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Characterization of *pal-1*, a Common Proviral Insertion Site in Murine Leukemia Virus-Induced Lymphomas of c-*myc* and *Pim-1* Transgenic Mice

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Insertional mutagenesis with Moloney murine leukemia virus (MoMLV) in c-*myc* and *Pim-1* transgenic mice permits the identification of oncogenes that collaborate with the transgenes in lymphomagenesis. The recently identified common insertion site *pal-1*, in MoMLV-induced lymphomas, is located in a region in which several independent integration clusters are found: *eis-1*, *gfi-1*, and *evi-5*. Proviral insertions of MoMLV in the different integration clusters upregulate the transcriptional activity of the *Gfi-1* gene, which is located within the *pal-1* locus. The *eis-1*/*pal-1*/*gfi-1*/*evi-5* locus serves as a target for MoMLV proviral insertions in pre-B-cell lymphomas of Eμ-*myc* transgenic mice (20%) and in T-cell lymphomas of *H-2K-myc* (75%) and Eμ-*pim-1* (93%) transgenic mice. Many tumors overexpress both *Gfi-1* as well as *Myc* and *Pim* gene family members, indicating that *Gfi-1* collaborates with *Myc* and *Pim* in lymphomagenesis. Proviral integrations in the previously identified insertion site *bmi-1* are, however, mutually exclusive with integrations in the *eis-1*/*pal-1*/*gfi-1*/*evi-5* locus. This finding suggests that *Bmi-1* and *Gfi-1* belong to the same complementation group in lymphoid transformation.

Cancer is the result of the sequential accumulation of oncogenic mutations in DNA. These alterations include inactivation of tumor suppressor genes and dysregulation and/or mutation of proto-oncogenes. Proviral tagging has proved to be a valuable system to identify oncogenes in experimental mouse model systems (22, 55). In mouse mammary tumor virus-induced mammary carcinomas, members of the *Wnt* gene family and of the *Fgf* growth factor family have been identified as targets for mouse mammary tumor virus activation (28, 32, 39, 41, 42, 44). In murine leukemia virus (MuLV)-induced myeloid leukemia, c-*myb* (37), *csfm*/*Csf-1* (4), *csfmr*/*fim-2*/c-*fms* (15), and *Evi-1* (36) were found to be activated and/or altered by proviral insertions. In MuLV-induced lymphomas, proviral insertions were detected in *evi-3* (23), *evi-5* (30), *fis-1*/*Cyclin D1* (25), *mlvi-1*/*mlvi-4*/*pvt-1*/c-*myc* (11, 17, 27), N-*myc* (56), *Pim-1* (12, 49), *Pim-2* (52), and *vin-1*/*Cyclin D2* (51). In addition to being used for the identification of genes involved in the initiation of tumorigenesis, retroviral insertional mutagenesis has been utilized to identify genes contributing to tumor progression. Both selection for specific growth properties of cells in vitro and transplantation of primary tumors in vivo have led to the identification of genes that appear to contribute to later stages in the transformation process. These genes include *Tpl-1*/*Ets-1* (5, 6), *Tpl-2*/*Cot-1* (3, 40), *Gfi-1* (14), *gfi-2*/interleukin-9 (IL-9) receptor gene (13), *Tiam-1* (18), *tic-1* (previously named *pim-2*) (9), and *Frat-1* (22a).

Proviral tagging in mice transgenic for oncogenes has been particularly useful for the identification of genes that can collaborate with the transgene in tumorigenesis. Infection of Eμ-*myc* transgenic mice (1a) with Moloney MuLV (MoMLV) results in a strongly accelerated pre-B-cell lymphomagenesis. From these tumors, the *Bmi-1* oncogene was identified (20, 59). The *Pim-1* and *Pim-2* oncogenes were also found to act as collaborators of *Myc*, sometimes in conjunction with *Bmi-1* (52, 59).

In an attempt to identify new collaborating oncogenes in MoMLV-induced tumors in Eμ-*myc* transgenic mice, another common insertion site, *pal-1* (proviral activation in lymphomas 1) was cloned. From chromosomal mapping of this common insertion site and subsequent physical mapping of the locus, it became apparent that the common insertion site *pal-1* is part of a larger region of 50 kb that comprises other independently identified common insertion sites. We show here that these other insertion sites, *evi-5* (30) and *eis-1*, are located next to, and *gfi-1* (14) is located within, the region of the *pal-1* integration locus. All independent proviral integrations in the *eis-1*/*pal-1*/*gfi-1*/*evi-5* locus result in the enhanced transcription of *Gfi-1*. We also demonstrate that proviral integrations in the *eis-1*/*gfi-1*/*pal-1*/*evi-5* locus are mutually exclusive with integrations in the *bmi-1* locus, indicating that these genes fall in the same complementation group of transformation.

MATERIALS AND METHODS

Transgenic mice and MoMLV infection. The generation of Eμ-*myc* transgenic mice has been described previously (60). Eμ-*myc* founder line 186 was backcrossed either to FVB/N and maintained as an inbred line or crossed to 129/OLA mice with a targeted mutation in one *Pim-1* allele (52). The Eμ-*pim-1* transgenic mice used in this study have been described previously (57). The *H-2K-myc* transgene was generated by fusing the *H2-K* promoter (33) to a 5.5-kb *Xba*I-*Bam*HI genomic mouse c-*myc* fragment containing exons 2 and 3, including the c-*myc* polyadenylation signal. The transgene was microinjected into the pronuclei of FVB/N zygotes, and these were transferred to $(B6 \times DBA)F_1$ foster mice. The transgenic founders were backcrossed to FVB/N. Genotyping was done by Southern analysis of genomic tail DNA as described by Laird et al. (24).

Lymphomas were induced by injecting 1-day-old mice with 10^4 to 10^5 infectious units of either MoMLV clone 1A (21) or supF-MoMLV (43). Mice were sacrificed when moribund; primary and infiltrated tumor tissues (thymus, spleen, mesenteric/peripheral lymph nodes, liver, and kidney) were frozen at −80°C. Single-cell suspensions were made from mesenteric lymph node tissue and used for flow cytometric analysis.

