

## Large-Scale Identification of Disease Genes Involved in Acute Myeloid Leukemia

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**Acute myeloid leukemia (AML) is a heterogeneous group of diseases in which chromosomal aberrations, small insertions or deletions, or point mutations in certain genes have profound consequences for prognosis. However, the majority of AML patients present without currently known genetic defects. Retroviral insertion mutagenesis in mice has become a powerful tool for identifying new disease genes involved in the pathogenesis of leukemia and lymphoma. Here we have used the Graffi-1.4 strain of murine leukemia virus, which causes predominantly AML, in a screen to identify novel genes involved in the pathogenesis of this disease. We report 79 candidate disease genes in common integration sites (CISs) and 15 genes whose family members previously were found to be affected in other studies. The majority of the identified sequences (60%) were not found in lymphomas and monocytic leukemias in previous screens, suggesting a specific involvement in AML. Although most of the virus integrations occurred in or near the 5' or 3' ends of the genes, suggesting deregulation of gene expression as a consequence of virus integration, 18 CISs were located exclusively within the genes, conceivably causing gene disruption.**

Acute myeloid leukemia (AML) is characterized by a block in myeloid differentiation that results in the accumulation of leukemic myeloid cells in the bone marrow and peripheral blood. For AML cases with specific chromosomal translocations, the identification and functional characterization of fusion genes located at translocation breakpoints have resulted in the discovery of pathways involved in leukemic transformation (34). However, it also has become clear that these defects themselves are not sufficient to cause acute leukemia, supporting the theory that the disruption of multiple regulatory mechanisms is required to fully transform hematopoietic stem and progenitor cells toward AML. At present, cytogenetic parameters are used successfully in clinics for risk stratification of leukemia. For instance, AML cases with t(8;21), t(15;17), and inv(16) chromosomal abnormalities are classified as low risk, whereas cases with 3q26, 5q, and 7q abnormalities generally are classified as high risk, with unfavorable treatment outcomes resulting in early relapse and decreased overall survival (34, 47). Importantly, for the majority of AML cases (>60%), tentatively classified as intermediate risk, the genetic parameters predictive for therapy outcome have not been identified (14, 15). Furthermore, in the approximately 20 to 40% of AML patients without chromosomal abnormalities, the molecular pathogenesis remains entirely unknown (33).

Retroviral insertion mutagenesis in mice has become a powerful and rapid method for the identification of new genes involved in cancer (23). This approach has benefitted greatly from both human and mouse genome programs and the recently developed genome database search programs (20, 29). Studies aimed at finding novel genes involved in leukemia thus

far have been carried out with virus strains that have a propensity to induce lymphoid malignancies or myelomonocytic tumors (21, 26, 32, 37, 44, 54). To focus this strategy on myeloid leukemia, we have used the Graffi-1.4 (Gr-1.4) virus, which has been demonstrated to induce leukemia with predominantly myeloid or mixed-lineage early hematopoietic phenotypes (9, 48). As with the Moloney and Cas-Br-M viruses used in previous studies, the Gr-1.4 virus does not contain oncogenic sequences but can deregulate or disrupt gene expression due to proviral integration.

Recently, Erkeland et al. reported on a new Gr-1.4 virus common integration site (CIS) that is located in the Yin Yang 1 (YY1) promoter and that causes the deregulation of YY1 gene expression, resulting in defective myeloid differentiation (9). Here we report 94 candidate leukemia disease genes that are targeted by the Gr-1.4 virus. While some of the affected genes in the Gr-1.4 virus-induced leukemia overlapped with those identified in lymphoma screens, suggesting a more general involvement in leukemogenesis and lymphogenesis, the majority of integrations appeared to affect novel genes that may be more specifically involved in the development of myeloid malignancies.

### MATERIALS AND METHODS

**MuLV Gr-1.4-induced leukemia.** Newborn mice were injected subcutaneously with 100  $\mu$ l of cell culture supernatant from murine leukemia virus (MuLV) Gr-1.4-producing NIH 3T3 cells (a gift from E. Rassart, Department des Sciences Biologiques, Université du Québec à Montréal, Montréal, Québec, Canada). Mice were treated and analyzed for the development of leukemia as previously described (9). Chromosomal DNA was isolated from leukemic cells for PCR-based screening (9).

**Cytological analysis and immunophenotyping of leukemic cells.** For morphological analysis, blood smears and cytospin preparations were fixed in methanol, stained with May-Grünwald-Giemsa stained, and examined by using an Axio-scope microscope (Carl Zeiss BV, Weesp, The Netherlands). Single-cell suspensions of different organs were analyzed by flow cytometry with a FACScan flow cytometer (Becton Dickinson and Co., Mountain View, Calif.). Cells were la-

