Identification of *Evi-3*, a Novel Common Site of Retroviral Integration in Mouse AKXD B-Cell Lymphomas

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We have identified a novel common site of ecotropic viral integration called ecotropic viral integration site 3 (*Evi-3*) in B-cell lineage lymphomas of the AKXD recombinant inbred strains of mice. A number of virally induced pre-B-, B-, myeloid, and T-cell lymphomas were screened for viral rearrangements at *Evi-3*; rearrangements were found in pre-B- and B-cell lymphomas but not in other hematopoietic tumors. Genetic mapping studies localized *Evi-3* to mouse chromosome 18, distinct from proto-oncogene and common viral integration loci identified previously in the mouse. Each proviral integration at *Evi-3* is contained within a 200-bp region that lies inside a CpG island. All but one of the proviruses have integrated in the same 5'-to-3' transcriptional orientation. Transcripts from *Evi-3* are expressed in a developmentally regulated manner in B cells. Taken together, these data suggest that *Evi-3* represents a novel proto-oncogene involved in mouse B-cell lymphomas.

Proto-oncogenes can be altered by a number of different mechanisms to induce or potentiate tumorigenesis. For example, slowly transforming retroviruses cause neoplasms by inserting near cellular proto-oncogenes (19, 45). The cellular proto-oncogenes altered by the viral integration represent novel or previously identified loci. Thus, slowly transforming retroviruses provide useful retrotransposon tags for identifying novel genes causally associated with neoplastic disease. These loci are identified by isolating a unique-sequence probe that flanks a single proviral insertion either by molecularly cloning a cellular DNA-proviral DNA junction fragment (6, 41) or by amplifying cellular DNA sequences that flank the provirus, using inverse PCR from an anchored primer in the proviral long terminal repeat (51). The flanking unique-sequence cellular DNA probe is used to screen a number of tumors for virally associated rearrangements at that locus. Because retroviruses can integrate into many sites in the host genome, the detection of rearrangements at the same locus in several independent tumors suggests that the locus encodes a gene whose alteration by viral integration gives the cells a growth advantage. These loci are called common sites of retroviral integration. Many new common sites of retroviral integration have already been identified in mouse mammary tumors and in mouse and rat hematopoietic tumors (see reference 50 for a review).

Several recombinant inbred (RI) strains of mice have been developed as model systems for identifying and studying genes causally associated with hematopoietic diseases (see reference 27 for a review). One series of RI strains that is particularly useful for studying the molecular genetic basis of the development of murine lymphomas consists of the AKXD RI strains (17, 39, 41, 42). These strains were derived by crossing mice from the inbred strains AKR/J and DBA/2J. AKR/J mice have a high incidence of T-cell lymphomas, whereas DBA/2J mice

rarely develop lymphomas. The high incidence of T-cell lymphomas in AKR/J mice is associated with the expression of two endogenous ecotropic murine leukemia viruses (24). In contrast, the single endogenous ecotropic proviral locus carried by DBA/2J mice is defective and is poorly expressed (11), which likely accounts for the low lymphoma incidence in this strain.

The AKXD RI strains differ among themselves and from the parents in lymphoma susceptibility, lymphoma cell type, average age of onset of disease, and somatic virus content (17, 38, 40, 42). Of 21 highly lymphomatous AKXD RI strains, only six strains developed predominantly T-cell lymphomas like those of the AKR/J parent. Seven strains developed predominantly B-cell lymphomas, seven strains developed both T- and B-cell lymphomas, and one strain developed myeloid leukemias (17, 42). Analysis of the proviral content of the AKXD tumors is consistent with induction of the tumors by viral integration and subsequent activation of cellular proto-oncogenes. All tumors contain somatically acquired proviruses, and most are monoclonal in origin (17, 38, 42). Notably, a novel set of proto-oncogene loci may be altered by viral integration in the AKXD B-lineage lymphomas, since the B-lineage lymphomas of the AKXD RI strains had very few rearrangements at known proto-oncogene loci (25, 39).

We have used the AKXD B-lineage lymphomas to identify and molecularly clone a novel common site of retroviral integration that we have designated ecotropic viral integration site 3 (*Evi-3*). We used an interspecific backcross (IB) to map *Evi-3* to a distinct location on mouse chromosome 18. Interestingly, proviral insertions at *Evi-3* are found only in B-lineage lymphomas, and transcripts have been identified in B cells. Taken together, these results suggest that *Evi-3* represents a novel putative proto-oncogene involved in mouse B-cell lymphomagenesis.