IPCR and construction size-selected library. The template for inverse PCR (IPCR) was prepared by digesting 3 μg of genomic tumor DNA with restriction enzyme *Hha*I in a volume of 100 μl for 3 h at 37°C and then incubated at 68°C

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for 10 min to inactivate the restriction enzyme; genomic fragments were ligated overnight at room temperature at a concentration of 10 ng/μl with T4 DNA ligase. Subsequently the ligase was heat inactivated (10 min at 68°C), and template DNA was linearized with *Xba*I restriction enzyme. For the first round of amplification, 50 ng of genomic DNA was used in combination with primers AB827 (5′-TCCATGCCTTGCAAAATGGC-3′) and AB946 (5′-GCGGCGGC CGCATGACCCTGTGCCTTATT-3′). Amplification was performed in a Hybaid Thermal Reactor, using denaturation (30 s, 94°C), annealing (45 s, 58°C), and extension (60 s, 72°C) steps for 35 cycles. For the nested PCR, 1/100 μl of the first round was used as a template together with primers AB949 (5′-CGCG TCGACCTTGCCAAACCTACAGGT-3′) and AB946, using the same amplification conditions for 25 cycles. The obtained fragments were subcloned in pBluescript SK+ (Stratagene), using the *Sal*I and *Not*I restriction sites in primers AB949 and AB946.

The common insertion site *eis-1* was cloned from a mesenteric lymph node tumor (q160), obtained after two serial subcutaneous transplantations of 5×10^6 mesenteric lymph node tumor cells from a supF-MoMLV-infected *H-2K-myc* transgenic mouse, into syngeneic recipients. Two size-selected libraries were constructed by using *Eco*RI-digested DNA from *H-2K-myc* tumor q160. Size selected fractions, containing DNA fragments of 13 to 17 and 5 to 7 kb, respectively, were purified by electroelution. The fractions containing the desired proviral insertion (15 and 6.5 kb) were identified by Southern blot hybridization with the MoMLV U3 long terminal repeat (LTR) probe (12) and inserted into the *Eco*RI site of Charon 4A (15 kb) or LambdaZAPII (6.5 kb) (Stratagene). The phage was packaged by using Gigapack Gold (Stratagene), and 10^5 recombinant phages were screened. Restriction analysis of the phage inserts revealed that the host DNA in both inserts corresponded to the same genomic *Eco*RI fragment. The 15-kb *Eco*RI fragment contained, next to flanking genomic sequences, an intact MoMLV provirus, whereas the 6.5-kb *Eco*RI fragment contained only a single LTR, generated by in vivo recombination of the ecotropic provirus.

Genomic DNA and RNA isolation and analysis. High-molecular-weight DNA was prepared from frozen mouse tissues as described previously (54). For Southern analysis, 15 μg of genomic DNA of each tumor was digested with a suitable restriction enzyme as recommended by the supplier (Boehringer Mannheim) and separated with 1× TAE (40 mM Tris-HCl [pH 7.9], 5 mM sodium acetate, 1 mM EDTA) running buffer on a 0.7% agarose gel. For Northern analysis, 15 μg of total RNA, isolated by the LiCl-urea method, was separated on a 1% formaldehyde agarose gel (46). The genomic DNA or total RNA was transferred to nitrocellulose (Schleicher & Schuell) or Hybond-N nylon (Amersham Life Science) membranes as recommended by the supplier. The Southern and Northern blots were hybridized with the appropriate random priming labeled [α-^{32}P]dATP probe at 42°C, using a hybridization solution containing 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 5× Denhardt solution, 50 mM Na_2HPO_4-NaH_2PO_4 (pH 6.8), 5 mM EDTA, and 150 μg of herring sperm DNA per ml. Final wash steps were performed in 0.1× SSC and 0.1% sodium dodecyl sulfate at 42°C.

Probes. The probes used were as follows: *pal-1* probe m12, 561-bp IPCR probe; *pal-1* probe A, 1.4-kb *Bgl*II-*Eco*RI fragment; *pal-1* probe B, 1.1-kb *Sac*I-*Eco*RI fragment; *pal-1* probe C, 1-kb *Pst*I fragment; *evi-5* probe EviBP, 1-kb *Bam*HI-*Pst*I fragment; *eis-1* probe EisKP, 0.7-kb *Kpn*I-*Pst*I fragment; *eis-1* probe EisHK, 0.7-kb *Hin*dIII-*Kpn*I fragment; MoMLV U3 LTR probe, 180-bp *Hpa*II fragment (12); *pim-1* probe A, 0.9-kb *Bam*HI fragment (12); *pim-2* probe for 5′ integration cluster, 0.5-kb *Bam*HI fragment (52); *pim-2* probe for the 3′ integration cluster, 0.7-kb *Sac*I fragment (52); N-*myc* probe, 3.5-kb *Pst*I fragment containing exon 2 and part of exon 3 (56); c-*myc* probe, 1.2-kb *Hin*dIII-*Eco*RI cDNA fragment; *bmi-1* open reading frame (ORF) probe, 1.3-kb *Xho*I-*Eco*RI *Bmi-1* cDNA fragment (a gift from M. Alkema); rat *Gfi-1* cDNA probe, 2.4-kb *Eco*RI fragment (14).

Library screening and sequence analysis. To obtain genomic clones from the *eis-1*, *pal-1*, *gfi-1*, and *evi-5* loci, probe EisKP, probe m12, a rat *Gfi-1* cDNA probe, and an *evi-5* probe (a gift from N. Copeland) were used to screen a genomic SVJ129 library (Stratagene). Independent overlapping lambda phage clones SVJ129λ2B, SVJ129λ14, SVJ129λ1, SVJ129λ2A, SVJ129λ23, and SVJ129λ17 were isolated. The 5′ part (835-bp *Nsi*I-*Eco*RI fragment) of rat *Gfi-1* cDNA was used as a probe to screen a BALB/c thymus cDNA library (Stratagene). Positive clones were in vivo excised as recommended by the supplier and sequenced. Sequencing of mouse *Gfi-1* cDNA and genomic clones was performed on double-stranded templates, using synthetic primers purchased from Pharmacia. Sequencing reactions were performed with a Promega T7 polymerase sequencing kit, with 7-deaza-dGTP to resolve compressions in G/C-rich regions. Searches of the GenBank database were performed by using the BLAST, FASTA, and WORDSEARCH programs on the National Center for Biotechnology Information file server. Amino acid alignments were generated with PILEUP. Sequences were analyzed with the Genetics Computer Group package of programs.

Nucleotide sequence accession numbers. The sequences for mouse *Gfi-1* 2.8-kb cDNA and the 5′ end of the 2.4-kb cDNA have been deposited in GenBank under accession numbers U58972 and U58973.