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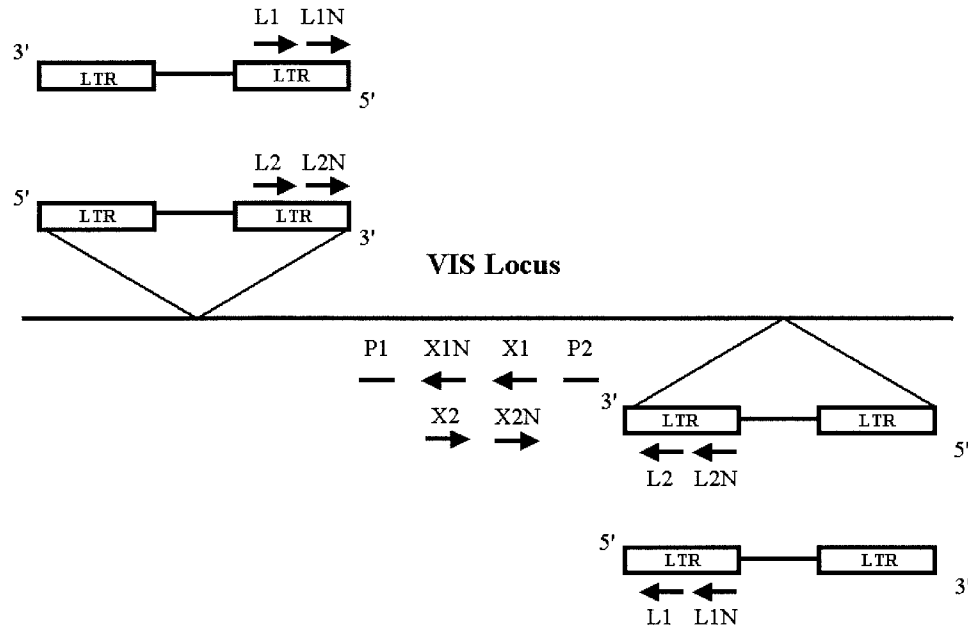


FIG. 1. Directed PCR of chromosomal DNA to determine the commonality of virus integration sites (VIS) identified by inverse PCR. Primers X1, X1N, X2, and X2N were designed from a locus that was identified as a virus integration site by inverse PCR. To amplify flanking genomic sequences and to determine the localization and orientation of the integrated provirus, four different nested PCRs were performed. First, primers X1 and X2 were combined with MuLV Gr-1.4 LTR primers L1 and L2. The products were amplified by a nested PCR with primers X1N and X2N in combination with L1N and L2N. The specificity of the amplified bands was checked by Southern blot analysis with probes P1 and P2.

beled as described previously (25) with the following rat monoclonal antibodies: ER-MP54, ER-MP58, M1/70 (Mac-1), F4/80, RB68C5 (GR-1), ER-MP21 (transferrin receptor), TER119 (glycophorin A), 59-AD2.2 (Thy-1), KT3 (CD3), RA3 6B2 (B220), and E13 161-7 (Sca1). Immunodetection was performed with goat anti-rat antibodies coupled to fluorescein isothiocyanate (Nordic, Tilburg, The Netherlands).

**Inverse PCR of MuLV Gr-1.4-induced leukemia.** The inverse PCR strategy was recently described in detail (9). Briefly, genomic DNA from primary tumors was digested with *HhaI* (CGCG). After circularization by ligation (rapid ligation kit; Roche Diagnostics, Mannheim, Germany), an initial PCR was performed with MuLV Gr-1.4 (long terminal repeat [LTR])-specific primers L1 (5'-TGCA AGATGGCGTTACTGTAGCTAG-3') and L2 (5'-CCAGGTTGCCCAAAG ACCTG-3'). Cycling conditions were 1 min at 94°C, 1 min at 62°C, and 3 min at 72°C for 30 cycles. For the second, nested PCR, primers L1N (5'-AGCCTTAT GGTGGGTCTTTC-3') and L2N (5'-AAAGACCTGAAACGACCTTGC-3') (15 cycles) were used. The PCR mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 200 μM deoxynucleoside triphosphates, 10 pmol of each primer, and 2.5 U of *Taq* polymerase (Pharmacia, Uppsala, Sweden). The PCR fragments were analyzed on a 1% agarose gel.

**Detection of virus integration by a specific nested PCR.** To determine the localization of the Gr-1.4 provirus in specific virus-targeted genes in an extended panel of leukemias, a nested PCR was performed with DNA from primary tumors. For the first PCR, virus integration site locus-specific primers X1 and X2 were used in combination with MuLV Gr-1.4 LTR-specific primers L1 and L2 (Fig. 1). Cycling conditions were 1 min at 94°C, 1 min at 62°C, and 3 min at 72°C for 30 cycles. For the second PCR, nested virus integration site-specific primers X1N and X2N were used in combination with nested LTR-specific primers L1N and L2N under the same conditions (Fig. 1). The PCR products obtained were analyzed by Southern blotting. To verify the correct nature of the amplified bands, the blots were hybridized with radiolabeled gene-specific probes P1 and P2 (Fig. 1) at 45°C in Church buffer (0.5 M phosphate buffer [pH 7.2], 7% [wt/vol] sodium dodecyl sulfate, 10 mM EDTA) overnight. Signals were visualized by autoradiography according to standard procedures (34a).

**Nucleotide sequence analysis.** PCR products were sequenced by using an ABI 3100 sequencer (Applied Biosystems, Neuwerkerk aan den IJssel, The Netherlands) with MuLV Gr-1.4-specific forward primer L1. Virus flanking genomic sequences were analyzed by using GenBank (National Center for Biotechnology Information), a Celera discovery system (Celera Genomics, Rockville, Md.) (19,

29), and Ensembl (Wellcome Trust Genome Campus, Hinxton, Cambridgeshire, United Kingdom) (20).

## RESULTS

**Gr-1.4-induced leukemias.** Eighty-nine newborn FVB/N mice were inoculated with Gr-1.4. When moribund, mice were sacrificed, and hematopoietic organs were isolated. Six mice died without signs of leukemia and were excluded from further investigation. Standard blood cell analysis was performed, and values were compared with the mean value for 10 normal FVB/N mice (24). Most of the leukemic mice had increased numbers of peripheral white blood cells and decreased numbers of platelets and red blood cells compared to normal controls (data not shown). Blast percentages in the bone marrow ranged from 24 to 90%, with an average of 48%. Leukemic cells from 76 mice were immunophenotyped. The major immunophenotypic features of these leukemias are given in Table 1. Based on these criteria, the leukemias were classified as T-lymphoid; mixed lymphoid, erythroid, and myeloid; myeloid; myelomonocytic; or erythroid. Fifty-nine leukemias were analyzed morphologically. Representative examples are shown in Fig. 2. Almost all of the mice showed splenomegaly, with 25% showing thymus enlargement, 20% showing lymph node enlargement, and 55% showing liver involvement. In 5% of the mice, leukemic cells were present in the bone marrow and blood, without overt peripheral organ involvement.

