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Characterization of Somatically Acquired Ecotropic and Mink Cell Focus-Forming Viruses in Lymphomas of AKXD Recombinant Inbred Mice

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The DNA of lymphomas from 12 AKXD recombinant inbred mouse strains was analyzed to determine the presence of somatically acquired ecotropic and mink cell focus-forming proviruses. Mink cell focus-forming proviruses were associated primarily with T-cell lymphomas, whereas ecotropic proviruses were associated with lymphomas of B-cell and myeloid lineages. A model based on the results is proposed to explain the variation in lymphoma types observed in different AKXD strains.

The AKXD recombinant inbred (RI) strains represent a valuable RI family for identifying and studying genes that affect susceptibility to lymphomas. These strains were derived by crossing inbred strains AKR/J and DBA/2J, which differ dramatically in their disease incidence. AKR/J is a prototypic high lymphoma strain; most animals develop T-cell lymphomas between 7 and 9 months of age (29). DBA/2J is a weakly lymphomatous strain (33).

The high incidence of lymphomas in AKR/J mice is associated with the expression of two endogenous ecotropic murine leukemia virus loci, *Emv-11* and *Emv-14* (12, 19). DBA/2J mice also carry an endogenous ecotropic proviral locus, *Emv-3*; however, this provirus has a small mutation that inhibits its expression (5). Mink cell focus-forming (MCF) viruses have also been identified in the thymic DNA of preleukemic and leukemic AKR/J mice (12, 14, 15). MCF viruses are not found in the germ line but appear to be generated by multiple recombinations involving an ecotropic virus and at least two nonecotropic viruses (24). One recombination takes place between an ecotropic virus and a xenotropic virus to generate a recombinant virus with xenotropic sequences in the U3 region of the viral long terminal repeat (LTR). The xenotropic virus donating the U3 sequences has been mapped to chromosome 1 and is present in the germ line of both the AKR/J and DBA/2J strains (17, 24). Xenotropic virus sequences are also found in the gp70 region of the MCF envelope (*env*) gene. The gp70 sequences are not derived from the same xenotropic virus that donates the U3 region, and the number of proviruses that may donate the *env* region is unknown. In contrast to the endogenous ecotropic viruses of AKR/J mice, which are weakly leukemogenic, MCF viruses are highly leukemogenic and are thought to be causally associated with the development of T-cell lymphomas in this strain. MCF viruses are thought to exert their oncogenic effect by integrating near and altering the expression of cellular proto-oncogenes (6, 8, 11, 22, 30).

Thirteen AKXD RI strains were recently studied to determine their susceptibility to lymphomas (23). Twelve of 13 strains analyzed had a high incidence of lymphoma development. However, the average age of onset of lymphomas varied among the different strains, suggesting that the AKXD strains have segregated for several loci that affect lymphoma susceptibility.

The AKXD lymphomas were classified according to cell type by histopathological data and detailed molecular analyses. Molecularly, lymphomas were categorized by rearrangements detected in immunoglobulin heavy (IgH) and kappa [IgG(κ)] light chain genes as well as in genes encoding the β chain of the T-cell receptor (T_β). The molecular data were particularly useful for identifying lymphomas of potential stem cell origin (lymphoms containing no gene rearrangements), pre-B-cell lymphomas [IgH but not IgG(κ) or T_β rearrangements], and phenotypically mixed lymphomas (lymphomas displaying characteristics of two different cell lineages). Two strains (AKXD-6 and AKXD-17) died predominantly from T-cell lymphomas, similar to the AKR/J progenitor strain. Three strains (AKXD-13, AKXD-14, and AKXD-27) died predominantly from B-cell lymphomas, and one strain (AKXD-23) died predominantly from myeloid tumors. The remaining six strains (AKXD-3, AKXD-7, AKXD-9, AKXD-15, AKXD-18, and AKXD-22) were susceptible to both T- and B-cell lymphomas (23) (Table 1). The results indicate that the AKXD strains have also segregated multiple loci that affect lymphoma type.