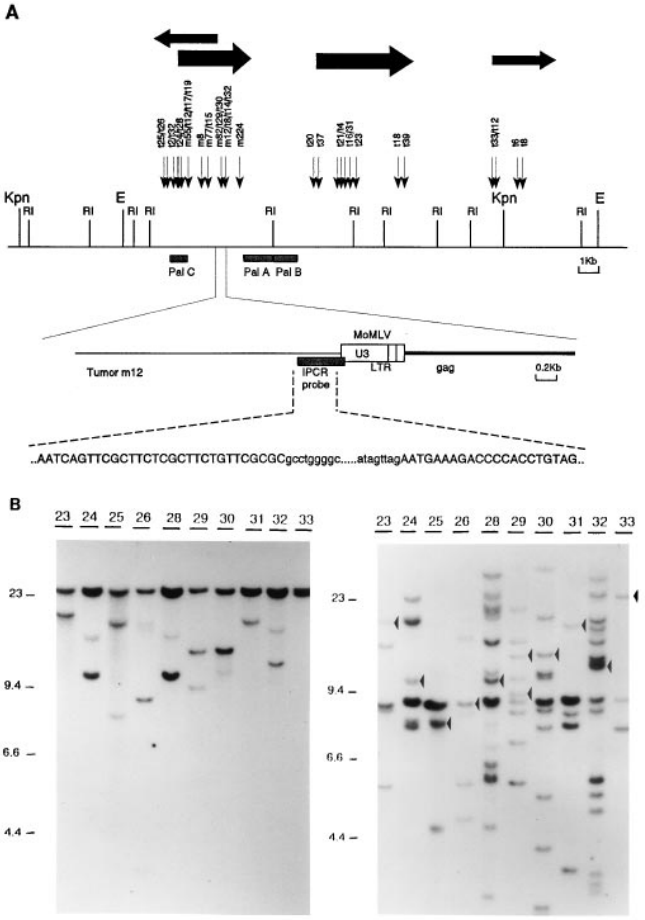

FIG. 1. (A) Restriction map of the *pal-1* locus. The different proviral integrations of MoMLV in Eμ-*myc* (m) and Eμ-*pim-1* (t) lymphomas are indicated. The large arrows indicate the orientation of the proviral insertion clusters. Three different DNA probes (pal A, pal B, and pal C) were used to detect and map proviral insertions in the *pal-1* locus. The common insertion site *pal-1* was cloned from tumor m12 by using IPCR to generate a flanking genomic probe. The IPCR probe and a part of the MoMLV provirus are shown with the 5′ LTR (U3, R, U5) and the *gag* region. The viral primer sequences in the generated IPCR probe are in uppercase, and the flanking genomic sequences are in lowercase. Abbreviations for the restriction endonucleases: Kpn, *Kpn*I; RI, *Eco*RI; E, *Eco*RV. (B) *pal-1* rearrangements in T-cell lymphomas of Eμ-*pim-1* transgenic mice due to proviral insertions of MoMLV. The endogenous fragment is 24 kb as detected with the pal C probe on *Kpn*I-digested tumor DNA; each smaller fragment is an independent rearrangement in the *pal-1* locus (left). The proviral insertions in each lymphoma were detected by the U3 LTR probe (right) on the identical *Kpn*I-digested tumor DNA samples. A *Kpn*I restriction site is present in the LTR of MoMLV. Depending on the proviral orientation, the rearranged band of the *pal-1* locus can be superimposed on the U3 LTR band, as indicated by the arrow. The number of the tumor sample is indicated above the lane. The marker is lambda phage DNA digested with *Hin*dIII. Sizes are indicated in kilobases.

RESULTS

***pal-1* is a common insertion site in B- and T-cell lymphomas.** To identify new proto-oncogenes which collaborate with c-*myc* in lymphomagenesis, proviral tagging was performed in Eμ-*myc* transgenic mice (59). Infection with the ecotropic MoMLV results in an acceleration of lymphomagenesis. All mice succumbed between 33 and 100 days postinfection from primarily pre-B-cell lymphomas. IPCR (50) was used to obtain cellular DNA sequences flanking the 5′ end of the MoMLV proviruses from Eμ-*myc* tumor 12 (m12), which had no integration in *pim-1* or *bmi-1* (Fig. 1A). A unique IPCR probe of 561 bp was generated and used to screen Southern blots containing tumor

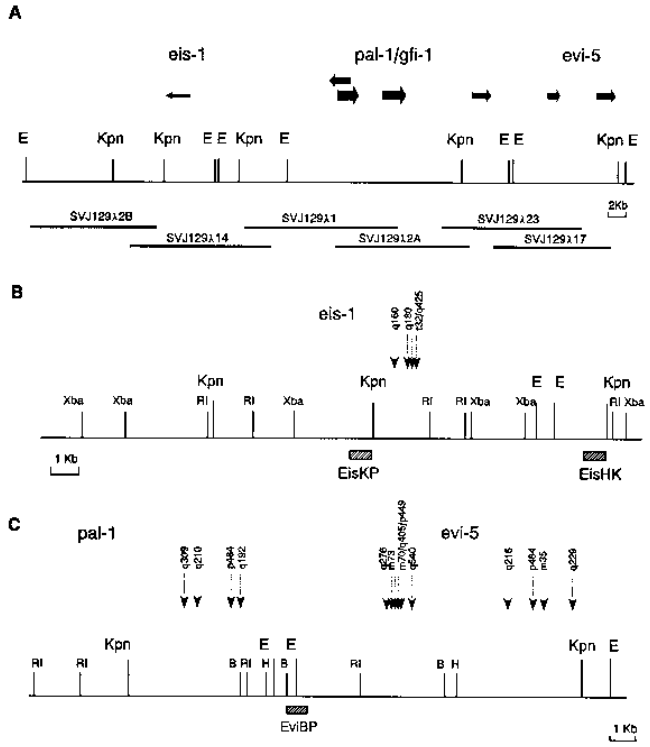

FIG. 2. Schematic representation of the combined *eis-1*/*pal-1*/*gfi-1*/*evi-5* locus. (A) The *eis-1* and *evi-5* insertion sites are juxtaposed to the *pal-1* insertion site. Restriction map and proviral integration clusters of MoMLV are indicated. The orientation of the arrow above the restriction map indicates the orientation of majority of the proviruses within the specific cluster. Six overlapping genomic phage lambda clones (SVJ129λ2B, SVJ129λ14, SVJ129λ1, SVJ129λ2A, SVJ129λ23, and SVJ129λ17), covering the complete *eis-1*/*pal-1*/*evi-5* locus, were isolated. (B) Detailed restriction map of the *eis-1* common insertion site. Two DNA probes (EisKP and EisHK) were used to detect proviral rearrangements in the *eis-1* locus. (C) Detailed restriction map of the *evi-5* locus. Proviral insertions in the *evi-5* locus were detected with probe EviBP. In panels B and C, the small arrows indicate the positions of the individual proviral insertions in the tumors from Eμ-*myc* (m, p), *H-2K-myc* (q), and Eμ-*pim-1* (t) transgenic mice. Abbreviations for restriction endonucleases: Xba, *Xba*I; RI, *Eco*RI; Kpn, *Kpn*I; E, *Eco*RV; H, *Hin*dIII.