MATERIALS AND METHODS

Mice. The AKXD RI strains were aged by B. A. Taylor at The Jackson Laboratory (Bar Harbor, Maine) as described previously (42). NFS/N mice were obtained from the colonies of the National Institutes of Health (Bethesda, Md.) (22). The

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IB (C57BL/6J $\times$ *Mus spretus*)F₁ $\times$ C57BL/6J was performed at the NCI-Frederick Cancer Research and Development Center as described previously (12). Subsets of a total of 205 mice were used for the mapping studies.

Cell lines and tumors. The HAFTL-1, JP-2, AAC6, HAFTL-2, Y1C1, JP7, BAMC1, HS1C5, HRC3, FEUC, FEU2NC1, KFFTL1, JP-5, BASC6C2, BAC14, JP-4 (2), JP-4 (1), FEMCL, and JP-1 cell lines are pre-B-cell lines derived from tumors that arose in mice infected with acutely transforming retroviruses carrying *fes*, *ras*, *abl*, and *src* oncogenes (23), and DNAs from these cell lines were kindly provided by Wendy Davidson (National Cancer Institute, Bethesda, Md.). The NFS-70, NFS-112, NFS-467, NFS-1135, NFS-1437, NFS-25, and NFS-1.0 cell lines are B-lineage cell lines derived from tumors that arose in NFS/N mice infected with Cas-Br-M murine leukemia virus or derivatives or in NFS/N mice congenic for AKR or C58 proviral loci (15, 22). BALB/c-1427 is a B-cell line derived from a tumor that arose in retrovirally infected BALB/c mice (43), and SJL-4 is a B-cell line derived from a tumor that arose in an SJL-*lpr/lpr* mouse (43). The cell type of each of these cell lines was determined by a combination of histopathological, molecular, and cell surface antigen analyses (15, 22, 43). Cell culture conditions have been previously described in detail (14).

The cell type of AKXD tumor DNAs was classified by histopathological and molecular analyses (17, 38). Tumors were grouped as pre-B cell if they had rearrangements at the immunoglobulin heavy-chain (*IgH*) locus, B cell if they had rearrangements at both the *IgH* and immunoglobulin kappa light-chain (*IgK*) loci, T cell if they had rearrangements at the T-cell receptor β -chain (*Tcrb*) locus, stem cell if they had rearrangements at none of the three loci, and myeloid if they had myeloid characteristics histopathologically and had rearrangements at none of the three loci. Mixed tumors had the molecular characteristics of both T and B cells.

Probes. Probes for the *IgH*, *IgK*, and *Tcrb* loci, for the ecotropic virus-specific envelope (*env*) gene (pEco), and for the mink cell focus-forming (MCF) virus *env* gene (pAKV-5) have been described elsewhere (40, 42).

The probes for kinesin heavy chain (*Kns*), N-cadherin (*N-cad*), tumor progression locus 2 (*Tpl-2*), and transthyretin (*Ttr*; previously called prealbumin) have been described previously (26, 37). The probe for mapping *Evi-3* was a randomly primed gel-purified insert from the plasmid p3-1, a 4.6-kb *SacI*-*KpnI* mouse genomic fragment cloned into pBluescript KS+.

The probe for the *vav* proto-oncogene is a 2.5-kb cDNA cloned into the *EcoRI* site of pBluescript KS+ and was a gift of Schula Katzav and Mariano Barbacid (Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, N.J.). The probe for glyceraldehyde phosphate dehydrogenase has been described elsewhere (43, 46).

DNA isolation and Southern blot analysis. High-molecular-weight DNAs were prepared from frozen mouse tissues (24, 42). Restriction endonuclease digestions, agarose gel electrophoresis, Southern blot transfers, and hybridizations were performed as previously described (17). Each Southern blot was washed under nonstringent conditions for three times with 1 $\times$ SSCP (0.12 M NaCl, 15 mM Na₃C₆H₅O₇, and 20 mM NaPO₄-0.1% sodium dodecyl sulfate at 65°C).

Library construction and screenings. A subgenomic library was constructed with DNA from tumor A008 by digesting the DNA to completion with *SacI* and electrophoresing the material through a SeaKem LE agarose gel (FMC) in Tris acetate buffer (36) to size select DNA fragments (9 to 18 kb), which were isolated by freezing and phenol extraction (6). The fraction containing the desired somatically acquired retrovirus

was identified by Southern blot hybridization to aliquots of the fractions, and the appropriate fraction was ligated to *SacI*-digested, dephosphorylated Lambda Dash arms (Stratagene). From this subgenomic library of 40,000 plaques, 39 plaques were positive when hybridized with the gel-purified randomly primed ecotropic virus-specific *env* pEco insert. Eight phage were purified and screened for the appropriate sequences, and all contained the same 10.5-kb *SacI* cell DNA-proviral DNA junction fragment.

