysis of neoplasms induced by Cas-Br-M MuLV tumor extracts. *J. Immunol.* **137,** 679–688.

Holmes, K. L., Palaszynski, E., Fredrickson, T. N., Morse, H. C. D., and Ihle, J. N. (1985). Correlation of cell-surface phenotype with the establishment of interleukin 3-dependent cell lines from wild-mouse murine leukemia virus-induced neoplasms. *Proc. Natl. Acad. Sci. USA* **82,** 6687–6691.

Honjo, T., Nakai, S., Nishida, Y., Kataoka, T., Yamawaki-Kataoka, Y., Takahashi, N., Obata, M., Shimizu, A., Yaoita, Y., Nikaido, T., and Ishida, N. (1981). Rearrangements of immunoglobulin genes during differentiation and evolution. *Immunol. Rev.* **59,** 33–67.

Hunter, T. (1991). Cooperation between oncogenes. *Cell* **64,** 249–270.

Ihle, J. N., Morishita, K., Matsugi, T., and Bartholomew, C. (1990). Insertional mutagenesis and transformation of hematopoietic stem cells. *Prog. Clin. Biol. Res.* **352,** 329–337.

Ikuta, K., Kina, T., MacNeil, I., Uchida, N., Peault, B., Chien, Y. H., and Weissman, I. L. (1990). A developmental switch in thymic lymphocyte maturation potential occurs at the level of hematopoietic stem cells. *Cell* **62,** 863–874.

Introna, M., Luchetti, M., Castellano, M., Arsura, M., and Golay, J. (1994). The myb oncogene family of transcription factors: Potent regulators of hematopoietic cell proliferation and differentiation. *Semin. Cancer Biol.* **5,** 113–124.

Jolicoeur, P., Nicolaiew, N., DesGroseillers, L., and Rassart, E. (1983). Molecular cloning of infectious viral DNA from ecotropic neurotropic wild mouse retrovirus. *J. Virol.* **45,** 1159–1163.

Jonkers, J., and Berns, A. (1996). Retroviral insertional mutagenesis as a strategy to identify cancer genes. *Biochim. Biophys. Acta* **1287,** 29–57.

Kreider, B. L., Orkin, S. H., and Ihle, J. N. (1993). Loss of erythropoietin responsiveness in erythroid progenitors due to expression of the Evi-1 myeloid-transforming gene. *Proc. Natl. Acad. Sci. USA* **90,** 6454–6458.

Ledbetter, J. A., and Herzenberg, L. A. (1979). Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. *Immunol. Rev.* **47,** 63–90.

Leenen, P. J., Kroos, M. J., Melis, M., Slieker, W. A., van Ewijk, W., and van Eijk, H. G. (1990a). Differential inhibition of macrophage proliferation by anti-transferrin receptor antibody ER-MP21: Correlation to macrophage differentiation stage. *Exp. Cell Res.* **189,** 55–63.

Leenen, P. J., Melis, M., Slieker, W. A., and Van Ewijk, W. (1990b). Murine macrophage precursor characterization. II. Monoclonal antibodies against macrophage precursor antigens. *Eur. J. Immunol.* **20,** 27–34.

Leslie, K. B., Lee, F., and Schrader, J. W. (1991). Intracisternal A-type particle-mediated activations of cytokine genes in a murine myelomonocytic leukemia: Generation of functional cytokine mRNAs by retroviral splicing events. *Mol. Cell. Biol.* **11,** 5562–5570.

Moreau-Gachelin, F., Tavitian, A., and Tambourin, P. (1988). Spi-1 is a putative oncogene in virally induced murine erythroleukaemias. *Nature* **331,** 277–280.

Morishita, K., Parganas, E., Bartholomew, C., Sacchi, N., Valentine, M. B., Raimondi, S. C., Le Beau, M. M., and Ihle, J. N. (1990). The human Evi-1 gene is located on chromosome 3q24–q28 but is not rearranged in three cases of acute nonlymphocytic leukemias containing t(3;5)(q25;q34) translocations. *Oncogene Res.* **5,** 221–231.

