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Identification of a Common Ecotropic Viral Integration Site, *Evi-1*, in the DNA of AKXD Murine Myeloid Tumors

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AKXD-23 recombinant inbred mice develop myeloid tumors at a high frequency, unlike other AKXD recombinant inbred strains which develop B-cell lymphomas, T-cell lymphomas, or both. AKXD-23 myeloid tumors are monoclonal, and their DNA contains somatically acquired proviruses, suggesting that they are retrovirally induced. We identified a common site of ecotropic proviral integration that is present in the DNA of all AKXD-23 myeloid tumors that were analyzed and in the DNA of all myeloid tumors that occur in AKXD strains other than AKXD-23. We designated this locus *Evi-1* (ecotropic viral integration site 1). Rearrangements in the *Evi-1* locus were also detected in the DNA of a number of myeloid tumors and myeloid cell lines isolated from strains other than AKXD. In contrast, few *Evi-1* rearrangements were detected in the DNA of T- or B-cell tumors. *Evi-1* may thus identify a new proto-oncogene locus that is involved in myeloid disease.

Mice from 21 of 23 AKXD recombinant inbred mouse strains derived from AKR/J, a strain with a high incidence of lymphoma, and DBA/2J, a strain with a low incidence of lymphoma, develop hematopoietic neoplasms at a high frequency (27; D. J. Gilbert, unpublished data). However, the average age of onset and the cell type of hematopoietic neoplasms varies from strain to strain, suggesting that in these strains a number of genes that affect susceptibility to lymphoma and disease type have been segregated during inbreeding. Mice from six of the AKXD strains died predominantly of T-cell lymphomas, mice from six strains died predominantly of B-cell lymphomas, and mice from one strain died predominantly of myeloid tumors. Mice from eight strains were equally susceptible to lymphomas of T- or B-cell origin.

Most AKXD lymphomas are monoclonal, as evidenced by clonal integrations of somatically acquired proviruses into their DNA (26; D. J. Gilbert, unpublished data). This finding is consistent with the hypothesis that viral integration is causally associated with lymphoma induction. Newly acquired clonal proviral integrations have also been observed by many laboratories in which lymphomas that arise in other highly viremic, highly lymphomatous mouse strains are studied (1, 2, 43, 44). While somatic integrations of mink cell focus-forming (MCF) proviruses have most often been associated with T-cell lymphomas (4, 5, 9, 23, 33), somatic ecotropic proviruses are usually associated with B-cell and myeloid tumors (1, 2, 44). A similar situation exists in AKXD strains (26; D. J. Gilbert, unpublished data).

DNAs from 258 AKXD hematopoietic neoplasms were recently characterized to determine whether they contain virally induced rearrangements in seven known or putative proto-oncogene loci found previously to serve as common sites of proviral integration in the DNA of murine or rat lymphomas (M. L. Mucenski, D. J. Gilbert, B. A. Taylor, N. A. Jenkins, and N. G. Copeland, Oncogene Res., in press). These loci included *Myc*, *Myb*, *Fis-1*, *Pim-1*, *Pvt-1*, *Mlvi-1*, and *Mlvi-2*. With the exception of *Myb*, virally induced rearrangements were identified in all loci. Most of these rearrangements were detected in the DNA of T-cell lymphomas; few rearrangements were detected in the DNA of B-cell lymphomas or myeloid tumors. These data suggest that the repertoire of cellular proto-oncogenes activated by viral integration into the DNA of these tumor cells may vary according to the cell lineage from which they were derived. The large number of well-characterized AKXD lymphomas representing diverse pathological types provides a valuable data base for identifying cellular loci that are altered by proviral integration. In the experiments described here, we began to characterize the DNA of AKXD lymphomas that contain single somatically acquired proviruses to determine whether new common sites of viral integration can be identified that may represent new proto-oncogene loci that are involved in a diverse variety of hematopoietic diseases.

MATERIALS AND METHODS

Mice. AKXD recombinant inbred mice were inbred and aged by B. A. Taylor at The Jackson Laboratory (Bar Harbor, Maine). NFS/N mice were obtained from the colonies of the National Institutes of Health (Bethesda, Md.). The origins and characteristics of the congenic strains NFS.C58-*C58v-1*, NFS.AKR-*Akv-1*, and NFS.C57BR-*H-2*^{k} were as described previously (8); these mice were bred in our colony (J. W. Hartley) at the National Institutes of Health.