To molecularly clone the unrearranged genomic DNA at *Evi-3*, the p3-1 probe was used to screen a genomic library made of C57BL/6J DNA that was partially digested with *Sau3A1* cloned into Lambda Dash (Stratagene). A screen of 10⁶ plaques gave 8 positive plaques, and 4 of these were subsequently purified to give overlapping clones that spanned 25 kb of genomic DNA.

RNA isolation and Northern (RNA) analysis. RNAs were prepared from cell lines and C57BL/6J adult mouse tissues by using RNazol (Cinna/Biotex). RNAs were poly(A)⁺ selected twice, using oligo(dT) spin columns (Pharmacia). Five micrograms of poly(A)⁺ RNA was size fractionated on 1.2% formaldehyde gels (33) and blotted onto Zetabind (AMF Cuno). Northern blots were hybridized and washed by the method of Church and Gilbert (10).

Statistical analysis. Standard errors of the recombination frequencies from the results of the IB were determined as described by Green (18), using the computer program SPRETUS MADNESS. Gene order was determined by minimizing the number of multiple crossovers among the loci analyzed (pedigree analysis) (see reference 2 for a review) and confirmed by the maximum-likelihood analysis (5).

RESULTS

Identification of four potential common viral integration sites in AKXD B-cell lineage lymphomas. A total of 121 AKXD B-lineage tumors from 21 AKXD R1 strains were analyzed to identify tumors that contained single somatically acquired proviruses (17, 40). DNAs from the B-lineage lymphomas were digested with *PvuII* or *SacI* and subjected to Southern blot hybridization analysis with two AKR ecotropic murine leukemia virus *env* gene probes. One probe, pEco, specifically detects ecotropic proviruses (9), and the other, pAKV-5, hybridizes to both ecotropic and class I MCF proviruses (21). These two viral classes are causally associated with the majority of AKXD lymphomas analyzed (17, 40). Each probe detects a 3' proviral DNA-cellular DNA junction fragment in *PvuII*- or *SacI*-digested DNA. DNAs from B-cell lymphomas that contained single somatically acquired proviruses were also analyzed with two other restriction endonucleases, *BclI* and *EcoRI*, to confirm the presence of a somatically acquired provirus. If viral integration has occurred in the same genomic region, we would expect to see the cellular DNA-proviral DNA junction fragments of similar sizes in several different tumors. At least four different sites among the B-cell tumors analyzed had similar-size cell DNA-proviral DNA junction fragments with at least two of the four restriction endonucleases (data not shown), suggesting that these four sites represent common sites of retroviral integration. One of these potential sites was chosen for molecular cloning to confirm its status as a common integration site.

Molecular cloning of *Evi-3* sequences. A 10.5-kb *SacI* cellular DNA-proviral DNA junction fragment was cloned from a lymphomatous spleen containing a single somatically acquired provirus. Cellular DNA sequences located 3' of this ecotropic provirus were analyzed for the presence of repetitive se-

FIG. 1. (A) Restriction fragment map of the *Evi-3* proviral integration site and of representative AKV ecotropic and MCF class II retroviruses (8, 9). The black line below the map represents the unique-sequence p3-1 cellular DNA probe. The shaded line represents the retrovirus sequences. The long terminal repeat of the provirus is shown as a hatched box. The numbers below the figure show the restriction fragment sizes for the genomic preintegration site and the expected sizes after integration of an ecotropic provirus or an MCF class II provirus. Abbreviations for restriction endonucleases: S, *SacI*; B, *BclI*; K, *KpnI*; H, *HindIII*; X, *XbaI*; V, *EcoRV*; E, *EcoRI*. (B) *Evi-3* rearrangements in the AKXD-27 B-lineage lymphomas. *SacI*-digested tumor DNA from 13 AKXD-27 animals was Southern blotted and hybridized with the p3-1 probe. The germ line *SacI* fragment is 15.0 kb, and rearrangements of 10.5 kb are consistent with the integration of an ecotropic retrovirus, whereas the 6.6-kb fragments are consistent with the integration of a class II MCF retrovirus. Note that each of the retrovirus-induced rearrangements in AKXD-27 is in a B-lineage lymphoma.

quences by Southern filter hybridization (data not shown), and surprisingly, all of the 3' flanking DNA sequence was free of repetitive elements. Thus, a unique-sequence 4.6-kb *SacI*-*KpnI* fragment (p3-1; Fig. 1A) was subcloned into pBluescript KS+ (Stratagene). Spleen and lymph node tumor DNAs digested with *SacI* or *EcoRI* from 258 AKXD mice were analyzed for DNA rearrangements that would be consistent with the integration of a retrovirus, using the *Evi-3* probe. Eleven rearrangements were found in the AKXD tumor DNAs, showing that *Evi-3* represents a common site of retroviral integration (summarized in Table 1).