Morishita, K., Parganas, E., Matsugi, T., and Ihle, J. N. (1992). Expression of the Evi-1 zinc finger gene in 32Dc13 myeloid cells blocks granulocytic differentiation in response to granulocyte colony-stimulating factor. *Mol. Cell. Biol.* **12,** 183–189.

Morishita, K., Parker, D. S., Mucenski, M. L., Jenkins, N. A., Copeland, N. G., and Ihle, J. N. (1988). Retroviral activation of a novel gene encoding a zinc finger protein in IL-3-dependent myeloid leukemia cell lines. *Cell* **54,** 831–840.

Mucenski, M. L., Taylor, B. A., Ihle, J. N., Hartley, J. W., Morse, H. C. d., Jenkins, N. A., and Copeland, N. G. (1988). Identification of a common ecotropic viral integration site, Evi-1, in the DNA of AKXD murine myeloid tumors. *Mol. Cell. Biol.* **8,** 301–308.

Nakamura, T., Largaespada, D. A., Lee, M. P., Johnson, L. A., Ohyashiki, K., Toyama, K., Chen, S. J., Willman, C. L., Chen, I. M., Feinberg, A. P., Jenkins, N. A., Copeland, N. G., and Shaughnessy, J. D., Jr. (1996a). Fusion of the nucleoporin gene NUP98 to HOXA9 by the chromosome translocation t(7;11)(p15;p15) in human myeloid leukaemia. *Nat. Genet.* **12,** 154–158.

Nakamura, T., Largaespada, D. A., Shaughnessy, J. D., Jr., Jenkins, N. A., and Copeland, N. G. (1996b). Cooperative activation of Hoxa- and Pbx1-related genes in murine myeloid leukaemias [see comments]. *Nat. Genet.* **12,** 149–153.

Nason-Burchenal, K., and Wolff, L. (1993). Activation of c-myb is an early bone-marrow event in a murine model for acute promonocytic leukemia. *Proc. Natl. Acad. Sci. USA* **90,** 1619–1623.

Nazarov, V., and Wolff, L. (1995). Novel integration sites at the distal 3′ end of the c-myb locus in retrovirus-induced promonocytic leukemias. *J. Virol.* **69,** 3885–3888.

Ott, D. E., Keller, J., Sill, K., and Rein, A. (1992). Phenotypes of murine leukemia virus-induced tumors: Influence of 3′ viral coding sequences. *J. Virol.* **66,** 6107–6116.

Pevny, L., Lin, C. S., D'Agati, V., Simon, M. C., Orkin, S. H., and Costantini, F. (1995). Development of hematopoietic cells lacking transcription factor GATA-1. *Development* **121,** 163–172.

Pevny, L., Simon, M. C., Robertson, E., Klein, W. H., Tsai, S. F., D'Agati, V., Orkin, S. H., and Costantini, F. (1991). Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. *Nature* **349,** 257–260.

Rasheed, S., Pal, B. K., and Gardner, M. B. (1982). Characterization of a highly oncogenic murine leukemia virus from wild mice. *Int. J. Cancer* **29,** 345–350.

Setoguchi, M., Higuchi, Y., Yoshida, S., Nasu, N., Miyazaki, Y., Akizuki, S., and Yamamoto, S. (1989). Insertional activation of N-myc by endogenous Moloney-like murine retrovirus sequences in macrophage cell lines derived from myeloma cell line-macrophage hybrids. *Mol. Cell. Biol.* **9,** 4515–4522.

Shannon, K. M., O'Connell, P., Martin, G. A., Paderanga, D., Olson, K., Dinndorf, P., and McCormick, F. (1994). Loss of the normal NF1 allele from the bone marrow of children with type 1 neurofibromatosis and malignant myeloid disorders [see comments]. *N. Engl. J. Med.* **330,** 597–601.

Shen-Ong, G. L., Morse, H. C. d., Potter, M., and Mushinski, J. F. (1986). Two modes of c-myb activation in virus-induced mouse myeloid

tumors [published erratum appears in *Mol. Cell. Biol.* (1986) **6**:2756]. *Mol. Cell. Biol.* **6,** 380–392.