Cell lines. DNA from 37 cell lines representing several distinct hematopoietic lineages and stages of differentiation was analyzed for *Evi-1* rearrangements. Thirteen cell lines (NFS-24, NFS-31, NFS-32, NFS-35, NFS-42, NFS-48,

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NFS-50, NFS-60, NFS-70, NFS-78, NFS-107, NFS-112, and NFS-126) originated from neoplastic spleens of NFS/N or (NFS/N × DBA/2N)F_1 mice inoculated with biologically cloned Cas-Br-M murine leukemia virus (MuLV) or splenic cell extracts prepared from mice inoculated with Cas-Br-M MuLV as neonates (12, 13, 42; J. N. Ihle, unpublished data). Cell line NFS-61 originated from an (NFS/N × NFS.C58-*C58v-1*)F_1 mouse inoculated with C2S L1 MCF virus, a biologically cloned MCF virus isolated from a tumor induced by the C2S ecotropic virus; cell line NFS-124 originated from an (NFS.C57BR-*H-2*^{k} × NFS.AKR-*Akv-1*)F_1 mouse inoculated with C2S L1 MCF virus. C2S is a biologically cloned ecotropic virus that was recovered from feral Lake Casitas mice (8). Cell line NFS-58 was derived from an (NFS/N × DBA/2N)F_1 mouse inoculated with C2S virus; cell line NFS-41 was derived from a tumor that arose spontaneously in an (NFS.C58-*C58v-1* × NFS.CBA-*Rmcf*^{r} *Hm*)F_1 mouse (J. W. Hartley, unpublished data). Thirteen cell lines (5F4, DA-1, DA-2, DA-3, DA-4, DA-8, DA-13, DA-24, DA-25, DA-29, DA-31, DA-33, and DA-34) originated from tumor cells of BALB/cAnN or (CBA/N × BALB/cAnN) × CBA/N mice inoculated as neonates with Moloney MuLV (14, 17, 42; J. N. Ihle, unpublished data). Four cell lines (FL7-MYB, FL8-MYB, IFLJ2, and VFL-J2) were derived from BALB/cAnN or NIH Swiss fetal mouse liver cultures infected with retroviral constructs containing the v-*myb* oncogene or the v-*myc* and v-*raf* oncogenes (41). Two cell lines (FD-HF and FD-J2) were derived from an interleukin-3 (IL-3)-dependent cell line (FDC-P1) which was isolated from long-term bone marrow cultures from DBA/2N mice and were infected with retroviral constructs containing either the v-*myc* oncogene or both a hybrid v-*raf*-v-*mil* oncogene and a hybrid v-*myc* oncogene (29). The WEHI-3 cell line originated from a tumor which arose in a BALB/c mouse inoculated with mineral oil (40). Cell lines were maintained in RPMI 1640 medium and 10% fetal bovine serum or in medium containing 20 U of purified IL-3 per ml or 25% WEHI-3-conditioned medium as a source of IL-3. Cells were analyzed for cell surface markers with monoclonal antibodies and by fluorescence-activated cell sorting (12, 13, 21). The cellular response to growth factors was assessed by determining incorporation of [^{3}H]thymidine (16).

Myelogenous leukemias. The DNAs of 18 cases of primary myelogenous leukemia from NFS/N or NFS/N hybrid mice were also analyzed for *Evi-1* rearrangements. Nine tumors (36425, 36884, 37108, 37376, 37485, 37641, 37720, 37749, and 37995) developed in NFS/N mice that were inoculated as neonates with cloned C2S virus or extracts of tumors induced by the C2S virus. The remaining nine tumors arose in (NFS/N × NFS.C58-*C58v-1*)F_1 mice, which express high levels of ecotropic virus (7). Two of these tumors (36949 and 38071) arose spontaneously; the remaining seven tumors (37050, 37064, 37192, 37195, 37226, 37762, and 37822) arose in mice that were inoculated with C2S L1 MCF virus (8). All tumors were classified solely by histopathological analyses.

DNA isolation, restriction enzyme analysis, DNA transfers, and hybridization. High-molecular-weight DNA was extracted from frozen tissues that were stored at −70°C (19). DNA (5 μg per lane) was digested to completion with an excess of restriction enzyme under reaction conditions recommended by the manufacturers (Bethesda Research Laboratories, Inc., Gaithersburg, Md.; Amersham Corp., Arlington Heights, Ill.; and New England BioLabs, Inc., Beverly, Mass.). The completely digested DNAs were submitted to electrophoresis through 0.8% agarose gels, transferred to membrane filters (Zetabind; AMF Cuno, Meriden,