MCF murine leukemia viruses have been causally associated with T-cell lymphomas (6, 8, 11, 22, 30), whereas ecotropic viruses have been associated with B-cell and myeloid lymphomas (35). Both the tissue tropism and oncogenicity of several murine leukemia viruses have been shown to reside in sequences within the LTRs (9, 18, 21, 26, 34). These results suggest a model to account for the variation in lymphoma types observed in different AKXD strains. Our model predicts that, during inbreeding, the AKXD strains segregated several genes, including endogenous proviral loci, which affect the nature of recombinant proviruses formed in preleukemic animals. Different recombinant proviruses may have different oncogenic potential. To test this prediction, we have now analyzed the ecotropic and MCF proviral DNA content of the AKXD lymphomas.

For these experiments, DNAs from brain and two lymphomatous tissues (usually spleen, lymph node, or thymus) of each animal were analyzed. Brain DNA served as a control to discriminate proviruses carried in the germ line

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TABLE 1. Correlation between somatically acquired proviral DNA content and *Rmcf* genotype in AKXD lymphomas

AKXD strain[a]	Mean age of onset of lymphomas (days, ± SEM)	*Rmcf*[b]	Predominant lymphoma type (%)[c]	Somatically acquired proviruses[d]		
				Ecotropic (69.3%)	MCF (6.4%)	Both (24.3%)
17	285 ± 17	s	T (90.9)	0/11	4/11	7/11
6	310 ± 23	s	T (84.6)	2/13	3/13	8/13
14	339 ± 17	s	B (83.3)	12/12	0/12	0/12
27	345 ± 31	s	B (76.9)	10/13	2/13	1/13
18	380 ± 21	r	T, B (45.5, 54.5)	5/11	0/11	6/11
13	380 ± 14	r	B (68.8)	14/16	0/16	2/16
3	412 ± 26	r	T, B (33.3, 40.0)	15/15	0/15	0/15
22	440 ± 39	r	T, B (57.1, 42.9)	6/7	0/7	1/7
23	445 ± 16	s	M (70.0)	9/10	0/10	1/10
9	448 ± 20	r	T, B (38.5, 54.9)	8/13	0/13	5/13
15	471 ± 25	r	T, B (33.3, 55.6)	9/9	0/9	0/9
7	486 ± 22	r	T, B (40.0, 50.0)	7/10	0/10	3/10

[a] Strains are listed in order of decreasing lymphoma susceptibility as determined by the mean age of onset of lymphomas in days (23).
[b] MCF resistance gene: r, resistant; s, sensitive.
[c] Tumors were classified by histopathological data and molecular analyses as described before (23): T, T cell; B, B cell; M, myeloid. Numbers within parentheses represent the percentage of tumors within a strain belonging to a particular cell lineage.
[d] Number of lymphomas containing particular proviral class/total number of animals within an AKXD strain analyzed. Percentage given reflects number of lymphomas containing particular proviral class of 140 (number of AKXD lymphomas containing somatically acquired proviruses).

versus those acquired somatically. By analyzing two lymphomatous tissues from each animal, we were able to determine whether the tumors were monoclonal in origin.

DNA (15 μg per tissue) was digested to completion with *Pvu*II, electrophoresed through 0.6% agarose gels, transferred to nitrocellulose, hybridized with a nick-translated probe (25), and washed as described previously (19). The two probes used, pEC0 and pAKV-5, were derived from the AKR ecotropic virus *env* gene. pEC0 specifically detects ecotropic proviruses (3), whereas pAKV-5 hybridizes with ecotropic and class I oncogenic MCF proviruses (14). Both probes detect 3′ proviral DNA-cellular DNA junction fragments in *Pvu*II-digested DNAs. For those DNAs that did not appear to contain somatic proviruses, a second analysis involving *Sst*I digestion (*Sst*I also produces detectable 3′ proviral DNA-cellular DNA junction fragments) was performed to identify somatic proviral fragments that might have comigrated with one of the endogenous ecotropic proviral DNA fragments after digestion with *Pvu*II. Representative Southern blot analysis of *Pvu*II-digested DNAs from three strains, AKXD-6, AKXD-13, and AKXD-7, is shown in Fig. 1 and 2. Lymphomas from several strains, for example, AKXD-6, usually contained both somatic ecotropic and MCF proviruses (Fig. 1). The bars indicate the location of endogenous ecotropic proviral sequences. Unlike strain AKXD-6, other strains, for example, AKXD-13 and AKXD-7, usually had lymphomas containing only somatic ecotropic proviruses (Fig. 2). Few tumors contained only somatic MCF proviruses (Table 1).