**Virus integration sites in Gr-1.4-induced leukemias.** PCR analysis in conjunction with database searches identified a total of 94 different virus integration sites out of 69 tumors, 38 of which were found in earlier screens. Examples of this latter

TABLE 1. Gr-1.4-induced leukemias<sup>a</sup>

Subtype	Leukemia type	Immunophenotype (markers)	No. of leukemias
I	T-lymphoid	MP21 <sup>+</sup> CD3 <sup>+</sup> Thy1 <sup>+</sup>	2
II	Mixed lymphoid, erythroid, and myeloid differentiation	Gr1 <sup>+</sup> F4/80 <sup>+</sup> Mac1 <sup>+</sup> Imm <sup>+</sup> CD3 <sup>+</sup> B220 <sup>+</sup> ( <i>gcsfr</i> <sup>+</sup> Ter119 <sup>+</sup> )	12
III	Myeloid differentiation	Imm <sup>+</sup> MP21 <sup>+</sup> (F4/80 <sup>+</sup> Gr1 <sup>+</sup> B220 <sup>+</sup> Mac1 <sup>+</sup> <i>gcsfr</i> <sup>+</sup> )	43
IV	Myelocytic or monocytic	Imm <sup>+</sup> Gr1 <sup>-</sup> <i>gcsfr</i> <sup>-</sup> (F4/80 <sup>+</sup> Mac1 <sup>+</sup> B220 <sup>+</sup> )	15
V	Erythroid	Ter119 <sup>+</sup> , MP21 <sup>+</sup> (Sca1 <sup>+</sup> )	4
Total			76

<sup>a</sup> All tumors were tested with the markers MP21, MP58, CD3, Thy1, Gr1, F4/80, Mac1, Sca1, B220, Ter119, and *gcsfr*. Markers that were consistently negative are not shown, unless they were informative for discrimination between myeloid subtypes III and IV. Imm<sup>+</sup> indicates positive staining for immature hematopoietic cell markers Sca1<sup>+</sup>, MP58<sup>+</sup>, and MP54<sup>+</sup> (Thy1<sup>+</sup>). Markers in parentheses were not present in all individual tumors.

group of CISs are p53 (39, 41), Notch-1 (12, 13, 31), Evi-1 (38), NF1 (Evi-2) (5), Lck-1 (1, 11, 35), Pim-1 (50), HoxA9 (Evi-6) (43), Fli-1 (4, 8), and N-Myc (18, 51). Notably, 79 of the 94 integrations were CISs, directly implicating the affected genes in leukemic transformation. The remaining 15 were included in this report because family members closely related to these genes were found in other studies. Fifty-six of the identified sequences were mapped near or in novel candidate leukemia genes. The products of the affected genes have been classified as receptors and signaling molecules (Table 2), as regulators of transcription (Table 3), and as having regulatory roles in other pathways (e.g., DNA stability and proteasomal targeting) or unknown functions (Table 4).

Approximately 15% of the virus integrations identified by this inverse PCR-based screen were determined to be common

(more than two integrations in a particular genomic locus) by the initial screen. To determine the frequency of common integrations more sensitively, we performed integration-specific PCRs with 49 Gr-1.4-targeted genes in multiple tumors (Tables 2 to 4); most appeared to be common. We sequenced the integrations in 19 genes derived from multiple (2 to 10) tumors; these included VDUP1, PrdxII, 11- to 19-lysine-rich leukemia (ELL), promyelocytic leukemia zinc finger (PLZF), and Edg3. In all instances, the integrations were located in the expected gene but at different positions, confirming the specificity of the Southern analysis and ruling out the possibility of cross-contamination by previously amplified PCR products. The genes flanking the virus integration sites were compared with data from the National Cancer Institute retrovirus-tagged cancer gene database (RTCGD) at <http://genome2.ncifcrf.gov>