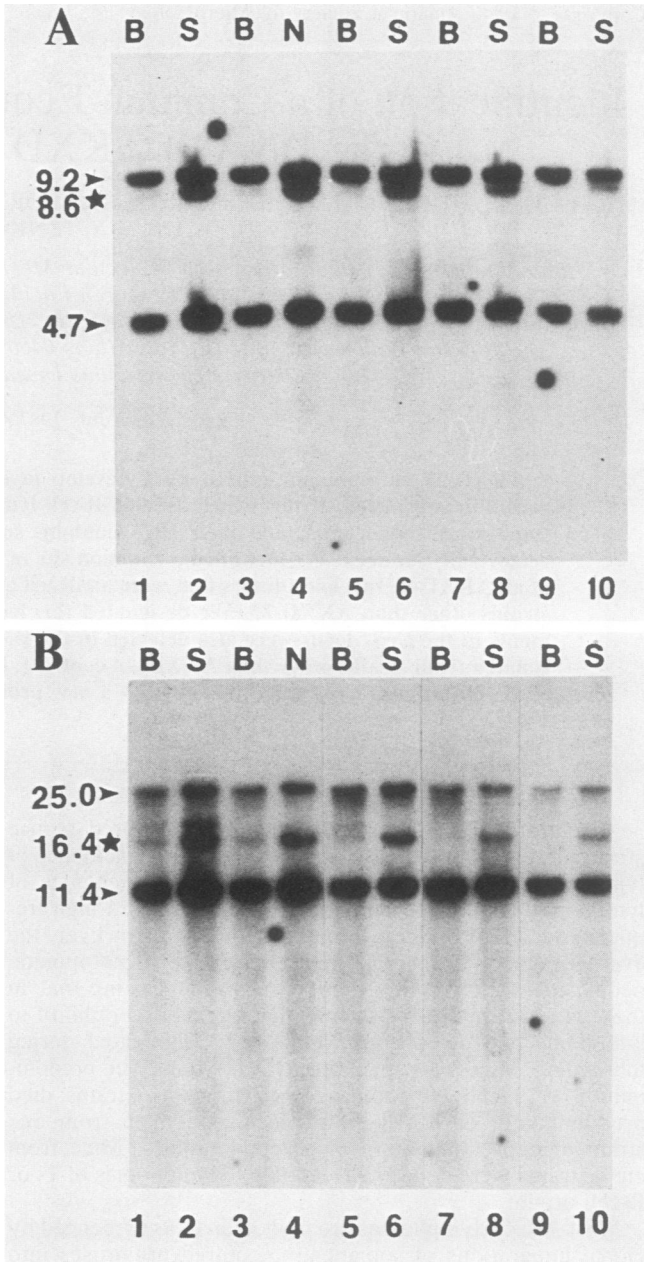

FIG. 1. Identification of a common site of ecotropic viral integration in the DNAs of AKXD-23 myeloid tumors. High-molecular-weight DNA was extracted from the brain (B) and at least two tumor-bearing tissues (S, spleen; N, lymph node) of each animal, one of which is shown in each panel. DNAs (5 μg per lane) were digested with either *Pvu*II (A) or *Eco*RI (B), submitted to electrophoresis through a 0.8% agarose gel, transferred to Zetabind, and hybridized and washed as described previously (27). The filters were hybridized with a nick-translated ecotropic virus-specific envelope probe, pEco (3, 30). DNA samples were loaded in the same order in both autoradiographs. Lanes 1 and 2, DNAs from tumor 112; lanes 3 and 4, DNAs from tumor 130; lanes 5 and 6, DNAs from tumor 163; lanes 7 and 8, DNAs from tumor 173; lanes 9 and 10, DNAs from tumor 172. Arrowheads indicate endogenous ecotropic proviral fragments, and stars indicate somatically acquired proviral fragments that appear to be located in the same-sized fragment in each tumor. The sizes (in kilobases) of hybridizing fragments were calculated by using ^{32}P-labeled *Hin*dIII-digested lambda DNA submitted to electrophoresis in parallel lanes of the same gels.

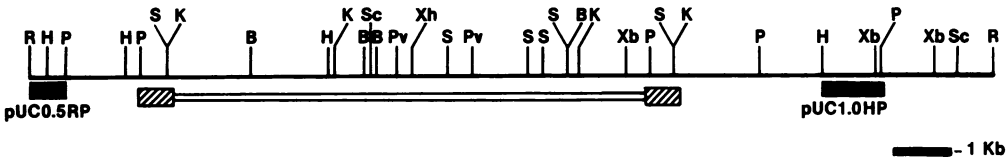

FIG. 2. Restriction map of the *Evi-1* viral integration site of tumor 130. The black line represents the 16.4-kb *Eco*RI fragment cloned from the DNA of tumor 130. The provirus is shown flanked by its LTRs (hatched boxes) below the restriction map, and unique sequences flanking cellular probes pUC0.5RP and pUC1.0HP are represented by black boxes. The provirus map is drawn 5′ to 3′ with respect to viral RNA. Abbreviations for restriction endonucleases are as follows: B, *Bam*HI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Pv, *Pvu*II; R, *Eco*RI; S, *Sma*I; Sc, *Sac*I; Xb, *Xba*I; Xh, *Xho*I.

Conn.), baked, prehybridized, hybridized, and washed as described previously (27). All filters were then autoradiographed at −70°C as described previously (19).