DNA from 26 independent pre-B-cell lymphomas, and five other tumors with a proviral integration in this region (m8, m55, m77, m82, and m224) were detected (Fig. 1A). This genomic region was named *pal-1*. To obtain lambda phage clones that covered the genomic region of the *pal-1* integration cluster, we screened several genomic libraries with the IPCR probe. Several independent phage clones were obtained, and a physical map was constructed (Fig. 2A). Three additional genomic probes (pal A, pal B, and pal C) were generated and used to map the different proviral integrations.

To investigate the involvement of *pal-1* in T-cell lymphomagenesis, we determined the proviral occupancy of *pal-1* in MoMLV-induced T-cell lymphomas in Eμ-*pim-1* and *H-2K-myc* transgenic mice. Previously it has been shown that overexpression of the *pim-1* oncogene in the lymphoid compartment of transgenic mice predisposes to T-cell lymphomas, albeit at a low frequency (57). Upon infection with MoMLV, there was a dramatic acceleration in the onset of T-cell lymphomas. In almost all of the tumors, either c-*myc* or N-*myc* was activated by proviral insertion. To determine whether *pal-1* is also a common insertion site in MoMLV-induced T-cell lymphomas in Eμ-*pim-1* transgenic mice, 43 tumor samples were analyzed by Southern analysis of *Kpn*I-digested DNA, using the pal C probe (Fig. 1B). Of these tumors, 84% (36 of 43) contain a proviral integration in the *pal-1* locus. Moreover, a number of tumors, such as t30 and t32 (Fig. 1B), harbor more than one integration in this locus, suggesting that within one tumor, different cell clones have acquired independent proviral integrations within the *pal-1* locus.

The mice of the *H-2K-myc* transgenic line H2MPA develop spontaneous T-cell lymphomas with an average latency period of 110 days (1). Infection with supF-MoMLV (43) accelerates the onset of T-cell lymphomas (average latency of 65 days). The T-cell lymphomas are either $CD4^+$ $CD8^+$ or $CD4^+$ $CD8^-$, as determined by flow cytometry with standard B- and T-cell surface markers. Northern analysis of a series of tumor RNA samples demonstrated that the endogenous c-*myc* gene was still a target for proviral insertion, although at a reduced frequency. Besides the proviral integrations present in the *pim-1* (13%) and *pim-2* (11%) loci, 59% (35 of 59) of the T-cell lymphomas carry a proviral insertion in the *pal-1* locus, as judged by Southern analysis on either *Kpn*I- or *Eco*RV-digested tumor DNA, using the pal A probe. These results show that proviral insertion in the *pal-1* locus can be detected in conjunction with *Pim-1* and c-*myc*/N-*myc* activation in both B- and T-cell tumors.

The common insertion sites *evi-5* and *eis-1* are located close to the *pal-1* locus. The *pal-1* locus was mapped to mouse chromosome 5, 2.5 centimorgans distal to *Bmp-3* (bone morphogen protein 3), using an interspecific backcross analysis with progeny derived from matings of (C57BL/6J × *Mus spretus*)F_1 mice with C57BL/6J mice (10a). No recombination was found between *pal-1* and *evi-5*, another common proviral insertion site in T-cell lymphomas of the recombinant inbred strain AKXD (30). To determine if *evi-5* and *pal-1* are part of the same common insertion cluster, we screened a mouse genomic phage library with a probe derived from the *evi-5* locus (generously provided by N. Copeland). One of the lambda phage clones obtained (SVJ129λ23) hybridized with both the *evi-5* and *pal-1* probes, indicating that the two insertion loci are close to each other. The map in Fig. 2 shows the relative positions of *pal-1* and *evi-5*. To determine if *evi-5* is also a common insertion site in T- and B-cell lymphomas, tumor panels of MoMLV-induced lymphomas in *H-2K-myc* and Eμ-*myc* were probed with the *evi-5* probe EviBP. Rearrangements in the *evi-5* locus were found in both T- and B-cell lymphomas. Comprehensive mapping of the proviral integrations indicated there are two distinct integration clusters within the *evi-5* locus (Fig. 2C).

Molecular cloning of proviral integrations from T-cell lymphomas in supF-MoMLV-infected *H-2K-myc* transgenic mice yielded a new common insertion site, *eis-1* (extra integration site 1). *eis-1* was mapped to the chromosomal region containing the *pal-1*/*evi-5* locus (10a). The *eis-1* probe did not cross-hybridize with the existing phage clones encompassing the *pal-1*/*evi-5* locus, suggesting that *eis-1* represented an independent integration cluster. Physical linkage between *eis-1* and *pal-1*/*evi-5* was established by isolation and characterization of additional overlapping phage clones (Fig. 2A). Several tumor panels were tested for the presence of proviral insertions in the *eis-1* locus. Only a few additional insertion sites were found in this locus, as depicted in Fig. 2B. In the *eis-1*/*pal-1*/*evi-5* locus, approximately 5% of the insertions map in the *eis-1* locus, 75% map in the three major integration clusters in the *pal-1* locus, and 20% map in the *evi-5* locus.

The *Gfi-1* gene is located within the *pal-1* locus and is upregulated as a result of proviral insertions in the *pal-1*/*evi-5* locus. At the time the chromosomal location was determined for *pal-1* and *evi-5*, it became apparent that this genomic region