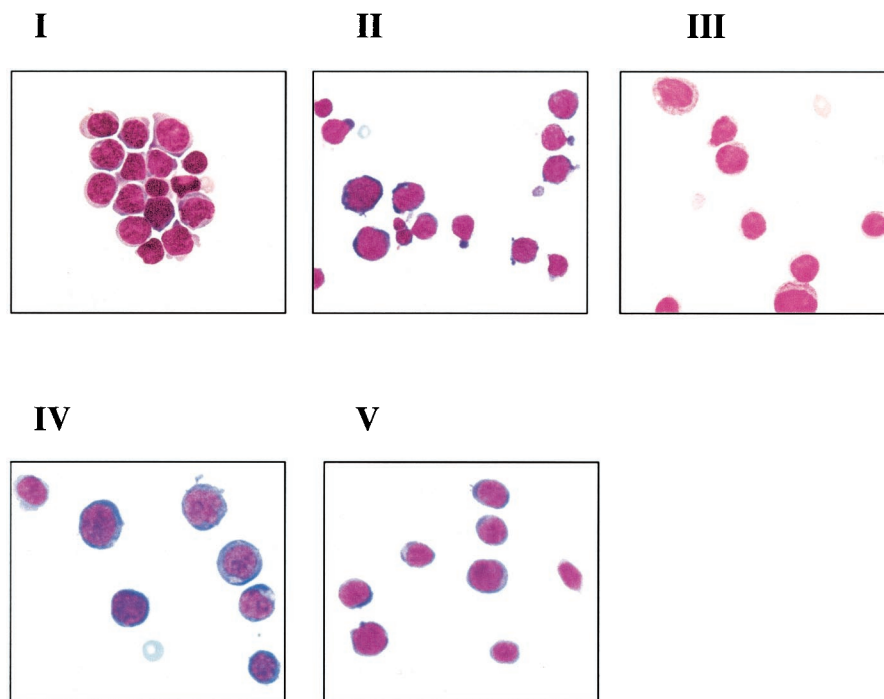


FIG. 2. Morphological features of MuLV Gr-1.4-induced leukemia. Micrographs were taken with a Zeiss Axioscope microscope (magnification,  $\times 1,000$ ). Morphological subtypes were lymphoblasts (I); mixed erythroblasts, myeloblasts, and lymphoblasts (II); myeloblasts (III); myelomonoblasts (IV); and erythroblasts (V).

TABLE 2. MuLV Gr-1.4 integration sites in receptors and signaling-related molecules

Gene <sup>d</sup>	Virus integration site localization	Database <sup>b</sup>	Tumors <sup>c</sup>	Subtype of (leukemia) <sup>d</sup>	Chromosome		Gr-1.4 virus unique CIS (x) or source <sup>e</sup>	Family member identified in other studies	Unique database identifier
					Mouse	Human			
IL-2R $\alpha$	7 kb upstream of gene	C, E	1#	IV	2A1	10p15.1	RTCGD		NM_008367
IL-2R $\beta$	500 bp upstream of gene	C, E	1	III	15E2	22q13.1	RTCGD		NM_008368
Notch-1	Exon 12, intron 14	C, E, N	2	II	2A3	9q34.3	RTCGD(31)	Notch-2	NM_008714
Flt3	1.5-2 kb upstream of gene	E	2	III	5G2	13q12.3	RTCGD		NM_010229
Tie-1	6-15 kb downstream of gene	C, E	2	III, V	4D1	1p34.2	x		NM_011587
c-Mpl	0.25-10 kb upstream of gene	C, E	2	III, V	4D1	1p34	x		NM_010823
Edg3 (G protein coupled)	18 kb upstream of gene	C, E	7/12§	II, III, IV	13B1	9q22.1	x	Edg1 (RTCGD)	NM_010101
Lck-1	Intron 1 (upstream of ATG)	C, E	1	III	4D2.3	1p34.3	RTCGD(50)	Pim-2, Pim-3	XM_110515
Pim-1	9 kb downstream of gene	C, E	1	VI	17B1	6p21.2	RTCGD	Akt-1	NM_008842
Akt-2	11-14 kb upstream of gene	C, E	4/13	II, IV	7A3	19q13.1	RTCGD	PTPN18, PTPNS1	NM_007434
PTPN5	Exon 1 (upstream of ATG)	C, E, N	1/24	III	7B3	11p15.1	x	(RTCGD)	NM_013643
PTPNI (PTPIB)	Intron 1 (downstream of ATG)	C, E, N	9/13	II, III, IV	2H3	20q13.13	RTCGD	PTPN18, PTPNS1	NM_011201
DUSP10 (MKP-5)	1 kb upstream of gene	C, E	1	III	1H4	1q41	RTCGD	DUSP2	NM_022019
Inpp5b	0.3 kb upstream of gene	C, E	6/13	I, III, V	4D1	1p34.3	x		NM_008385
Inpp4a	Intron 14	C, E, N	6/12	III, IV, V	1B	2q11.2	RTCGD		NM_030266
Dok-1 (p62)	26-28 kb from gene	C, E, N	10/10	I, II, III	6D1	2p13	x	Dok1 (pending) (RTCGD)	NM_001381
Phospholipase D3 (Pld3)	25 kb upstream of gene	E	5/13	II, III, IV	7A3	19q13.1	RTCGD	Plaa, 2310004B05Rik	NM_011116
Dapp-1	0.8 kb upstream of gene to intron 1 (downstream of ATG)	C, E	11/13	II, III, IV	3H2	4q24	RTCGD		NM_011932
Edaradd	17 kb downstream of gene	C, E	1	III	13A2	1q43	RTCGD		NM_133643
Semaphorin 4b	3 kb upstream of gene	C, E	1	V	7D2	15q26.1	RTCGD	Semaphorin 7a	XM_133534
Insulin receptor substrate 2	2.5 kb downstream of gene	C, E	2	III, IV	8A2	13q34	RTCGD		XM_146235
NF1 (Evi-2)	Intron 35	C, E	2	III	11B5	17q11.2	RTCGD (5)		NM_010161
PKI-gamma	66 kb upstream of gene	C, E	1	IV	2H3	20q13.12	RTCGD		U97170
Tao-2	3-4 kb upstream of gene	C, E	5/13	II, III, IV	7F4	16p13.11	RTCGD		NM_004783
Shc3	41 kb downstream of gene	C, E	7/12	II, III, IV	13B1	9q22.1	x		NM_009167
Calcyphosine	Intron 4 (downstream of ATG)	C, E	2/13	II	15A2	5p13.3	x		NM_029341
TNF-related protein 6	2.5 kb downstream of gene	C, E	1	III	15E2	22q13.1	RTCGD	Many	AF329842
Lymphotoxin beta (TNF-C)	8.3 kb upstream of gene	C, E	1	III	17B2	6p21.3	RTCGD	Many	NM_008518
Phosphodiesterase 1	Intron 3, intron 4 (downstream of ATG)	C, E	6/13	I, II, III, IV	10A3	6q22.31	x	Phosphodiesterase 4b (RTCGD)	NM_006208
Soes-2	0.75 kb upstream of gene to intron 1 (downstream of ATG)	C	8/39	II, III, IV, V	10C3	12q22	x	Soes-1, Soes-7, Ash-2 (RTCGD)	NM_003877
ARF-related protein	1.5 kb upstream of gene, intron 1 (upstream of ATG)	C, E	8/13	II, III, IV	2H4	20q13.13	x	ARF6, ARF2 (RTCGD)	AF217796
RAP-1	18 kb upstream of gene	C, E	1	III	15F2	12q12	x	RAP1a (RTCGD)	AF103905