RESULTS

Identification of a common site of ecotropic viral integration in AKXD-23 myeloid lymphomas. A total of 179 AKXD hematopoietic neoplasms from 12 AKXD recombinant inbred strains were screened to identify tumors that contained single somatically acquired proviruses. DNA from these lymphomas were digested with *Pvu*II or *Sac*I and subjected to Southern blot hybridization analysis with the use of two AKR ecotropic MuLV envelope (*env*) gene probes. One probe, which we designated pEco, specifically detects ecotropic proviruses (3), whereas pAKV-5 hybridizes to both ecotropic and class I oncogenic MCF proviruses (11). These two viral classes appear to be causally associated with the majority of AKXD lymphomas analyzed (26; D. J. Gilbert, unpublished data). Both probes detect 3′ proviral DNA-cellular DNA junction fragments in *Pvu*II- or *Sac*I-digested DNA. DNAs from lymphomas that appeared to contain single somatically acquired proviruses were also analyzed with two other restriction enzymes, *Bcl*I and *Eco*RI, to confirm the presence of a single somatically acquired provirus. Of 179 lymphomas that were analyzed, 140 lymphomas contained somatically acquired proviruses. Importantly, 31 tumors appeared to contain single new integration sites (data not shown). Of these proviruses, 30 were of ecotropic virus origin; only 1 appeared to be of MCF virus origin.

Among the DNAs examined, most, if not all, of the AKXD-23 myeloid tumors DNAs (seven AKXD-23 myeloid tumors were analyzed) appeared to contain proviruses that were integrated into the same chromosomal domain. Representative results for DNAs cleaved with *Pvu*II or *Eco*RI and hybridized with the ecotropic virus-specific probe pEco are shown in Fig. 1. Brain DNA was included as a control to discriminate between endogenous and somatically acquired proviruses. In all but the first tumor, which appeared to contain two somatically acquired ecotropic proviruses, only a single somatic ecotropic provirus was detected (Fig. 1). This provirus was identified as a 8.6-kilobase (kb) *Pvu*II or a 16.4-kb *Eco*RI fragment in each DNA examined. Faintly hybridizing fragments of similar size were sometimes detected in brain DNA, possibly due to the infiltration of leukemic cells into the brain tissue of some animals. Identical hybridization patterns were also obtained when we used probe pAKV-5, which detects both oncogenic class I MCF proviruses and ecotropic proviruses (data not shown). Therefore, these tumors do not appear to contain somatically acquired class I MCF proviruses. These results strongly suggest that each tumor contains a somatically acquired ecotropic provirus integrated into the same chro-

TABLE 1. Cell type specificity of *Evi-1* rearrangements in the DNAs of AKXD tumors

Strain	No. of tumors	No. of tumors with DNA rearrangements/total no. of tumors for the following tumor types[a]:							
		Stem	Pre-B cell	B cell	T cell	Myeloid	T cell, B cell[b]	T cell, myeloid[b]	B cell, myeloid[b]
3	15	0/1	0/2	0/4	0/5	—[c]	0/3	—	—
6	13	—	0/1	0/1	0/11	—	—	—	—
7	10	—	—	0/5	0/4	—	0/1	—	—
9	13	—	0/6	0/1	0/5	1/1	—	—	—
13	16	1/1	0/1	1/10	0/2	—	0/1	—	1/1
14	12	—	0/1	0/9	0/1	—	0/1	—	—
15	9	—	—	0/5	0/3	1/1	—	—	—
17	11	—	0/1	—	0/10	—	—	—	—
18	11	—	—	0/6	0/5	—	—	—	—
22	7	—	0/1	0/2	0/4	—	—	—	—
23	10	—	—	0/1	0/1	7/7	—	0/1	—
27	13	—	1/5	0/5	0/3	—	—	—	—

[a] Tumors were classified by histopathological data and molecular analyses, as described previously (27). Molecular classification was based on rearrangements detected in the immunoglobulin heavy- (IgH) and kappa light-(IgGκ) chain genes for B cells and rearrangements in the beta chain of the T-cell receptor (Tβ) for T cells. Stem cell tumor DNAs were not rearranged in the IgH, IgGκ, or the Tβ loci; pre-B-cell tumor DNAs were rearranged only in the IgH locus; B-cell lymphoma DNAs were rearranged in both the IgH and IgGκ loci; T-cell lymphoma DNAs were frequently rearranged at the IgH locus and always rearranged at the Tβ locus; and myeloid tumor DNAs were infrequently rearranged only at the IgH locus. Histopathological analysis was used to identify myeloid tumor DNAs. Boxed values indicate that tumors were identified as containing *Evi-1* rearrangements.

[b] Mixed lymphoma-type tumors displaying phenotypes characteristic of more than one lineage.

[c] —, Tumor cells of a particular cellular lineage were not detected within a strain.