DNA rearrangements at *Evi-3* in AKXD B-lineage lymphomas. Each of the 11 tumors containing proviral insertions at *Evi-3* was classified as either pre-B or B cell by our histopatho-

logical and molecular analyses (Table 1). No rearrangements were found in T-cell, myeloid, or stem cell tumors. About 9% (11 of 121) of the pre-B- and B-cell lymphomas of the AKXD RI strains contained rearrangements at *Evi-3*. Note that the tumors were classified as pre-B cell or mature B cell on the basis of the molecular status of *IgH* (pre-B-cell) and *IgK* (B-cell) gene rearrangements. Thus, some of the tumors classified as mature B molecularly may not be expressing immunoglobulin on their cell surface and may not be accurately classified to the intermediate B-cell stages.

The frequency of *Evi-3* rearrangements in AKXD B-cell and pre-B-cell tumors varied markedly between different AKXD strains. Seventy percent (7 of 10) of the B- and pre-B-cell tumors from the AKXD-27 strain showed rearrangements at

TABLE 1. Distribution of *Evi-3* rearrangements^a

Mouse strain	No. of indicated tumor type ^b					
	Stem	Pre-B	B	T	Myeloid	Mixed ^c
AKXD-2		2	1/8	1		
AKXD-3	1	2	4	5		3
AKXD-6		1	1	11		
AKXD-7			5	4		1
AKXD-8			4	3		1
AKXD-9		2/6	1	5	1	
AKXD-10	1	1	10	3		
AKXD-11	1	2	6			
AKXD-12		1	3	8		
AKXD-13	1	1	10	2		2
AKXD-14		1	1/9	1		1
AKXD-15			5	3	1	
AKXD-16		5	6	2		3
AKXD-17		1		10		
AKXD-18			6	5		
AKXD-21				17		
AKXD-22		1	2	4		
AKXD-23			1	1	7	1
AKXD-24		2	2	9		
AKXD-26			2	14		1
AKXD-27		4/5	3/5	3		
CWD/Agl			12^d			
NFS/N ^e		1/6	0/1			

^a Cell type and strain distribution of *Evi-3* rearrangements in hematopoietic tumors from 21 AKXD strains, CWD/Agl, and retrovirally infected NFS mice.

^b Boldface numbers show tumors with rearrangements at *Evi-3* and then the total number of tumors screened in that category.

^c Includes tumors with mixed T- and B-cell characteristics.

^d These 12 CWD/Agl tumors have been classified by histopathological analysis and cell surface marker analysis (1). Nearly 100% of the tumors were positive for surface immunoglobulin, indicating that they are mature B cells.

^e Although these tumors are listed under mouse strains, they are actually cell line derivatives of primary tumors from NFS/N mice that were infected with Cas-Br-M retrovirus or derivatives (22) or were spontaneous tumors of ecotropic virus congenic NFS/N mice (15).

the *Evi-3* locus (Table 1; Fig. 1B). In contrast, of six other AKXD strains that developed predominantly B-lineage lymphomas, only three had tumors with rearrangements at *Evi-3*: the AKXD-9 strain had rearrangements in two of six pre-B cell tumors, the AKXD-2 strain had rearrangements in one of eight B-cell tumors, and the AKXD-14 strain had rearrangements in one of nine B-cell tumors.

DNA rearrangements at *Evi-3* in other B-lineage lymphomas. Seven cell lines established from retrovirus-induced hematopoietic tumors that arose in NFS/N or NFS/N ecotropic virus congenic mice (15, 31, 43), and that represent various B-cell lineages, were also analyzed for virus-induced rearrangements at the *Evi-3* locus. Two other lymphoma cell lines, SJL-4, and BALB-1427, derived from retrovirus-induced B-cell lymphomas (43), were also analyzed for rearrangements at *Evi-3*. In addition, DNAs from 19 cell lines derived by transformation with acute retroviruses carrying the *fos*, *ras*, *abl*, or *src* oncogene (23) were analyzed for rearrangements at *Evi-3*. Only the NFS-467 cell line from a retrovirus-infected NFS mouse revealed a rearrangement at *Evi-3*. No rearrangements were found in the cell lines established by infection with acutely transforming retroviruses.