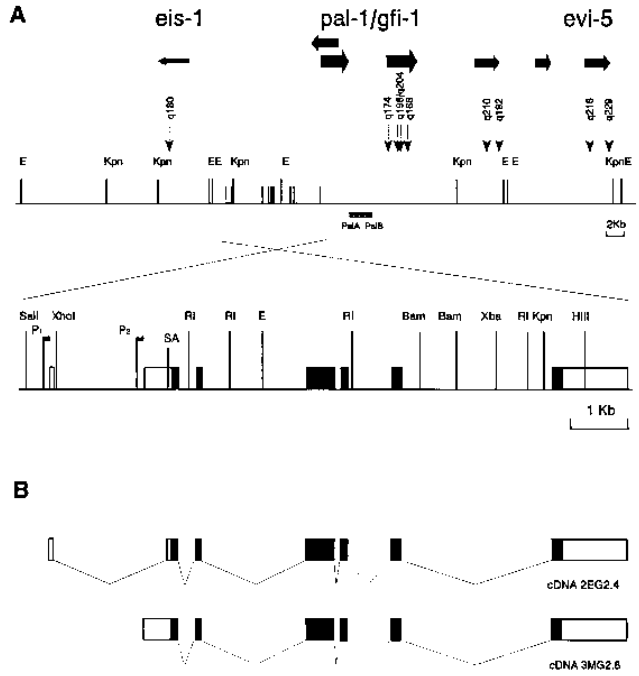

FIG. 3. (A) Location of the *Gfi-1* gene within the *pal-1* locus. In the restriction map of the *eis-1*/*pal-1*/*evi-5* locus, the position of the mouse *Gfi-1* gene is in the reverse transcriptional orientation, to the left of the pal A and pal B probes. Proviral insertions that activate *Gfi-1* expression are dispersed over the complete *eis-1*/*pal-1*/*evi-5* locus. The locations of some proviral insertions as present in *H-2K-myc* tumors (q) are indicated. The *Gfi-1* exon-intron structure is shown below at a larger scale. The alternative exon 1A is located to the left of the *Xho*I site. There are two transcription start sites, possibly regulated by two promoters, P_1 and P_2, which result either in a 2.4-kb or a 2.8-kb mRNA. The noncoding exons are drawn as open boxes, and the coding exons are drawn as black boxes. Restriction enzymes: E, *Eco*RV; Kpn, *Kpn*I; SalI, *Sal*I; XhoI, *Xho*I, RI, *Eco*RI; Bam, *Bam*HI; Xba, *Xba*I; HIII, *Hin*dIII. SA, splice acceptor. (B) The *Gfi-1* gene is encoded by six exons. The alternative exon (1A) is represented in the shorter cDNA clone 2EG2.4. The second full-length cDNA that was cloned from the BALB/c mouse thymus cDNA library (cDNA 3MG2.8) contains a longer 5′ UTR but encodes the same protein as shown in Fig. 4.

also cross-hybridized with the rat *Gfi-1* cDNA. *Gfi-1* (growth factor independence 1) was cloned as a common retroviral insertion site from rat T-cell lymphoma cell lines which had progressed during their propagation in vitro toward IL-2-independent growth (14). Indeed, the *Gfi-1* gene appeared to be located within the *pal-1* locus. To determine the exact genomic organization of *Gfi-1*, we cloned the mouse *Gfi-1* cDNA. Since *Gfi-1* is expressed in normal rat thymus, a mouse thymus cDNA library was screened with a probe derived from the 5′ end of the rat cDNA. We obtained two full-length mouse cDNA clones which differed in their 5′ sequences (Fig. 3B). The cDNA clone 3MG2.8 represents the larger endogenous transcript of 2.8 kb, and the second clone, 2EG2.4, corresponds to the smaller transcript of 2.4 kb. The differences in the 5′ untranslated region (UTR) (Fig. 3A and Table 1) are the result of an alternative untranslated first exon (1A) which splices into the first exon (exon 1B), 48 nucleotides (nt) before the translation start site (Fig. 4). The presence of this alternative first exon implicates transcriptional regulation through two different promoters. In addition to exons 1A and 1B, there are five other exons which comprise the remainder of the gene and span a genomic region of 10 kb. The nucleotide sequence of the smaller cDNA 2EG2.4 is 91% identical to the published sequence of rat *Gfi-1*, and the inferred amino acid sequence is

TABLE 1. Exon-intron boundaries in the mouse *Gfi-1* gene[a]

	Exon	Intron	Exon
ex.1A	...CCCCGA**AGg**taggttt	TCTCTC**AG**AACTCAGT	...ex.1B
ex.1B	...CAGAGC**AGg**tgcgaag	ttccgc**ag**AGGGCGGC	...ex.2
ex.2	...GTCTCC**AGg**aagcctt	cttcac**ag**CGTCGGAG	...ex.3
ex.3	...GCAGCA**AGg**tgaggct	gccctc**ag**GTGTTCTC	...ex.4
ex.4	...ACTCCC**AGg**taagatc	ctctgc**ag**GAACGCAG	...ex.5
ex.5	...CCACAC**AGg**tgagcta	ccttgc**ag**GTGAGAAG	...ex.6

[a] Exon sequences are in uppercase, and intron sequences are in lowercase. Conserved nucleotides at the splice donor and splice acceptor sites are in boldface. The splice acceptor site in exon 1B (ex.1B) is within this first alternative exon and will be used only when transcription starts at P_1.

97% similar between mouse and rat *Gfi-1*, with only a few conservative substitutions in the region outside the six zinc finger DNA binding motifs (Fig. 5). The six zinc fingers have the conserved $C\text{-}X_2\text{-}C\text{-}X\text{-}K\text{-}X\text{-}F\text{-}X_8\text{-}H\text{-}X_{3\text{–}4}\text{-}H\text{-}X_7$ sequence which is reminiscent of the classical C_2H_2 type of zinc finger DNA binding motif present in many transcription factors. The fourth and fifth zinc fingers have a conserved stretch of 7 amino acids (aa) (the H/C link) that is shared by the Krueppel-like subfamily of zinc finger proteins (7, 45). The amino-terminal end of *Gfi-1* contains a small region of homology to three other zinc finger proteins, Slug, Xsna, and IA-1, the SGI (Xsna/Slug, Gfi, IA-1) domain. The vertebrate gene *Slug* (38) and the *Xenopus* gene X*sna* (47) encode zinc finger proteins that are related to snail, a protein that is required for mesoderm formation in *Drosophila melanogaster* (8). IA-1 was cloned as a novel human insulinoma-associated cDNA, using a substraction library approach (16).

Proviral integrations in the *pal-1* locus activate the *Gfi-1* gene, resulting in a three- to sixfold-higher expression in T- and B-cell lymphomas. Since the endogenous expression of *Gfi-1* in T cells is higher than in B cells, the most profound effect of overexpression is seen in pre-B-cell tumors (data not shown). Previously, it had been demonstrated that *Gfi-1* is activated by promoter insertion (14). In our analysis, enhancer activation is the most frequent mode of activation of *Gfi-1*. Proviral insertions are predominantly upstream of *Gfi-1* in the opposite transcriptional orientation. Integrations at a large distance from *Gfi-1* in the *evi-5* locus also result in an enhanced expression of *Gfi-1* (Fig. 3 and 6). All proviral insertions in the *eis-1* locus, with the exception of tumor q180, also activate *Gfi-1* (Fig. 6). At this moment it is not clear whether the insertion in tumor q180 affects the activity of another gene in this region. A number of other genes are present in the *eis-1*/*pal-1*/*gfi-1*/*evi-5* locus, but their exact role in the MoMLV-induced lymphomas is unknown. There are a few T-cell lymphomas, such as q186, that do not contain a proviral insertion in the *eis-1*/*pal-1*/*gfi-1*/*evi-5* locus but still show a significant upregulation of the *Gfi-1* gene. Possibly there is yet an other integration cluster outside the 50-kb region covered by the different probes. Alternatively, transcription of *Gfi-1* might be induced in *trans*.