Shen-Ong, G. L., and Wolff, L. (1987). Moloney murine leukemia virus-induced myeloid tumors in adult BALB/c mice: Requirement of c-myb activation but lack of v-abl involvement. *J. Virol.* **61,** 3721–3725.

Shimizu, K., Ichikawa, H., Tojo, A., Kaneko, Y., Maseki, N., Hayashi, Y., Ohira, M., Asano, S., and Ohki, M. (1993). An ets-related gene, ERG, is rearranged in human myeloid leukemia with t(16;21) chromosomal translocation. *Proc. Natl. Acad. Sci. USA* **90,** 10280–10284.

Side, L. E., Emanuel, P. D., Taylor, B., Franklin, J., Thompson, P., Castleberry, R. P., and Shannon, K. M. (1998). Mutations of the NF1 gene in children with juvenile myelomonocytic leukemia without clinical evidence of neurofibromatosis, type 1. *Blood* **92,** 267–272.

Siglin, J. C., Weghorst, C. M., Rodwell, D. E., and Klaunig, J. E. (1995). Gender-dependent differences in hepatic tumor promotion in diethylnitrosamine initiated infant B6C3F1 mice by alpha-hexachlorocyclohexane. *J. Toxicol. Environ. Health* **44,** 235–245.

Silver, J., and Buckler, C. E. (1986). A preferred region for integration of Friend murine leukemia virus in hematopoietic neoplasms is closely linked to the Int-2 oncogene. *J. Virol.* **60,** 1156–1158.

Springer, T., Galfre, G., Secher, D. S., and Milstein, C. (1979). Mac-1: A macrophage differentiation antigen identified by monoclonal antibody. *Eur. J. Immunol.* **9,** 301–306.

Storch, T. G., and Chused, T. M. (1984). Sex and H-2 haplotype control the resistance of CBA-BALB hybrids to the induction of T cell lymphoma by Moloney leukemia virus. *J. Immunol.* **133,** 2797–2800.

Tanigawa, T., Robb, L., Green, A. R., and Begley, C. G. (1994). Constitutive expression of the putative transcription factor SCL associated with proviral insertion in the myeloid leukemic cell line WEHI-3BD. *Cell Growth Differ.* **5,** 557–561.

Tomonari, K. (1988). A rat antibody against a structure functionally related to the mouse T-cell receptor/T3 complex. *Immunogenetics* **28,** 455–458.

Valk, P. J., Hol, S., Vankan, Y., Ihle, J. N., Askew, D., Jenkins, N. A., Gilbert, D. J., Copeland, N. G., de Both, N. J., Lowenberg, B., and Delwel, R. (1997a). The genes encoding the peripheral cannabinoid receptor and alpha-L-fucosidase are located near a newly identified common virus integration site, Evi11. *J. Virol.* **71,** 6796–6804.

Valk, P. J., Vankan, Y., Joosten, M., Jenkins, N. A., Copeland, N. G., Lowenberg, B., and Delwel, R. (1999). Retroviral insertions in Evi12, a novel common virus integration site upstream of Tra1/Grp94, frequently coincide with insertions in the gene encoding the peripheral cannabinoid receptor Cnr2. *J. Virol.* **73,** 3595–3602.

Valk, P. J. M., Joosten, M., Vankan, Y., Lowenberg, B., and Delwel, R. (1997b). A rapid RT-PCR based method to isolate complementary DNA fragments flanking retrovirus integration sites. *Nucleic Acids Res.* **25,** 4419–4421.

Vogelstein, B., and Kinzler, K. W. (1993). The multistep nature of cancer. *Trends Genet.* **9,** 138–141.

Wolff, L. (1997). Contribution of oncogenes and tumor suppressor genes to myeloid leukemia. *Biochim. Biophys. Acta* **1332,** F67–F104.

Yoshida, K., Nemoto, K., Nishimura, M., and Seki, M. (1993). Exacerbating factors of radiation-induced myeloid leukemogenesis. *Leuk. Res.* **17,** 437–440.