Chromosomal mapping of *Evi-3*. To determine whether a gene or genes at *Evi-3* is a novel proto-oncogene, the chromosomal location of *Evi-3* was determined by IB mapping. The IB involving C57BL/6J and *M. spretus* has been analyzed for over 1,000 markers distributed over each mouse autosome and the X chromosome (see references 12 and 13 for reviews). The

FIG. 2. (A) Segregation of alleles in 154 (C57BL/6J $\times$ *M. spretus*)F₁ $\times$ C57BL/6J offspring. Each column represents the chromosome identified in the N₂ progeny that was inherited from the (C57BL/6J $\times$ *M. spretus*)F₁ female parent. Black boxes represent the presence of a C57BL/6J allele; white boxes represent the presence of an *M. spretus* allele. Numbers at the bottom show the total number of animals inheriting each type of chromosome. Locus symbols are given at the left. (B) Linkage map of the proximal end of mouse chromosome 18 showing the map locations of *Evi-3* and flanking markers. The loci are aligned at the centromere at *Kns* (26), and the map distances (in centimorgans) determined in our IB are given at the immediate left. Loci that have been mapped in humans are underlined, and the human map locations are given at the far left (26, 37).

markers analyzed include many proto-oncogenes, growth factors and their receptors, transcription factors, and common sites of viral integration. DNAs from C57BL/6J and *M. spretus* were digested to completion with several restriction endonucleases and analyzed by Southern blot analysis for informative restriction fragment length polymorphisms (RFLPs), using the p3-1 probe. A 4.8-kb fragment was found in *Pst*I-digested C57BL/6J DNA, whereas a 6.8-kb fragment was found in *Pst*I-digested *M. spretus* DNA. Thus, the segregation of this RFLP identifying *Evi-3* was monitored in the offspring from the IB. Linkage was detected to markers on the proximal end of mouse chromosome 18, including *Kns*, *N-cad*, *Tpl-2*, and *Ttr* (26, 37). Between 161 and 205 animals were analyzed for each linked marker. To give gene order, the segregation distribution patterns of RFLPs identifying *Evi-3*, *Kns*, *N-cad*, *Tpl-2*, and *Ttr* were analyzed in each of 154 offspring from the IB (Fig. 2A). After determining gene order, we analyzed up to 188 animals for each locus in pairwise combinations to give recombination fractions between loci as *Kns*-0/188-*Tpl-2*-4/188-*Evi-3*-1/187-*N-cad*-3/161-*Ttr*. The recombination distances expressed in centimorgans $\pm$ standard error are (*Kns*, *Tpl-2*)-2.1 $\pm$ 1.1-*Evi-3*-0.5 $\pm$ 0.5-*N-cad*-1.9 $\pm$ 1.1-*Ttr*. Thus, *Evi-3* maps to a distinct location on chromosome 18, indicating that it identifies a novel locus (Fig. 2B).

Location and orientation of proviral insertions at *Evi-3*. To determine the location, type, and orientation of each of the proviruses within this region, digestion with the restriction endonucleases *Sac*I, *Eco*RV, *Bcl*I, *Eco*RI, *Kpn*I, and *Xba*I and subsequent Southern blot analyses were used to examine brain, spleen, and lymph node DNA from the animals with proviral insertions detected with *Sac*I and *Eco*RI previously. Brain DNA was used as a control, since it is rarely infiltrated with tumor tissue (17). Whenever possible, two lymphomatous tissues from each animal were examined to demonstrate the monoclonality of the tumor. *Sac*I and *Xba*I cleave asymmetrically within ecotropic proviruses; therefore, these endonucleases were used to determine the orientations of proviruses within the locus (9). *Kpn*I and *Eco*RV restriction sequences are found within the proviral long terminal repeats; therefore,

FIG. 3. Positions and orientations of integrated proviruses in the *Evi-3* locus. Horizontal arrows indicate the proviral orientation from a 5'-to-3' direction of transcription in each tumor, and the vertical arrows extending from these tumors to the restriction map indicate locations of the proviral integration sites within the genomic region. These sites mapped to two spots on each side of the *KpnI* site. The type of provirus integrated into *Evi-3* is given in parentheses as AKV ecotropic (E), MCF (M), or Cas-Br-M ecotropic (C). A 2.7-kb region surrounding the proviral integrations is magnified in the diagram above, showing the locations of the rare-cutting endonucleases *BssHII*, *EagI*, *NaeI*, and *SacI*. These endonuclease sites define a CpG island that extends over 2 kb of DNA, and this CpG island is represented on both restriction maps as a hatched box. Flanking unique-sequence cellular DNA probes 1 and 2 are represented by black lines below the map. Restriction endonuclease abbreviations are as given in the legend to Fig. 1.

these endonucleases were used to more precisely determine the locations of the proviruses. *EcoRI* and *BclI* do not cleave within ecotropic proviruses and therefore were used to determine the type of proviral rearrangement as well as the structural integrity of the provirus.