<sup>a</sup> IL-2R $\alpha$ , interleukin 2 receptor alpha; PKI-gamma, phosphokinase gamma; TNF, tumor necrosis factor; ARF, ADP-ribosylation factor.  
<sup>b</sup> Sequences from inverse PCR were analyzed with Cebra (C), Ensembl (E), and National Center for Biotechnology Information (N) BLAST search programs, and the exact integration site was localized.  
<sup>c</sup> Number of tumors found positive for integration by inverse PCR only (out of 69 analyzed) # or by inverse PCR followed by directed PCR§ (positive out of total analyzed).  
<sup>d</sup> As defined in Table 1 and Fig. 2.  
<sup>e</sup> Source (reference) for other studies in which the Gr-1.4 virus integration site was previously found.

TABLE 3. MuLV Gr-1.4 integration sites in transcriptional regulators

Gene <sup>a</sup>	Virus integration site localization	Database <sup>b</sup>	Tumors <sup>c</sup>	Subtype of tumor <sup>d</sup>	Chromosome		Gr-1.4 virus unique CIS (x) or source <sup>e</sup>	Family member identified in other studies	Unique database identifier
					Mouse	Human			
JunB	10 kb upstream of gene	C	14/14	I, II, III, IV	8C3	19p13.2	RTCGD		NM_008416
Fli-1	Between exon 1a and exon 1 (upstream of ATG)	C, E, N	7	III, V	9A5.2	11q24	RTCGD (8)		NM_008026
p53	Introns 1, 5, and 6; exons 5, 6, and 7; 1.4 kb upstream of gene	C, E, N	7	III, V	11B4	17p13.1	RTCGD(39)		NM_011640
Evi-1	2-14 kb upstream of gene	C, E	2	III, IV	3A3	3q26	RTCGD(38)		NM_007963
n-Myc	Intron 1 upstream of gene	C, E, N	1	II	12A2	2p25.1	RTCGD(51)	Pvt1, c-Myc (RTCGD)	NM_008709
COE (OLF-1/EBF)	14 kb upstream of gene	C, E	4/48	II, III, IV	11B1.1	5q34	RTCGD		AF208502
Elf-4 (MEF)	0.25-5.5 kb upstream of gene, intron 1 (downstream of ATG)	C, E	11/12	II, III, IV	XA3.2	Xq26.1	RTCGD		NM_019680
YY1	0.5-1.5 kb upstream of gene	E, N	14/20	I, III	12F2	14q32.2	RTCGD (9)		NM_009537
Sax-1 (NKX-1.1)	0.3 kb upstream of gene, intron 1, exon 2 (upstream of and downstream of ATG)	C, E, N	1	III	7F4	10q26.12	x	NKX-1.2 (RTCGD)	NM_009123
NK-2.3 homeobox	6 kb upstream of gene	C, E	2	III, IV	19D1	10q24.32	x	NKX-1.2 (RTCGD)	AF155583
Evi-6 (Hoxa9)	2 kb upstream of gene	C, E	1	IV	6B3	7p15.2	RTCGD(43)	Hoxa7, Hoxb4 (RTCGD)	NM_010456
mPLZF	1.5 kb upstream of gene to intron 1 (upstream of ATG)	C, E	3/59	III	9B	11q23	x		NM_010823
ELL	Exon 1, intron 1 (downstream of ATG)	C, E	11/15	II, III, IV	8C1	19p13.11	x		NM_007924
Cesanne	0.8 kb, 16 kb upstream of gene	C, E	2/13	III, IV	3F2	1q23.1	x		NM_020205
Nuclear hormone receptor NR1D1	8-10 kb upstream of gene	C, E	11/12	II, III, IV	11D	17q21.1	x		BC008989
Retinoid X receptor alpha	84 kb upstream of exon 1, 2 kb upstream of gene, intron 1 (upstream of ATG)	C	3/12	II, III, IV	2A3	9q34.2	x		NM_011305
n-Cor-1	Intron 1 (downstream of ATG)	E	3/13	III	11B2	17p11.2	x	n-Cor-2 (RTCGD)	NM_011308
HDAC1	14 kb upstream of gene	C, E	1/13	III	4D2.3	1p34.3	x	HDAC5, HDAC9 (RTCGD)	NM_008228
HDAC7A	0.1-8 kb upstream of gene, introns 3 and 4 (downstream of ATG)	C, E	3/13	II, III	15F2	12q12	x	HDAC5, HDAC9 (RTCGD)	NM_019572
Smadcc2	0.55 kb upstream of gene	C, E	1	III	10D3	12q13.3	x	Smadcc1 (RTCGD)	NM_003075
HMG-17	28 kb upstream of gene, 1-2 kb downstream of gene	C, E	3/13	II, III	8C1	19p13.11	x	HMG-a1, HMG-a2, HMG-b1, HMG-cl HMG-Cr (RTCGD)	AL590390

<sup>a</sup> COE, Collier/OLF-1/EBF; EBF, early B-cell factor; MEF, myeloid elf1-like factor; mPLZF, mouse PLZF.  
<sup>b</sup> See Table 2, footnote b.  
<sup>c</sup> See Table 2, footnote c.  
<sup>d</sup> See Table 2, footnote d.  
<sup>e</sup> See Table 2, footnote e.