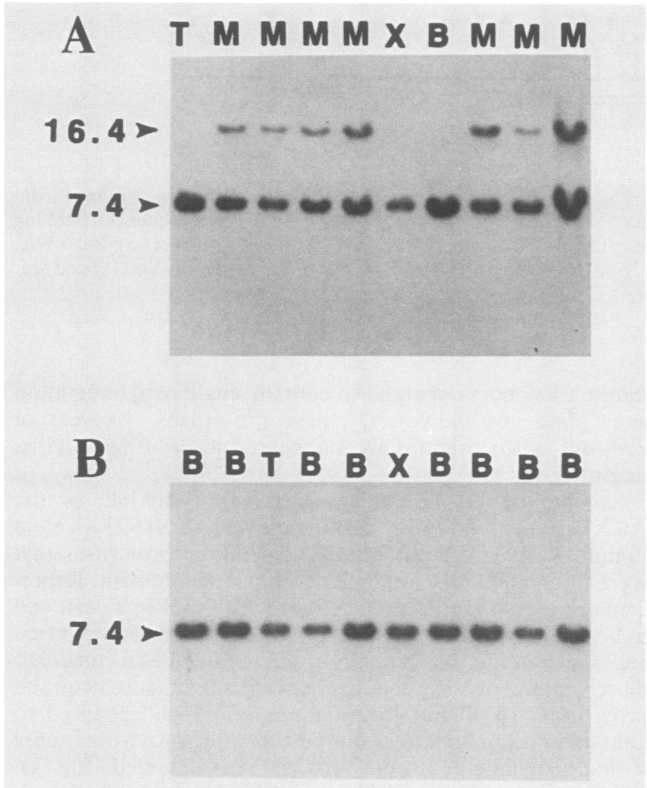

FIG. 3. *Evi-1* rearrangements in the DNAs of AKXD-23 (A) and AKXD-14 (B) tumors. *Eco*RI-digested DNA from 10 AKXD-23 and 10 AKXD-14 tumors was analyzed for *Evi-1* rearrangements by Southern blot analysis (as described in the legend to Fig. 1 and previously [27]) and hybridization with the unique sequence cellular probe pUC1.0HP from the *Evi-1* locus. Each tumor was classified with respect to cell type (T, T-cell; B, B-cell; X, mixed; M, myeloid) based on molecular analysis, histopathological analysis, or both (27). Tumor classifications are listed at the top of each lane. The sizes (in kilobases) of hybridizing fragments were calculated by using ^{32}P-labeled, *Hin*dIII-digested lambda DNA that was submitted to electrophoresis in parallel lanes of the same gels.

mosomal domain. Additional Southern blot analysis of *Bcl*I- and *Sac*I-digested DNAs supported this finding (data not shown). We designated this common site of ecotropic viral integration *Evi-1* (ecotropic viral integration site 1).

Molecular cloning of *Evi-1* sequences. To confirm the existence of a common integration site, we molecularly cloned unique cellular DNA sequences homologous to the *Evi-1* locus from one of the tumor DNAs. DNA from tumor 130 (Fig. 1, lanes 3 and 4) was digested to completion with *Eco*RI and submitted to electrophoresis through a 0.8% low-melting-point agarose gel. Size-selected DNA fragments (15 to 20 kb) were isolated, and a subgenomic library was constructed in the lambda phage vector EMBL-4. Three recombinant phage carrying the expected 16.4-kb insert were identified with the ecotropic virus-specific probe pEco. The 16.4-kb genomic insert from one recombinant phage was subcloned into the *Eco*RI site of plasmid pBR325, and its restriction map was determined (25, 36). The restriction map of this clone is shown in Fig. 2. The restriction map of the ecotropic provirus cloned from lymphoma 130 was identical to that of other nondefective ecotropic proviruses, suggesting that no substantial deletions or rearrangements were present.

Cellular DNA sequences located both 5′ and 3′ of this ecotropic provirus were isolated and screened for the absence of repetitive sequences and were subcloned into pUC18. Two such subclones (pUC0.5RP and pUC1.0HP) were isolated; these were located 5′ and 3′ to the proviral integration site, respectively (Fig. 2).

Characterization of *Evi-1* rearrangements in the DNA of AKXD lymphomas. We screened *Eco*RI-digested DNA from 140 AKXD lymphomas for rearrangements in the *Evi-1* locus by Southern blot analysis using the pUC1.0HP probe. These included DNAs from 2 stem cell tumors, 18 pre-B-cell tumors, 49 B-cell tumors, 54 T-cell tumors, 9 myeloid tumors, and 8 tumors of mixed-cell type (Table 1). Representative Southern blot results for the DNA of tumors from two strains (AKXD-23 and AKXD-14) are shown in Fig. 3. The pUC1.0HP probe detected a germ line fragment (preintegration site) of approximately 7.4 kb and a rearranged fragment (due to insertion of a 9.0-kb ecotropic provirus) of approximately 16.4 kb. No rearrangements were detected in the DNAs of the B-cell, T-cell, or mixed-cell tumors from AKXD-14 mice, whereas rearrangements were detected in the DNAs of all AKXD-23 myeloid tumors analyzed. Rearrangements were not detected in the DNAs of the AKXD-23 T-cell, B-cell, or mixed-cell lymphomas (Fig. 3).