Of 179 lymphomas analyzed, 140 (78.2%) contained somatically acquired proviruses (Table 1). Lymphomas not containing somatic proviruses may represent polyclonal tumors, spontaneous nonvirally induced tumors, or tumors containing proviral sequences not detectable with the hybridization probes used. Lymphomas lacking somatically acquired proviruses were not analyzed further. Most (82.9%) of the 140 lymphomas containing somatic proviruses appeared to be monoclonal in origin, based on the fact that the same pattern of somatically acquired proviruses was observed in the DNA of multiple organs of individual animals (Fig. 1 and 2; data not shown). In contrast to AKR/J lymphomas, which all contain somatic MCF proviruses, only 43 (30.7%) of the AKXD lymphomas contained MCF proviruses (Table 1). Most of these tumors (34 of 43 lymphomas; 79.1%) also contained somatically acquired ecotropic proviruses. Ninety-seven (69.3%) lymphomas contained only somatically acquired ecotropic proviruses (Table 1).

By using molecular probes specific for B and T cells, the

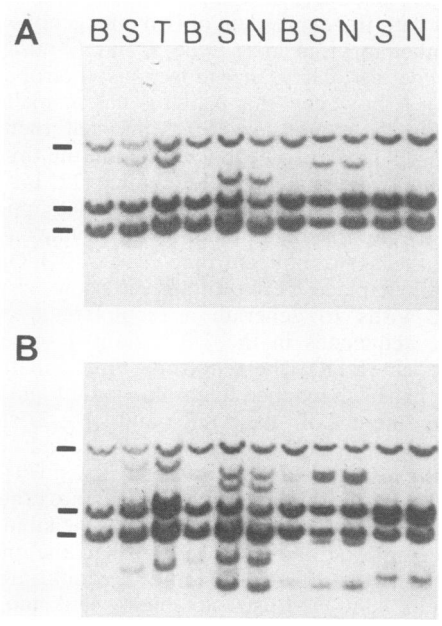

FIG. 1. Characterization of somatically acquired proviruses in lymphomas of AKXD-6 mice. High-molecular-weight DNA was extracted from the brain (B) and at least two lymphomatous tissues of each animal (S, spleen; N, lymph node; T, thymus). DNA (15 μg per lane) was digested with *Pvu*II, submitted to electrophoresis through a 0.6% agarose gel, and transferred to nitrocellulose as described in the text. (A) The filters were first hybridized with a nick-translated ecotropic virus-specific envelope probe, pEC0 (Eco probe) (3, 25). (B) After autoradiography, the filters were erased and hybridized with an envelope probe, pAKV-5 (MCF probe), that hybridizes to ecotropic viruses as well as class I oncogenic MCFs (14). Bars indicate endogenous ecotropic proviruses.

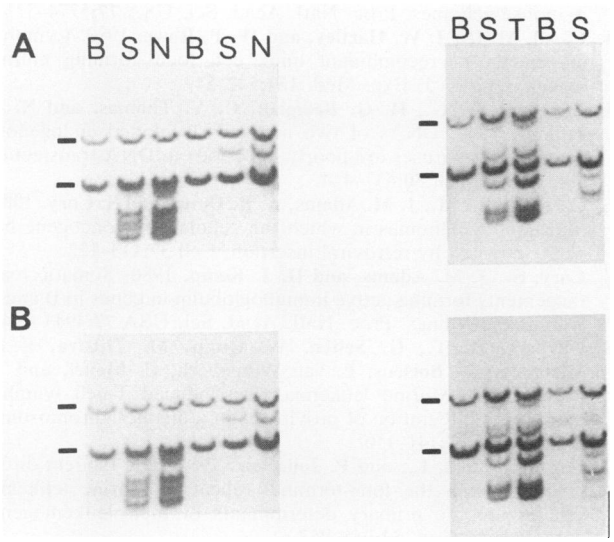

FIG. 2. Characterization of somatically acquired proviruses in lymphomas of AKXD-13 and AKXD-7 mice. Proviruses were identified as described in the legend to Fig. 1. (A) Eco probe; (B) MCF probe.