Proviral insertions in the *pal-1* locus are mutually exclusive with integrations in the *bmi-1* locus. Proviral integrations in *pim-1* and *pim-2* loci overlap with proviral integrations in the *eis-1*/*gfi-1*/*pal-1*/*evi-5* locus in MoMLV-induced tumors of Eμ-*myc* and *H-2K-myc* transgenic mice. However, in the Eμ-*myc* mice, no overlap was observed between proviral integrations in the *bmi-1* and the *eis-1*/*gfi-1*/*pal-1*/*evi-5* loci (59). To further substantiate this notion, 59 additional lymphomas from Eμ-*myc* transgenic mice were analyzed. The analysis showed that 22 had a proviral insertion in the *bmi-1* locus and 12 had one

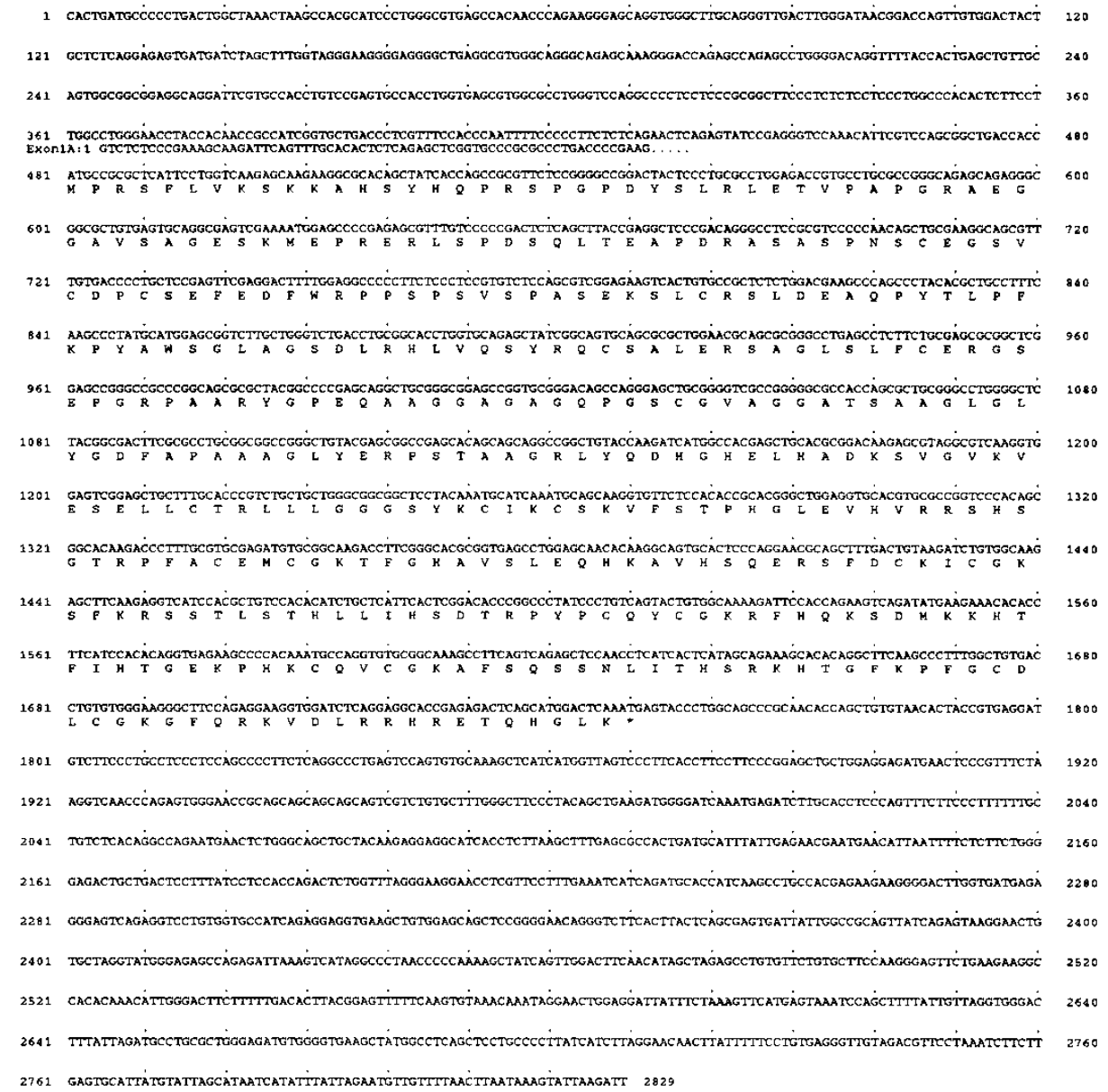

FIG. 4. Mouse *Gfi-1* cDNA sequence. The complete sequence of cDNA3MG2.8 contains a 5′ UTR of 480 nt. The amino acid sequence is shown below the DNA sequence of the ORF. The cDNA2EG2.4 has an identical sequence in the ORF and 3′ UTR, but differs in the 5′ UTR, due to the presence of an alternative first exon (1A) of 68 nt which splices to exon 1B′, 48 nt before the translation initiation site.

or more integrations in the *pal-1*/*evi-5* locus. No tumor carried integrations in both loci. This finding indicates that activation of *Bmi-1* and activation of *Gfi-1* are mutually exclusive and suggest that the two genes act on identical or similar targets relevant for lymphomagenesis.

The graph in Fig. 7 represents the combined data on the different proviral insertion sites, as detected in MoMLV-induced lymphomas in the various transgenic models. In almost 100% of the T-cell lymphomas in the Eμ-*pim-1* transgenic mice, integrations in both the *myc* and the *eis-1*/*gfi-1*/*pal-1*/*evi-5* complementation groups were observed. It is evident that neither the *pim-1*/*pim-2* nor the *eis-1*/*gfi-1*/*pal-1*/*evi-5*/*bmi-1* locus is involved in 100% of the tumors, suggesting that other genes belonging to these complementation groups might be identified in these tumors.