Results of Southern blot analysis for the DNA from cells of all 140 AKXD tumors are summarized in Table 1. Rearrangements in the *Evi-1* locus were detected in the DNAs from cells of 13 of 140 (9.3%) AKXD tumors. Rearrangements were detected in the DNAs from cells of all nine AKXD myeloid tumors analyzed. Seven of these tumors were from AKXD-23, one was from AKXD-6, and one was from AKXD-15 mice (Table 1). The DNA of one mixed-cell tumor from strain AKXD-13 that displayed phenotypic characteristics of both the B-cell and myeloid lineage also contained an *Evi-1* rearrangement. *Evi-1* rearrangements were not detected in the DNAs of T-cell lymphomas. However, *Evi-1* rearrangements were occasionally detected in the DNAs of B-cell lymphomas. The DNA of one of two tumors classified as a stem cell lymphoma also contained an *Evi-1* rearrangement.

***Evi-1* rearrangements can be detected in the DNAs of myeloid tumors or cell lines from strains other than AKXD.** The DNAs of 37 cell lines established from hematopoietic neoplasms that arose in NFS/N or NFS/N hybrid mice (see

TABLE 2. Cell-type specificity of *Evi-1* rearrangements in the DNAs of NFS/N- and NFS/N hybrid-derived cells lines

Cell lineage[a]	Cell lines
Stem cell	NFS-42, NFS-50
Pre-B cell	NFS-32, NFS-70
B cell	NFS-112, IFLJ2
T cell	NFS-24, 5F4, DA-2, and DA-25
Myeloid	NFS-60[b], NFS-78[b], NFS-31, NFS-35, NFS-58, NFS-61, NFS-107, NFS-124, NFS-126, DA-1, DA-3, DA-4, DA-8, DA-13, DA-24, DA-29, DA-31, DA-33, DA-34, FDHF, FDJ2, VFLJ2, and WEHI-3
Myeloid-erythroid precursor	NFS-48[b]
Myeloid and B cell	NFS-41
Fetal liver cell lines	FL7-MYB, FL8-MYB

[a] The cell lineage was determined by histopathological analysis, dependence on IL-3 for growth, and cell surface markers.
[b] Cell lines with *Evi-1* rearrangements.

TABLE 3. Origin and characteristics of NFS/N- or NFS/N hybrid-derived tumors containing *Evi-1* rearrangements

Primary tumor	Mouse inoculated	Virus inoculated[a]	Tumor pathology[b]
37064	(NFS/N × NFS.C58-*C58v-1*)F_1	C2S L1 MCF	Myelogenous leukemia
37376	NFS/N	C2S tumor extract	Myelogenous leukemia
37720	NFS/N	C2S tumor extract	Myelogenous leukemia
37641	NFS/N	C2S	Myelogenous leukemia
NFS-48	NFS/N	Cas-Br-M tumor extract	Erythroleukemia
NFS-60	(NFS/N × DBA/2N)F_1	Cas-Br-M	Myelogenous leukemia
NFS-78	NFS/N	Cas-Br-M tumor extract	Myelogenous leukemia

[a] Biologically cloned Cas-Br-M or cell extracts prepared from splenic tumors that arose in NFS/N mice inoculated with Cas-Br-M MuLV were isolated as described previously (12). C2S, C2S tumor extract, and C2S L1 MCF virus were prepared as described previously (8).
[b] Primary tumor classification was done by histopathological analysis.

above) and that represented various cellular lineages were also analyzed for rearrangements in the *Evi-1* locus (Table 2). DNAs of three of these cells lines (NFS-48, NFS-60, and NFS-78) contained *Evi-1* rearrangements (Table 2). The origin and phenotypic characteristics of these three cell lines are summarized in Tables 3 and 4. These cell lines were isolated from primary tumors induced by neonatal inoculation of NFS/N or (NFS/N × DBA/2N)F_1 mice with the biologically cloned wild mouse ecotropic virus Cas-Br-M or with tumor extracts prepared from primary tumors induced by Cas-Br-M virus (Table 3). The NFS-48 cell line was derived from an erythroleukemia that had histologic myeloid characteristics, whereas those that produced the NFS-60 and NFS-78 cell lines were myelogenous leukemias. The cell lines derived from these tumors were also analyzed for myeloid-specific markers (Table 4). NFS-48 did not express *Mac-1* or *Thy-1*, suggesting that these cells are an early erythroid-myeloid progenitor. NFS-60 and NFS-78 had a myeloblastic morphology and expressed *Thy-1*, indicating that they are at an early stage in the myeloid lineage. Consistent with these classifications, none of the cell lines expressed the marker detected by the RB6-8C5 monoclonal antibody (12). All three cell lines were IL-3 dependent for growth in vitro. *Evi-1* rearrangements were not detected in the DNAs of cell lines representing other hematopoietic lineages (Table 2).