somatically acquired proviruses could be associated with specific lymphoma types. MCF proviruses were predominantly associated with T-cell lymphomas (Tables 1 and 2). Of 43 lymphomas identified containing MCF proviruses, 38 (88.4%) were classified as being of T-cell origin based upon rearrangements in the T_β locus. Four of the five non-T-cell lymphomas containing MCF proviruses were classified as pre-B-cell lymphomas based upon rearrangements in the IgH locus. However, because immunoglobulin H rearrangements are not restricted to B cells (7, 10, 16, 20, 27), these tumors may have been misclassified and may actually be of T-cell origin. MCF proviruses were identified in all of the T-cell lymphomas obtained from AKXD-17 and AKXD-6 mice which, similar to the AKR/J progenitor strain, die predominantly of T-cell lymphomas. Of 33 T-cell lymphomas from strains other than AKXD-17 and AKXD-6, 17 also contained MCF proviruses. However, 16 T-cell lymphomas contained only somatically acquired ecotropic proviruses.

Although MCF proviruses were associated with T-cell lymphomas, ecotropic proviruses were associated with stem cell, pre-B-cell, B-cell, and myeloid lymphomas (Table 2). These results are consistent with the model proposed previously to account for the variability in the types of lymphoma observed in different AKXD strains. At present, we do not know whether the ecotropic proviruses identified in non-T-cell lymphomas are recombinant proviruses. Some of these proviruses may have undergone recombination outside the regions probed in Southern blots (Fig. 1 and 2), for example, within the LTR sequences. The association of somatic ecotropic proviruses with lymphomas of many diverse lineages (including T-cell lymphomas lacking MCF proviruses) could be explained by such recombinations. Experiments are in progress to test this possibility.

Several genetic loci have been identified in mice that can act in *trans* to affect virus replication. The AKR/J and DBA/2J strains differ at one of these loci, the MCF resistance gene, *Rmcf* (13). *Rmcf* is a dominant gene found on mouse chromosome 5 that affects susceptibility of cells to MCF infection (13) and is thought to act by viral interference (1, 13). Mice carrying the resistant allele (*Rmcf*^{r}) are 30- to 100-fold less susceptible to MCF viral infection than mice carrying the sensitive allele (*Rmcf*^{s}). DBA/2J mice carry the *Rmcf*^{r} allele, whereas AKR/J mice carry the *Rmcf*^{s} allele (13). The AKXD strains represent a rare opportunity to study the effect of the *Rmcf* locus on lymphomagenesis.

The reduced number of somatic MCF proviruses found in AKXD lymphomas can, in part, be attributed to the presence of the resistant *Rmcf*^{r} allele. Large numbers of MCF provirus-containing lymphomas were identified in only two strains, AKXD-17 and AKXD-6 (Table 1). Both strains are homozygous for the *Rmcf*^{s} allele. Ecotropic proviruses were the predominant provirus found in strains homozygous for the *Rmcf*^{r} allele (AKXD-13, AKXD-3, AKXD-22, and AKXD-15) (Table 1). However, several strains (AKXD-18, AKXD-9, and AKXD-7) produced tumors containing MCF proviruses, even though they were homozygous for the *Rmcf*^{r} allele. This result is not unusual, since *Rmcf*^{r} reduces, but does not eliminate, susceptibility to MCF infection (13). Three AKXD strains, AKXD-14, AKXD-17, and AKXD-23, carry the *Rmcf*^{s} allele; however, few somatic MCF proviruses were identified in their lymphomas (Table 1). One interpretation of these results is that a single endogenous provirus, carried by AKR/J but not by DBA/2J mice, is responsible for donating the majority of nonecotropic proviral sequences to the class I MCF *env* gene. A single xenotropic provirus has been shown to donate the U_3 LTR sequences to class I MCF proviruses (24); therefore, it may be possible that a single endogenous provirus is also responsible for donating the *env* sequence to MCF proviruses.