A restriction fragment map of the unrearranged locus was generated from overlapping genomic clones that spanned approximately 25 kb. The digests of brain and tumor DNAs were compared with the genomic restriction fragment maps. Notably, *SacI* and *XbaI* digests revealed similar-size restriction fragments in all but three of the tumors with proviral insertions at *Evi-3*, consistent with the introduction of a provirus containing a *SacI* or *XbaI* recognition sequence in the same genomic site and in the same orientation in multiple independent tumors (Fig. 1B; summarized in Fig. 3). The *KpnI* digests revealed that the proviruses were tightly clustered on each side of a genomic *KpnI* site and within a 200-bp genomic region (Fig. 3). The *EcoRI* and *BclI* digests revealed that all but two of the animals gave restriction patterns consistent with those of full-length ecotropic proviruses (Fig. 3). The proviruses in the remaining tumors, A153 and A094, were subsequently classified as class II MCF proviruses on the basis of the locations of *SacI*, *XbaI*, *BclI*, and *EcoRI* restriction sites within the provirus (Fig. 1B and data not shown). Each of these class II MCF proviruses was oriented in the same transcriptional direction as the ecotropic proviruses.

The provirus in tumor A174 was oriented in the opposite transcriptional direction as the other proviruses. Analysis of the *EcoRV* restriction fragments in DNAs from this tumor

suggests that it contains a small deletion or rearrangement of the proviral long terminal repeat. Notably, *EcoRI* and *BclI* fragment sizes were consistent with the existence a full-length ecotropic provirus in this tumor. These results suggest that the deletion is likely to be less than 500 bp, but this provirus has not been characterized further.

Digests of the lymphomatous lymph node DNA from animal A188 showed that two proviral insertions had occurred at *Evi-3*, whereas the lymphomatous spleen from this animal had only one proviral insertion. Since the germ line unrearranged band was nearly absent in the lymphomatous node (data not shown), our interpretation of this result is that a second provirus has integrated into the other *Evi-3* allele during the expansion of tumor cells in the lymph node. One of these proviruses was full length, whereas the other appeared to have a 1.5-kb deletion. These results also indicate that the tumors in the spleen and lymph node in animal A188 are not monoclonal.

The NFS-467 cell line with a proviral integration at *Evi-3* was also analyzed further. The proviral rearrangement in the B-cell line NFS-467 is consistent with integration in the same genomic site and is in the same 5'-to-3' orientation as in the proviruses integrated at *Evi-3* in the AKXD tumors. This tumor arose in an NFS mouse infected with a recombinant defective Cas-Br-M retrovirus (Cas NS-1) carrying the *v-cbl* oncogene. The defective virus was pseudotyped with replication-competent helper Cas-Br-M ecotropic virus (22, 32). The restriction map is consistent with the map of the helper virus, not of the Cas NS-1 retrovirus carrying *v-cbl*.

***Evi-3* lies within a CpG island.** In addition to the standard restriction endonucleases mentioned above, the bacteriophage lambda clones containing the genomic preintegration region were digested with several rare-cutting endonucleases (*NotI*, *SacII*, *EagI*, *NaeI*, and *BssHII*) that specifically cleave CpG sequences. A cluster of sites for these rare-cutting endonucleases identifies the presence of CpG islands that are associated with the 5' ends of mammalian genes (4). The restriction endonuclease digestions of the bacteriophage lambda clones with the rare-cutting enzymes revealed the presence of a putative CpG island within the lambda phage clones. Each of the proviral insertions at *Evi-3* occurred within this CpG island (Fig. 3).

Transcripts from *Evi-3* are found in B cells. Because of the extreme clustering of proviruses and the extent of unique-sequence DNA in the region, we generated unique-sequence probes that flanked and spanned the proviral integration site to identify transcripts. Each of these unique-sequence fragments (probes 1 and 2; Fig. 3) was examined for the presence of evolutionarily well-conserved sequences that might suggest the presence of a transcribed region. A zoo blot containing DNAs from many different vertebrate and nonvertebrate species was hybridized with each of the unique-sequence probes. The results of this analysis showed that probes 1 and 2 were evolutionarily well conserved, for they hybridized to DNAs from mouse, rat, mink, hamster, human, sheep, dog, cat, turkey, and chicken (data not shown).

The permanent NFS-467 cell line serves as a valuable source of mRNA and protein for identifying alterations associated with retroviral integration in a gene or genes at *Evi-3*, since the AKXD tumors containing *Evi-3* rearrangements were not saved for mRNA or protein analyses. Thus, we examined the NFS-467 cell line that contains a proviral insertion at *Evi-3* for transcripts. We also examined three other B-lineage tumor cell lines (NFS-112, NFS-70, and NFS-1.0) as well as adult mouse organs, including brain, thymus, spleen, kidney, testis, and heart, for transcripts. Probes 1 and 2 were hybridized individ-