TABLE 4. Other MuLV Gr-1.4 integration sites

Gene <sup>a</sup>	Virus integration site localization	Database <sup>b</sup>	Tumors <sup>c</sup>	Subtype of tumor <sup>d</sup>	Chromosome		Gr-1.4 virus unique CIS (x) or source <sup>e</sup>	Family member identified in other studies	Unique database identifier
					Mouse	Human			
Protein-arginine deiminase type I (Pdi-1, PAD11)	0.5 kb upstream of gene, intron 1 (upstream of ATG)	C, E	5/13	II, III, IV	4E1	1p36.1	RTCGD	Pdi-2 (RTCGD)	NM_011059
Myosin IC synaptic vesicle	Intron 1 (upstream of ATG)	C, E	12/39	II, III, IV	9D	15q22.2	RTCGD	Myosin 1B, myosin IIIA (RTCGD)	XM_207764
Glycoprotein 2A (Sv2A)	27 kb upstream of gene	E	1	III	3F2	1q23.1	RTCGD		NM_022030
Swap70	Intron 3 (downstream of ATG)	C, E	1	IV	8C3	19p13.2	RTCGD		NM_009302
PAFAH1B2	11 kb downstream of gene	E, N	1	III	9B	11q23.3	RTCGD		NM_008775
Netrin 1	Intron 1 (downstream of ATG)	C, E	1	ND	11B3	17p11.2	x		XM_193630
Multispanning membrane protein (TM9SF2)	1 kb upstream of gene	C, E	1	III	14E5	13q32.3	RTCGD		NM_080556
Histone H3.3A (H3F3A)	Exon 1 (downstream of ATG)	C, N	1	IV	1H4	1q41	x	H1b, H1c, H2 (RTCGD)	Z85979
Gene trap locus 13 (GTL1-13)	17 kb upstream of gene	C, E	2	III	2E1	11q12	x		NM_021512
Tumor differentially expressed Tdel	15 kb upstream of gene	C, E	1	IV	2H3	20q13.12	RTCGD		NM_012032
O-Methyltransferase	4.5-2.5 kb upstream of gene	E	2	III	14B	10q22.2	x		AK007659
CHD2	17.3-18.8 kb upstream of gene	C, E	11/13	I, II, III, IV	7D1	15q26.1	x		NM_001271
Blim	0.8-3 kb upstream of gene	C, E	4/30	II	7D2	15q26.1	x		NM_007550
VDUP1	1.6 kb upstream of gene to intron 1 (downstream of ATG), exon 8 (downstream of stop) to 3 kb downstream of gene	C, E, N	14/14	I, III, IV	3F2	1q21	x		AF282826
PrdxII	2.4 kb upstream of gene to intron 1 (upstream of ATG)	C, E	14/14	I, II, III, IV	8C3	19p13.2	x		NM_011563
Lymphocyte antigen (Y-6M, LY-6A.2/LY-6E.1; TAP)	6 kb downstream of gene	C, E	8/39	II, III, IV, V	10C3	12q22	x	Lymphocyte antigens 108, 74, and 6E (RTCGD)	NM_010738
Periactin	57 kb upstream of gene	E	5/13	II, III, IV	7A3	19q13.1	x		AJ222968
RNase H1 subunit	3 kb upstream of gene	C, E	14/14	I, II, III, IV	8C3	19p13.2	x		AK010292
Apoptosis inhibitory 5 (Api-5), fibroblast growth factor 2-interacting factor 2	6 kb downstream of gene, intron 1 (downstream of ATG)	C, E	5/25	I, III	2E1	11p11.2	x		NM_007466
Hematopoietic progenitor (CDCA4)	Intron 1 (upstream of ATG)	C, E	2/13	IV	14E5	13q23.3	x		AK010535
U1 snRNP binding protein	1 kb upstream of gene	C, E	2	II, III	5F	?	x		AK018232
Unnamed	Intron 1 (downstream of ATG)	C, E	3/13	II, III	15F2	12q12	x		AK002729
Unnamed	1.5 kb downstream of gene	C, E	5/13	II, III, IV	7F4	16p13.11	x		AK004043
Unnamed	0.1-1.5 kb upstream of exon 1	C, E	9/12	II, III, IV	8C1	19p13.11	x		AK013135
Unnamed	Intron 1 (downstream of ATG)	E	5/25	I, III	2E1	11p11.2	x		AK001752
Lim domain protein Ril	Intron 1 (downstream of ATG)	C, E	2/13	I, II	11B1.3	5q31.1	x		NM_019417
D6M5e	Intron 4 (downstream of ATG)	C, E, N	10/10	I, II, III	6D1	2p13	x		AF084364
Psmb-1	1 kb upstream of gene	C, E	1	ND	17A2	6q27	x	Psmb-8 (RTCGD)	NM_011185
Ubiquitin	13 kb upstream of gene	C, E	9/12	II, III, IV	8C1	19p13.11	x	Many (RTCGD)	NM_019639
Psmc-1	0.34 kb upstream of gene	C, E	1	IV	14C1	14q11.2	x	Psmb-8 (RTCGD)	AB007136
Ddx-21	2 kb downstream of gene	C, E	1	IV	10B4	10q22.1	x	Ddx-3, Ddx-6, Ddx-26 (RTCGD)	NM_019553
Actin-like	0.2 kb upstream of gene	C, E	1	III	10C2	12q23.1	x	β-Actin (RTCGD)	AK008409
Alpha-1 catenin	83 kb upstream of gene	C, E	1	III	18B3	5q31.2	x		NM_009818
VDAC-2	7.5 kb downstream of gene	C	1	III	14B	10q22.2	x	VDAC-1 (RTCGD)	NM_011695