Rearrangements in the *Evi-1* locus were also detected in the DNAs of 4 of 18 (22.2%) primary myelogenous leukemias (Table 1). The origin of these four tumors is summarized in Table 2. Three of the *Evi-1* rearrangements were detected in the DNAs of tumors that developed in NFS/N mice (37376, 37720, and 37641) inoculated with the ecotropic C2S virus or a tumor extract from tumors induced by the C2S virus, whereas the fourth rearrangement was detected in the DNA of a tumor that arose in an (NFS/N × NFS.C58-*C58v-1*)F_1 mouse (37064) inoculated with the C2S L1 MCF virus.

Location and orientation of the DNAs of proviruses integrated into *Evi-1*. The location of proviral integration sites within the *Evi-1* locus in AKXD tumors was determined by Southern analysis by using the restriction endonuclease *Kpn*I and the unique cellular sequence probe pUC1.0HP (Fig. 2). *Kpn*I was chosen for these studies because restriction sites for this enzyme are found in the long terminal repeats (LTRs) of most MuLV genomes (38). To distinguish between ecotropic proviruses and recombinant MCF proviruses, the restriction endonuclease *Eco*RI was used, because MCF proviruses have an *Eco*RI restriction site at position 6.9 of their genomic map (28), whereas ecotropic proviruses lack this site (24). Two restriction endonucleases (*Pvu*II and *Xba*II) that cleave asymmetrically within the MuLV genome were used to determine the orientation of each provirus. The results of this analysis are presented in Fig. 4.

All rearrangements within the *Evi-1* locus in AKXD tumors appeared to be caused by viral integration (Fig. 4). All viral integrations occurred within a 0.6-kb region. Eleven viral integrations were clustered within a 300-base-pair region. All proviruses lacked an *Eco*RI restriction site, indicating that they were ecotropic proviruses.

Similar results were found for the cell lines and myeloid tumors obtained from NFS/N or NFS/N hybrid mice that contained *Evi-1* rearrangements. All rearrangements were due to the integration of an ecotropic provirus within a 1.2-kb region. The orientation of the wild mouse ecotropic proviruses C2S and Cas-Br-M were not determined in these studies because enzymes such as *Pvu*II and *Xba*I do not cleave asymmetrically within these proviral genomes (20; S. K. Chattopadhyay, unpublished data).

TABLE 4. Characteristics of NFS/N- or NFS/N hybrid-derived cell lines containing *Evi-1* rearrangements

Cell line[a]	20αSDH (units of activity)[b]	*Thy-1*[c]	*Mac-1*[c]	8C5[c]	Cell type
NFS-48	2,503	−	−	−	Erythroid-myeloid progenitor
NFS-60	21,000	+	−	−	Myeloid
NFS-78	1,680	+	+	−	Myeloid

[a] All cell lines were dependent on IL-3 for growth.
[b] Results are expressed as previously described (42).
[c] *Thy-1*, *Mac-1*, and 8C5 expression were assessed by fluorescence-activated cell sorting, as described previously (13).

DISCUSSION

The AKXD recombinant inbred strains of mice die of many diverse retrovirally induced hematopoietic diseases and represent a valuable resource for identifying common sites of viral integration that may represent novel proto-oncogene loci. From the initial analyses of the DNAs of these tumors we identified a common site of ecotropic viral integration, *Evi-1*. Viral integration into the *Evi-1* locus was detected in the DNAs from cells of 13 of 140 AKXD tumors, including all 9 AKXD myeloid tumors analyzed. Few *Evi-1* rearrangements were detected in the DNAs of B- or T-cell lymphomas. *Evi-1* rearrangements were also detected in the