FIG. 4. Northern blot of twice-selected poly(A)⁺ RNA from four NFS cell lines hybridized to probe 1. Note the 14.0-kb transcript in all four cell lines and the 7.6-kb transcript in the NFS-112, NFS-70, and NFS-467 cell lines. A faint 6.0-kb transcript was also detected in all cell lines. The NFS-467 cell line has a retroviral integration at *Evi-3*. The NFS-70 cell line represents a progenitor B stage of development, the NFS-112 line represents a large precursor B stage, the NFS-467 line represents a small B-lymphocyte stage, and the NFS-1.0 cell line represents a follicular center cell B-lymphocyte stage (15, 23, 43). As a control for the amount of mRNA in each lane, the Northern blot was stripped and rehybridized with *vav*, a gene expressed ubiquitously in hematopoietic cells (shown beneath the Northern blot) (7). As a second control, the blot was stripped and rehybridized to glyceraldehyde phosphate dehydrogenase, a housekeeping enzyme that has a relatively constant level of mRNA through all parts of the cell cycle (46), and similar results were obtained (data not shown). Note that the NFS-467 lane contains much less RNA than the other lanes do.

usually to Northern blots of twice-selected poly(A)⁺ mRNA from each of these sources. Probe 1 detected transcripts in all of the B-cell lines (Fig. 4) as well as in adult mouse heart (data not shown). Note that probe 1 detects a 14.0-kb transcript in all of the cell lines examined. Probe 1 also detects a 7.6-kb transcript in the NFS-467, NFS-112, and NFS-70 cell lines but not in the NFS-1.0 cell line. Therefore, *Evi-3* is transcriptionally active in several B-lineage tumor cell lines.

DISCUSSION

AKXD mice succumb to a number of different hematopoietic diseases that are retrovirally induced. However, unlike the lymphomatous AKR/J parent, which dies mainly of T-cell lymphoma, many of the AKXD RI strains die of B-cell lymphoma. We have used these B-cell lymphomas to molecularly clone a novel common site of retroviral integration in mouse AKXD B-cell lymphomas called *Evi-3*. Proviral integrations at *Evi-3* were found in the DNAs of 11 of 258 AKXD tumors. Each of the rearrangements occurred in B-lineage cells and occurred in 9.1% of the pre-B and B-cell lymphomas in the AKXD strains. No rearrangements were found in either myeloid or T-cell tumors. Notably, 19% of the pre-B-cell lymphomas but only 5.5% of the B-cell lymphomas of the AKXD strains had proviral insertions at *Evi-3*.

The high incidence of B-lineage lymphomas with proviral insertions at *Evi-3* in strain AKXD-27 strongly suggests that

the genetic background in this strain is favorable for lymphoma development when a retrovirus has integrated at *Evi-3*. Previous studies have shown that several unlinked murine loci have an influence on tumor cell type and retrovirus content in the AKXD RI strains (17). Moreover, proviral insertions at a common site of retroviral integration, *Evi-1*, and the development of myeloid tumors are influenced by the genetic background of strain AKXD-23 (41). It is possible that in the AKXD RI strains, loci that have an influence on the development of tumors and the specific retroviral integration sites seen in the tumors have segregated. Tumorigenesis is a multistep process, and retroviruses can integrate into many sites in the genome. However, only those sites that will give the cells a growth advantage after alteration by proviral insertion will be represented in the monoclonal expansion of tumor cells. Examining the other AKXD strains that developed B-cell lymphomas for additional common sites of retroviral integration will show whether strain background plays a large role in the common proviral insertions seen in the tumors or whether the strain background is particularly specific to tumor development in strain AKXD-27 when a retrovirus has integrated at *Evi-3*.

Proviral insertions at *Evi-3* are not restricted to the AKXD tumors. A proviral insertion was detected in a cell line derived from a pre-B-cell lymphoma that occurred in retrovirally infected NFS mice (NFS-467). NFS-467 was derived by infection with a Cas-Br-M recombinant virus (NS-1) that carries the *v-cbl* oncogene. Because of known cooperativity among other oncogenes and common sites of retroviral integration (47, 51), we wanted to know whether *Evi-3* represented a secondary site necessary for tumor development in cooperation with the *v-cbl* oncogene. We screened four other primary B-cell lymphomas that had arisen after infection by NS-1, but none had viral integrations at *Evi-3* (28). We also screened all of the AKXD tumors for retroviral integrations in *c-cbl*, and no rearrangements were found (28). Although *Evi-3* and *v-cbl* may cooperate in tumor development in the lymphoma that gave rise to the NFS-467 cell line, these results suggest that *Evi-3* and *cbl* are not often involved in cooperative events. It will be interesting to determine whether an activated *v-cbl* is present in NFS-467.