DISCUSSION

We have utilized proviral tagging as a method to identify proto-oncogenes which can collaborate with the *Myc* and *Pim* oncogenes in lymphomagenesis. *pal-1* and *eis-1* were identified as two novel common insertion sites in MoMLV-induced tumors in *myc* and *pim* transgenic mice. Both *pal-1* and *eis-1* colocalize with two independently cloned common insertion sites, *gfi-1* and *evi-5*. The *evi-5* locus was cloned as a common site of retroviral integration in AKXD T-cell lymphomas (30), and *gfi-1* was cloned as a common integration site in IL-2-independent rat T-cell lymphoma cell lines (14). Detailed analysis demonstrates that the *Gfi-1* gene is located within the *pal-1* locus, that *eis-1* is immediately downstream of *Gfi-1*, and that the *evi-5* locus is upstream. The combined *eis-1*/*gfi-1*/*pal-1*/*evi-5*

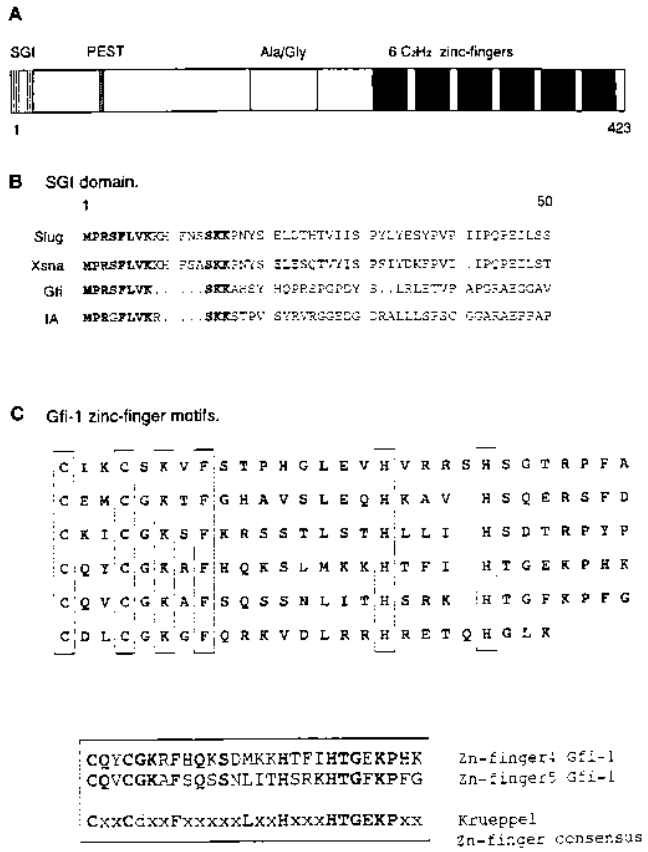

FIG. 5. (A) Schematic representation of the Gfi-1 protein. The complete protein is 423 aa long. The SGI domain is located in the first N-terminal 20 aa, the PEST domain is at aa 93 to 104, the Ala/Gly stretch is at aa 158 to 210, and the C-terminal half contains six zinc finger motifs, represented by six black boxes, with the consensus sequence C-X_2-C-X-K-X-F-X_8-H-X_{3-4}-H-X_7. (B) Amino acid alignment between the first 50 aa of Slug and the N-terminal region of XSna, IA-1, and Gfi-1. The SGI domain comprises the first 20 aa, with the highest homology in the first 9 aa. (C) Alignment of the amino acid composition of the individual zinc fingers in the Gfi-1 protein. The conserved amino acids are boxed. The region of the zinc finger that contacts the DNA (H/C link) in zinc fingers 4 and 5 has homology to the Krueppel zinc finger consensus sequence.

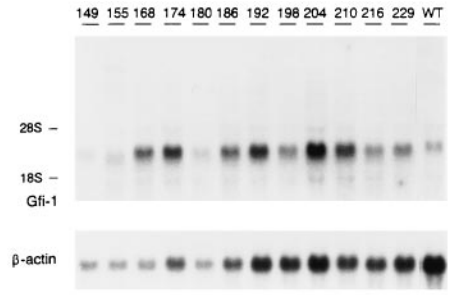

FIG. 6. Overexpression of *Gfi-1* in T-cell lymphomas as a result of proviral insertions in the *eis-1/pal-1/evi-5* locus. The Northern blot contained 15 μg of total RNA of the different lymphoma samples and was probed with the rat *Gfi-1* cDNA and β-actin probe. In addition to the endogenous thymus expression (wild type [WT]) of mouse *Gfi-1*, a higher level of expression of *Gfi-1* was found in the different lymphomas. Tumors q149, q155, and q186 have no proviral insertion in the *eis-1/pal-1/gfi-1/evi-5* locus; tumors q168, q174, q192, q198, q204, and q210 carry proviral insertions in the *pal-1/gfi-1* locus; q180 has proviral insertions in the *eis-1* locus; and q216 and q229 have proviral insertions in the *evi-5* locus. The positions of the two ribosomal bands (28S and 18S) are indicated.

locus spans a region of approximately 50 kb, containing several distinct integration clusters of MoMLV. The proviral occupancy of the *eis-1/gfi-1/pal-1/evi-5* locus in the different tumor panels varies between 14% in the pre-B-cell tumors of Eμ-*myc* transgenic mice to 93% in T-cell lymphomas of Eμ-*pim-1* transgenic mice. Many tumors, especially T-cell lymphomas, harbor two or more independent subclonal integrations either within the *pal-1* locus itself or within the *pal-1* and *evi-5* or *eis-1* loci. This implies that there is a high selection pressure for proviral integration in this region.

We have shown that most if not all proviral insertions in the *eis-1/gfi-1/pal-1/evi-5* locus can activate the *Gfi-1* gene. Although *Gfi-1* is already expressed in T cells, there is a clear upregulation of the full-length transcript in the different MoMLV-induced T-cell lymphomas, carrying a provirus in the *eis-1/gfi-1/pal-1/evi-5* locus. It is evident that *Gfi-1* can be activated by the enhancer of the MoMLV provirus over distances up to 25 kb. Although *Gfi-1* was originally cloned in an experimental setting, designed to identify genes involved in tumor progression, our data indicate that *Gfi-1* is also involved in the initiation of lymphomagenesis. Most proviral insertions in the *eis-1/gfi-1/pal-1/evi-5* locus are clonal, indicating that insertion in this locus is an early event in lymphomagenesis. It remains to be established whether *Gfi-1* is the only target gene in this locus, since there are several other genes present in this region, which are currently being characterized (48a). To determine the effects of *Gfi-1* overexpression on lymphomagenesis, we will generate transgenic mice that overexpress *Gfi-1* in the