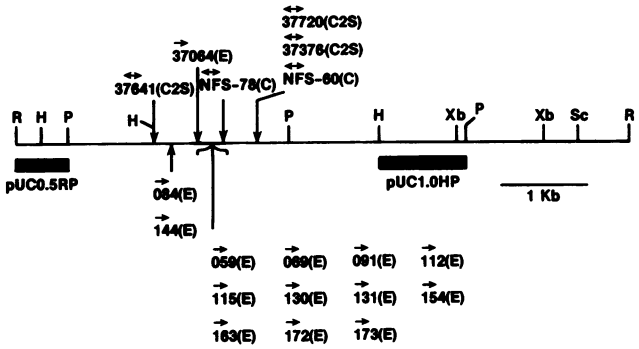

FIG. 4. Position and orientation of integrated proviruses in the *Evi-1* locus. The position and orientation of the proviruses integrated into the *Evi-1* locus was determined by genomic restriction enzyme analysis, as described in the text. Brackets and vertical arrows indicate proviral integration sites; horizontal arrows indicate the proviral orientation. Double-headed arrows indicate that the proviral orientation is not known. The type of provirus integrated into *Evi-1*, ecotropic (E), Cas-Br-M (C), or C2S, is given in parentheses. The position and orientation of the virus integrated into the *Evi-1* locus of the NFS-48 cell line could not be determined because the DNA supply of this cell line was exhausted. Proviral integrations listed below the map represent the DNAs of AKXD tumors, whereas those listed above the map represent the DNAs of NFS/N and NFS/N hybrid tumors and cell lines containing *Evi-1* rearrangements. Flanking cellular probes (pUC0.5RP and pUC1.0HP) are represented by black boxes. Restriction endonuclease abbreviations are the same as given in the legend to Fig. 3.

DNAs of cases of primary myelogenous leukemias and in myeloid cell lines derived from NFS/N or NFS/N hybrid mice injected as neonates with wild mouse ecotropic or MCF MuLV. Viral integration into the *Evi-1* locus is therefore not restricted to AKXD hematopoietic tumors. One myeloid cell line (NFS-60) containing a viral integration in the *Evi-1* locus also contains a viral integration within the *Myb* locus (34, 41, 42). This finding suggests that rearrangements in these two loci may cooperate in tumor initiation, tumor progression, or both. Results of detailed chromosomal mapping studies suggest that *Evi-1* does not represent any proto-oncogene or common viral DNA integration site previously mapped in mice (M. L. Mucenski, D. J. Gilbert, B. A. Taylor, N. A. Jenkins, and N. G. Copeland, Oncogene Res., in press), suggesting that the *Evi-1* locus may identify a new proto-oncogene that is involved in myeloid disease.

Only ecotropic proviruses were found to be integrated into the *Evi-1* locus in AKXD lymphomas. All proviruses were located within a 0.6-kb region and were oriented, when discernible, in the same transcriptional direction. Restriction enzyme mapping suggested that they are nondefective ecotropic proviruses. Subsequent DNA transfection of NIH 3T3 mouse cells with the DNA of one such provirus cloned from AKXD-23 myeloid tumor 130 confirmed this prediction (unpublished data). Similar results were found for the myeloid tumors and cell lines isolated from NFS/N or NFS/N hybrid mice infected with wild mouse ecotropic or MCF virus. Where determined, DNAs of C2S-, Cas-Br-M-, or *C58v-1*-encoded ecotropic viruses were integrated into the *Evi-1* locus in these tumors.

The tissue tropism and oncogenicity of several MuLVs has been shown to reside in sequences located within the LTRs (6, 18, 22, 31, 39). Therefore, the ecotropic proviruses found in the *Evi-1* locus might have contained recombinant LTRs with enhanced oncogenic potential for myeloid cells. The *env*-specific probes used to characterize these proviruses in this study would not necessarily detect these recombination events. To determine whether this was the case, we sequenced the 5′ and 3′ LTRs of the ecotropic provirus located within the *Evi-1* locus of AKXD-23 myeloid tumor 130 (data not shown) by using the dideoxy chain termination method (32). The sequences of both LTRs were identical to that of the prototypic endogenous AKR ecotropic provirus (data not shown). Only one copy of the 99-base-pair sequence duplicated in the U3 region of the exogenous ecotropic provirus AKR623 was found (10). We are currently analyzing the provirus from tumor 130 in more detail to determine whether it is recombinant in regions other than the LTR.

The sequence of the two noncontiguous cellular DNA fragments that flanked this provirus (a total of 802 base pairs) was also determined (data not shown). The 312 base pairs of cellular DNA immediately flanking the proviral integration site were A-T rich (67.6%), similar to that which has been reported for several other retroviral integration sites (15, 35, 37). Nonsense codons appeared frequently in all six reading frames of both cellular sequences. These sequences were also screened for homology to sequences found in the GenBank data base. No substantial regions of homology were found.

The pUC1.0HP and pUC0.5RP unique sequence probes from the *Evi-1* locus were also used in Northern blot analysis to screen for *Evi-1* transcripts in total RNA and poly(A)$^+$ mRNA from numerous cell lines representing several different hematopoietic cell lineages, including cell lines NFS-60 and NFS-78, which contain viral integrations within the *Evi-1* locus (unpublished data). No transcripts were detected. Overlapping λ clones spanning approximately 25 kb of the *Evi-1* locus were also identified and shown to cross-hybridize under reduced stringency conditions to DNA fragments with unique sequences from every species analyzed, including humans, suggesting that portions of each insert are evolutionarily well conserved (unpublished data). Highly conserved regions with unique sequences from both clones are being identified and will be used as probes in Northern analysis to determine whether DNA in the *Evi-1* region is transcribed.

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