The *Evi-3* locus maps to the proximal end of mouse chromosome 18 and is distinct from proto-oncogene and common viral integration site loci identified previously in the mouse. Thus, *Evi-3* is a novel common site of retroviral integration that is implicated in the development of mouse B-cell lymphomas. The map location of *Evi-3* in the mouse lies between a region of homology with human chromosome 18q, and a region of homology with human chromosome 10p (13, 26). Therefore, we predict that *Evi-3* will map to either 18q or 10p in the human. Human chromosome 18q is involved in chromosomal aberrations in many human tumors, including chronic lymphocytic leukemia, non-Hodgkin's lymphoma, and synovial sarcoma (30, 34, 44). Patients with chromosome 18q-syndrome, which involves a deficiency of the 18q21-qter region (29), have low levels of immunoglobulin A (20), indicating that one or more genes concerned with the synthesis of immunoglobulin lies within this region. Human chromosome 18q is also involved in the Lynch cancer family syndrome, which includes cancers of the colon and endometrium (35). Human chromosome 10p may be involved in hereditary multiple endocrine neoplasia type II (49) and is frequently affected by chromosomal aberrations in human adult T-cell leukemia (48) and acute nonlymphocytic leukemia (3). Thus, *Evi-3* may be involved in one of these human diseases.

Each of the proviral insertions at *Evi-3* is in a 200-bp region

that lies within a CpG island. CpG islands are regions of DNA with a high G+C content and a high frequency of CpG dinucleotides relative to the bulk genome (16). It is likely that CpG islands are maintained in generally CpG-depleted vertebrate genomes because of their hypomethylated status (4, 16). CpG islands are often associated with the 5' ends and promoter regions of mammalian genes (4) and often serve as landmarks to search for transcripts. The CpG island at *Evi-3* was originally identified as a cluster of restriction sites for endonucleases that specifically cleave CpG sequences. Its status as a hypomethylated island was subsequently confirmed by pulsed-field gel analysis of mouse genomic DNA, using methylation-sensitive restriction endonucleases (data not shown). Moreover, genomic sequence analysis of the *Evi-3* region reveals additional restriction endonuclease recognition sites for CpG sequences and confirms the extreme G+C richness (data not shown). It is noteworthy that the proviruses identified to date are clustered within 200 bp at *Evi-3*. This clustering is not spread out over the entire CpG island; instead, it lies in a small region within the island. This extensive clustering has not generally been observed for other common sites of retroviral integration (50). Our original screen for proviral integrations scanned 15 kb of genomic DNA for rearrangements; however, additional probes were used to screen an additional 10 kb of genomic DNA, and no further rearrangements were observed (25).

The extreme clustering of proviruses and their orientation mainly in a single transcriptional direction are consistent with the suggestion that these proviruses have oriented in the 5' end of a gene and possibly within exon sequences. This arrangement of proviruses is frequently seen for a promoter insertion mechanism of alteration of flanking genes (50). The observation of proviral insertions at *Evi-3* within a CpG island is also consistent with probable promoter insertion. These results directed our search for genes at the *Evi-3* locus.

Our studies show that a gene at *Evi-3* is expressed in four NFS B-cell tumor cell lines. These cell lines represent B cells fixed at different stages of B-cell differentiation: the NFS-70 cell line represents a progenitor B stage of development, the NFS-112 line represents a large precursor B stage, the NFS-467 line represents a more mature small B-lymphocyte stage, and the NFS-1.0 cell line represents a mature follicular center cell B-lymphocyte stage (15, 23, 43). It is interesting that the 7.6-kb transcript is present in the early stages of B-cell development (NFS-70 and NFS-112) but not at the more mature stage (NFS-1.0), indicating that the expression of this transcript may be regulated during the development and differentiation of B cells. Although the NFS-1.0 and NFS-467 cell lines are at similar (although not identical) stages of B-cell development, the NFS-467 cell line expresses the 7.6-kb transcript from *Evi-3* whereas the NFS-1.0 cell line does not. The retroviral insertion at *Evi-3* in NFS-467 does not appear to cause overexpression of transcripts; however, it is possible that the integrations have allowed misexpression of a transcript that is required for proper cell differentiation (Fig. 4 and unpublished observations). Thus, the proviral insertions may cause *Evi-3* transcripts to be expressed inappropriately, leading to the development of B-cell lymphoma.

The identification of *Evi-3* demonstrates that the AKXD B-lineage lymphomas are a valuable resource for identifying novel common sites of retroviral integration that probably represent proto-oncogenes involved in B-cell lymphomagenesis. The frequency of proviral integration at *Evi-3* (9.1%) in the AKXD B-cell lymphomas suggests that other common sites of viral integration that are involved in B-cell lymphomagen-

esis will be found in these tumors. These sites may also represent novel putative proto-oncogenes.

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