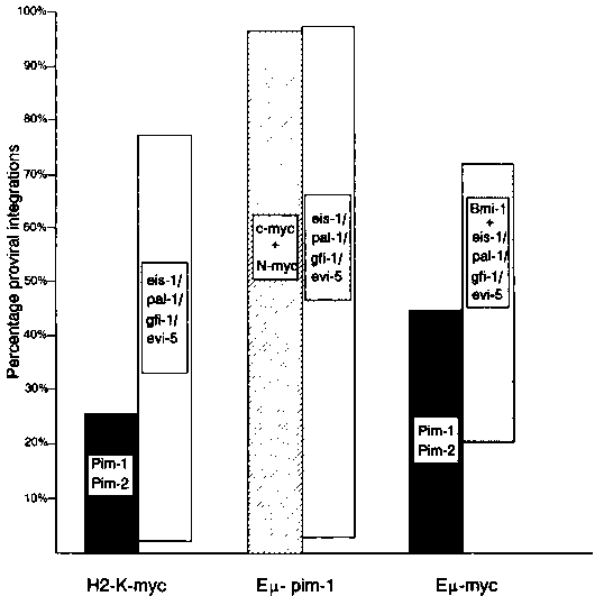

FIG. 7. Summary of the percentages of proviral insertions in the MoMLV-induced tumors in the different transgenic lines. In the *H-2K-myc* transgenic mice, 14 of 59 (24%) of T-cell lymphomas harbor either an integration in the *pim-1* or *pim-2* locus and 44 of 59 (75%) have an integration in the *eis-1/pal-1/gfi-1/evi-5* locus. In the Eμ-*pim-1* transgenic mice, 41 of 43 (95%) of T-cell lymphomas overexpress either C-*myc* or N-*myc* as a result of proviral integration, and 40 of 43 (93%) have a proviral integration in the *eis-1/pal-1/gfi-1/evi-5* locus. In the Eμ-*myc* transgenic mice, with either the wild-type alleles for *Pim-1* (n = 45) or one *Pim-1* null allele (*Pim-1*$^{-/+}$) (n = 40), 37 of 85 (44%) of the pre-B-cell lymphomas have a proviral integration in the *pim-1* or *pim-2* locus; proviral integrations in the *bmi-1* locus in 32 of 85 (38%) of lymphomas are mutually exclusive with proviral insertions in the *eis-1/pal-1/gfi-1/evi-5* locus (17/85 = 20%) and are therefore represented by one bar. The overlap between the different bars indicates the lymphomas which have proviral integrations in two independent common insertion sites.

lymphoid lineage and assess their predisposition to hematological malignancies.

The Gfi-1 protein encodes a protein of 423 aa and has the characteristic features of a transcription factor (14). The carboxy terminus of the protein contains six zinc finger DNA binding domains, of which two are homologous to the Krueppel zinc finger consensus sequence (7, 45). There is a region rich in alanine and glycine, also present in other transcriptional regulators, which might act as a transcriptional repressor domain (19, 31). Furthermore, the first 20 aa (SGI domain) of Gfi-1 are homologous to the amino termini of three other zinc finger proteins, Slug, Xsna, and IA-1 (16, 38, 47, 61). Although the exact role of this domain is not clear yet, it could be involved in the presumed transcriptional regulatory function of these proteins. Recently it has been shown that Gfi-1 binds DNA in a sequence-specific manner and indeed can act as a transcriptional repressor (62).

Extensive analysis of the MoMLV-induced pre-B-cell lymphomas in Eμ-myc transgenic mice showed that proviral integrations in the *bmi-1* locus are mutually exclusive with integrations in the *eis-1*/*gfi-1*/*pal-1*/*evi-5* locus. The *bmi-1* locus was originally identified as a common insertion site in Eμ-*myc* transgenic mice (20, 59). Proviral integration in the *bmi-1* locus leads to overexpression of the wild-type Bmi-1 protein. The *bmi-1* locus is found only as a common site of proviral integration in MoMLV-induced pre-B-cell tumors, as we did not find any proviral rearrangements in the *bmi-1* locus in the T-cell tumor panels analyzed (our unpublished results). However, the common insertion site *flvi-2*, which encompasses the gene *Bmi-1*, serves as a target for insertional mutagenesis in feline leukemia virus-induced thymic lymphosarcomas in cats (29). This would be in agreement with the observation that *bmi-1* transgenic mice are strongly predisposed to both B- and T-cell lymphomas (2a). Possibly the *bmi-1* locus is less accessible to proviral insertions in T cells than the *eis-1*/*gfi-1*/*pal-1*/*evi-5* locus.

Bmi-1 encodes a nuclear phosphoprotein of 324 aa which contains several motifs found in transcriptional regulators, including an unusual zinc ring finger motif shared by several diverse nuclear proteins. Bmi-1-specific motifs are conserved in the Posterior Sex Combs (Psc) protein from *D. melanogaster* (10, 58). Psc belongs to the Polycomb group, the members of which are involved in maintaining homeotic genes in their suppressed state after their initial expression during development. This would indicate that *Bmi-1* is involved in the regulation of *hox* gene expression, and this has been confirmed in mice overexpressing or lacking endogenous *Bmi-1*, resulting in either anterior or posterior transformation along the axial skeleton (2, 53).

The different common proviral integration loci in the various transgenic lines can be assigned to three distinct complementation groups. One complementation group is formed by the *Pim*, and the other is formed by the *Myc* gene family members. These complementation groups each contain proteins which are structurally and therefore functionally related. The *Pim* gene family consists of *Pim-1* and *Pim-2*. Both genes are targets for proviral activation in MoMLV-induced pre-B-cell and T-cell lymphomas and encode protein serine/threonine kinases (48, 52, 59). *Pim-2* is 61% identical to *Pim-1* in the catalytic kinase domain. Of the *Myc* gene family, both N-*myc* and c-*myc* serve as targets for proviral integration of MoMLV in wild-type and Eμ-*pim-1* transgenic mice (11, 56). Overexpression of both c-*myc* and N-*myc*, but also L-*myc*, predisposes to the development of lymphomas (1a, 26, 34, 35). We and others have shown that the *Pim* and *Myc* gene family members are effective collaborators in the development of lymphomas (34, 60).

The third complementation group consists of *Gfi-1* and *Bmi-1*, since proviral insertions in the *eis-1*/*pal-1*/*gfi-1*/*evi-5* locus and *bmi-1* locus are mutually exclusive. Both *Gfi-1* and *Bmi-1* can collaborate with the *Pim* and *Myc* gene family members in the process of lymphomagenesis. Provirus tagging in Eμ-L-*myc*/*pim-1* double transgenic mice has also demonstrated that the tripartite collaboration of *Myc*, *Pim*, and *Gfi-1* is effective in the generation of T-cell lymphomas (61). Interestingly, the gene products of *Gfi-1* and *Bmi-1* are not structurally related. Both proteins probably act as transcriptional regulators, and it will be interesting to determine whether they interact directly or act indirectly on the same or similar target genes in the process of lymphomagenesis.

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