Proteoglycan, secretory granule (PRG1)	C, E	1	I	10B4	10q22.1	x	PRG4 (RTCGD)	NM_011157
Potassium channel Kcnk5	C, E	1	II	14A3-B	6p21.2	RTCGD		NM_021542
FK506BP8	C, E	9/12	II, III, IV	8C1	19p13.11	x	FK506BP3, FK506BP10 (RTCGD)	NM_010223
MCT related	C, E	1	III	11B4	17p13.2	x	MCT4 (RTCGD)	AB041591
RNA binding motif 5	E, N	3/11	I, III	9F2	3p21.31	x		AJ309168
RNA binding motif 6	E, N	3/11	I, III	9F2	3p21.31	x		NM_011251

<sup>a</sup> TAP, T-cell-activating protein; MCT, monocarboxylate transporter.  
<sup>b</sup> See Table 2, footnote b.  
<sup>c</sup> See Table 2, footnote c.  
<sup>d</sup> See Table 2, footnote d. ND, not determined.  
<sup>e</sup> See Table 2, footnote e.

and other sources (Tables 2 to 4). The Gr-1.4 integrations that are included in this database or that were reported in other studies are indicated by the database name or references in Tables 2 to 4. Family members of Gr-1.4-targeted genes found in other studies are also indicated in those tables. Most of the virus integrations occurred in or near the 5' or 3' ends of the genes, suggesting that the levels of expression of these genes are deregulated as a consequence of virus integration. Eighteen CISs were located exclusively within the gene, conceivably causing gene disruption. This group comprises the previously reported virus targets Notch-1, NF1, PTPN1, Inpp4a, p53, SWAP70, and Kcnk5 and 11 new virus targets: calcyphosine, phosphodiesterase 1, ELL, NCOR-1, HDAC-7A, histone H3.3A, Api-5, Ril, D6Mm5e, and two unknown genes.

### DISCUSSION

In this study, we have used in vivo retroviral mutagenesis with the Gr-1.4 virus complex to identify novel disease genes specifically involved in the pathogenesis of AML. In comparable studies with other virus strains, e.g., Moloney, AKXD, or Cas-Br-M, the most prominently appearing tumor types are T- and B-cell lymphomas; in the case of BXH2, myelomonocytic tumors are prominent (21, 30, 32, 37, 54). In contrast, more than 80% of the leukemias induced by Gr-1.4 unequivocally exhibited immunophenotypic characteristics of myeloid cells, with immature morphological features (mainly myeloblasts), emphasizing the unique features of the Gr-1.4 virus complex as a tool for characterizing pathogenetic mechanisms in myeloid disease. Indeed, the majority of the CISs described here have thus far not been reported in extensive screens in lymphoma models, although some overlap was observed. The latter observation is not surprising in view of the fact that cell type-specific events usually impinge on downstream common regulatory pathways that can be affected in multiple tumor types.

Although most of the Gr-1.4 CISs have been linked to candidate disease genes based on their proximity to these genes, proviral integrations have been reported to influence gene expression over distances of more than 100 kb. Thus, we cannot exclude the possibilities that genes located more distantly from the CISs also are deregulated and that multiple genes are affected by a single CIS (44). In addition, three other aspects of retroviral screens as they are currently being performed must be emphasized. First, malignancies induced by replication-competent retroviruses are usually oligo- or polyclonal rather than monoclonal (9, 32). Although this characteristic gives rise to high frequencies of CISs in relatively small cohorts of mice, it complicates the search for cooperating events within one leukemic clone. Second, the sensitive PCR-based techniques used to identify virus integrations do not allow distinction between CISs present in a majority of the leukemic cells, initiating an early pathogenic event, and CISs present in only a minor population of the cells, probably affecting leukemia progression genes. Finally, a recent study emphasized that MuLV strains have a preference for integration near transcriptional start sites (62). Our data obtained with Gr-1.4 corroborate these conclusions and indicate that retroviral mutagenesis with MuLV preferentially, although not exclusively, identifies gene deregulation rather than gene disruption. Another important

conclusion drawn by Wu and colleagues is that there appear to be no integration hot spots (preferred integration sites in certain loci) for MuLV (62). Therefore, CISs found in viral screens are likely to play a role in leukemia.

Many of the Gr-1.4-affected genes appeared to be related to signaling; some have been linked to human leukemia already. For instance, the Tie-1 gene, which encodes a tyrosine kinase receptor that is normally expressed in vascular endothelial cells and hematopoietic stem cells, is overexpressed in chronic myeloid leukemia (59). Importantly, high Tie-1 levels inversely correlate with the survival of chronic myeloid leukemia patients in the early chronic phase (59). Markedly increased levels of Tie-1 also were detected in bone marrow samples from myelodysplastic syndrome and AML patients (58). Notch-1 is another example of a CIS associated with human disease. The human homologue of the Notch (Tan-1) gene is involved in chromosomal translocation t(7;9)(q34;34.3), which results in a truncated receptor in human T-lymphoblastic neoplasms. Notch-1 is a proviral integration site in mouse lymphoid leukemias (12, 31). Our data suggest that aberrant Notch signaling also may be involved in myeloid leukemia. Notably, the integrations in the Notch gene result in the constitutive formation of the truncated active form of Notch (31), which has been demonstrated to interfere with granulocyte colony-stimulating factor-induced myeloid differentiation in the 32D cell model (55). Edg3 (endothelial differentiation gene 3) is a G-protein-coupled receptor (GPCR) involved in cell proliferation and survival (2). The expression of Edg3 is down-regulated during the differentiation of HL60 human leukemia cells and is assumed to mediate sphingosine 1-phosphate-induced  $Ca^{2+}$  responses (49). Although the role of Edg receptors in normal hematopoiesis and malignancies is not yet clear, a closely related GPCR, cannabinoid receptor type 2, was found in a CIS in Cas-Br-M-induced leukemia (57). Notably, perturbed expression of cannabinoid receptor type 2 recently was found to interfere with myeloid differentiation, supporting the notion that this type of GPCR may contribute to the pathogenesis of AML by inducing a maturation block (27).