AKXD lymphomas contained relatively few somatic proviruses (3.3 proviruses per tumor) compared with AKR/J lymphomas (8.7 proviruses per tumor) (14). Furthermore, many lymphomas (including ones of pre-B-cell, B-cell, T-cell, and myeloid cell lineages) contained only a single detectable somatic provirus. Slightly more MCF proviruses (3.6 proviruses; average for tumors containing MCF or ecotropic and MCF proviruses) than ecotropic proviruses (2.4 proviruses; average for tumors containing only ecotropic or ecotropic and MCF proviruses) were identified per tumor. Unexpectedly, about the same average number of MCF proviruses were present in MCF provirus-containing lymphomas from AKXD strains homozygous for the *Rmcf*^{s} or *Rmcf*^{r} allele. The reason for this finding is unclear. Nevertheless, the finding of fewer somatically acquired MCF proviruses in AKXD lymphomas (regardless of *Rmcf* genotype) relative to AKR/J lymphomas suggests that the

TABLE 2. Correlation between lymphoma type and somatically acquired proviral DNA content in AKXD lymphomas

Lymphoma type[a]	Somatically acquired proviruses[b]		
	Ecotropic	MCF	Both
Stem cell	2/2	0/2	0/2
Pre-B cell	14/18	1/18	3/18
B cell	48/49	0/49	1/49
T cell	16/54	8/54	30/54
Myeloid	9/9	0/9	0/9
Mixed cell			
T, B	6/6	0/6	0/6
T, M	1/1	0/1	0/1
B, M	1/1	0/1	0/1

[a] Tumors were classified by histopathological data and molecular analyses as described before (23).

[b] Number of lymphomas containing particular proviral class/total number of lymphomas analyzed.

AKXD strains have segregated loci (other than *Rmcf*) that affect susceptibility to MCF virus infection.

The AKXD lymphomas were initially only analyzed for somatically acquired ecotropic and class I oncogenic MCF proviruses. The AKXD lymphomas were not screened for the presence of class II MCF proviruses. Both class I and class II MCF proviruses are *env* gene recombinants; however, only class I MCF proviruses have also undergone recombination in the U3 region of the viral LTR (32). Also, class I MCF proviruses retain ecotropic sequences within the N terminus of the p15E *env* region (2, 14, 24). Class I MCF proviruses have been isolated from highly lymphomatous strains, such as AKR/J and C58/J, and they accelerate the development of lymphomas when injected into susceptible hosts. Class II MCF proviruses have been isolated primarily from low leukemic strains and are usually nonpathogenic (4, 28). However, the recent demonstration that class II MCF proviruses may be causally associated with the development of spontaneous nonthymic lymphomas in CWD mice (31) prompted us to examine the AKXD lymphomas for the presence of class II MCF proviruses.

Potential class II MCF proviruses were identified by Southern blot analysis, using two probes: (i) a U3 probe, designated U3LTR, that is specific for ecotropic virus LTRs (24) (this U3 region is retained by class II MCF proviruses); and (ii) a probe, pAKV-3, from the 3′ region of the *pol* gene (14) (this region is retained by some class II MCF proviruses). Few fragments were identified with the U3 LTR or pAKV-3 probe that were not already identified by using the ecotropic and class I MCF virus-specific probes, indicating that class II MCF proviruses are not causally associated with most AKXD lymphomas (D. Gilbert, N. Jenkins, and N. Copeland, unpublished data). Of course, the AKXD lymphomas may contain other families of somatically acquired proviruses that were not detectable with the probes used in these studies.

A large panel of lymphomas, representing many different cell lineages, has been identified from the initial characterization of the AKXD RI strains. The AKXD lymphomas will be useful for identifying known cellular oncogenes that are activated by viral integration. In addition, detailed molecular analysis of AKXD lymphomas that contained only one somatically acquired provirus may aid the identification of new common sites of viral integration that represent previously undiscovered cellular proto-oncogenes.

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