Both protein and lipid phosphatases are crucial, mostly negative, regulators in growth factor signaling pathways (7, 64). Two members of the protein tyrosine phosphatase nonreceptor (PTPN) family affected by Gr-1.4 are PTPN1 and PTPN5. The virus integrations in the PTPN1 gene are localized in intron 1 and conceivably disrupt its function. The PTPN1 gene was found in a CIS in BXH2-induced leukemia (32). Because TYK2, JAK2, and STAT5—JAK/STAT signaling intermediates involved in growth control by hematopoietic growth factors—are all inactivated by PTPN1 (also known as PTP1B) (3, 16, 42), the disruption of PTPN1 is predicted to contribute to uncontrolled proliferation and survival of leukemic cells. Integrations in the PTPN5 gene occur in exon 1, upstream of the ATG translation start site. At present, little is known regarding the signaling function of PTPN5, which is alternatively termed STEP. Interestingly, recent data suggest that PTPN5/STEP shows a preferential affinity for p38 mitogen-activated protein kinase in a redox-sensitive manner, leaving the Erk kinases largely unaffected (40). Inhibition of p38 mitogen-activated protein kinase activity by pharmacological inhibitors has been shown to interfere with terminal neutrophilic differentiation (G.-J. van de Geijn et al., unpublished data) and to prevent

apoptosis in neutrophils (10). Conceivably, dephosphorylation of p38 by PTPN5/STEP may have similar effects and may promote the survival of immature myeloid cells. Other phosphatases that are targeted by Gr-1.4 are the inositol polyphosphate phosphatases Inpp4a and Inpp5b, which catalyze the hydrolysis of phosphatidylinositol polyphosphates at positions 4 and 5, respectively. Intriguingly, Inpp4a is a major target for transcription factor GATA-1 and inhibits the growth of megakaryocytes and NIH 3T3 fibroblasts (60). Inpp5b is required for normal sperm development and function (17), but how this enzyme affects normal or leukemic blood cell development is not known.

Reactive oxidant species (ROS) are generated by multiple cellular mechanisms, including metabolic processes, phagocytic responses to pathogens (oxidative burst), and membrane receptor-mediated signaling. The tight regulation of ROS concentrations in the cell is controlled by both catabolic and redox mechanisms and is important for signal transduction, as it influences protein kinase and phosphatase reactions as well as the activities of transcription factors (46). When ROS levels in a cell are too high, the resulting oxidative stress will affect normal protein function and genomic stability (46). Genes involved in the redox-controlled regulation of ROS levels that are commonly targeted by Gr-1.4 are those for vitamin D<sub>3</sub>-upregulated protein 1 (VDUP1) and peroxiredoxin 2 (PrdxII). VDUP1 interacts with thioredoxin (TRX) and thereby counteracts the ameliorating effects of TRX on stress-induced apoptosis via apoptosis signal-regulating kinase 1 (ASK-1) and the function of TRX as an antioxidant (28). The consequence of upregulation of PrdxII is as yet unclear, but it may interfere with the function of peroxiredoxin complexes and therefore inhibit the antistress activities of TRX (6). Importantly, PrdxII is a TRX reductase and has been found to be overexpressed in human breast cancer (45).

The ELL and PLZF genes are two examples of genes that are part of fusion genes in human AML as a result of chromosomal translocations. In translocation t(11;19)(q23;p13.1), the ELL gene is fused to the trithorax-like mixed lineage leukemia (MLL) gene (56). The ELL protein is an RNA polymerase II elongation factor (53). Recently, it was demonstrated that both MLL-ELL and ELL inhibit p53 activity via binding of the C-terminal part of ELL to the transactivating domain of p53 (61). The PLZF gene, involved in translocation t(11;17)(q23;q21), is fused in frame with RAR $\alpha$  (22, 36). PLZF is expressed in hematopoietic stem cells and normally is down-regulated during differentiation. The overexpression of PLZF plays a role in the maintenance of the immature state of hematopoietic progenitor cells and promotes cell survival (52, 63). The fact that PLZF is found as a common Gr-1.4-targeted gene indicates that the deregulation of PLZF expression can contribute to leukemic development independent of its fusion to RAR $\alpha$ . One of the major features of PLZF is its tight binding to transcriptional corepressors. Interestingly, several genes involved in transcriptional repression by chromatin modification, such as those for N-Cor-1 and histone deacetylases HDAC1 and HDAC7, are affected by Gr-1.4. Notably, family members of these proteins (N-Cor-2 and HDAC5) also were found in previous screens (54).

In conclusion, using high-throughput retroviral screens with the Gr-1.4 virus complex, we have identified novel pathways



involved in myeloid leukemia. Currently, we are studying the consequences of aberrant gene expression or function by using gene transfer methodology with 32D cells and primary bone marrow cultures (9). In addition, we are analyzing the significance of these pathways for human AML by screening expression levels in a panel of clinical samples with a gene expression array and quantitative reverse transcription-PCR technology. By following this combinatorial approach, we hope to develop new prognostic indicators that will discriminate further between low and high responses to therapy in different risk groups. Furthermore, the results obtained may open new avenues for developing specific and sensitive therapies based on the heterogeneous pathogenetic features of human AML.

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