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Association of Recombinant Murine Leukemia Viruses of the Class II Genotype with Spontaneous Lymphomas in CWD Mice

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We determined the phenotype and genotype of murine leukemia viruses associated with the development of spontaneous nonthymic lymphomas in the high-leukemia mouse strain CWD/J. By T_1 oligonucleotide fingerprint analysis of the viral RNA, the ecotropic viruses recovered from the spleen or thymus of preleukemic CWD/J mice were found to represent the progeny of the two endogenous ecotropic proviruses present in this strain. Polytropic murine leukemia viruses were produced by tissues from one-half of the leukemic mice, and fresh tumor cells from one of the two animals tested expressed recombinant envelope glycoproteins. The genomic structure of the recombinant viruses resembled those of class II polytropic viruses of NFS · Akv mice and differed from those of class I recombinant viruses that are commonly isolated from other high-leukemia strains such as AKR and HRS. Acquired retroviral sequences with the structural features of class II recombinant proviruses were detected in the DNA from each CWD/J tumor by the Southern blot technique. Finally, the injection of a mixture of CWD/J ecotropic and class II recombinant polytropic viruses into neonatal CWD/J mice accelerated the onset of lymphoma, whereas the endogenous ecotropic virus was inactive in these assays.

CWD is a recently described strain of mice that develops spontaneous nonthymic lymphomas at a high frequency (1; unpublished observations). As is the case for many other high-leukemia strains, CWD mice express endogenous murine leukemia viruses (MuLVs) early in life. Angel and Bedigian previously reported that the spleens from 93% of 3-month-old CWD/Agl mice expressed ecotropic MuLVs, whereas the splenocytes from all animals over the age of 7 months released xenotropic viruses (1).

In the studies reported here, it was found that ecotropic viruses from CWD mice were derived from the two endogenous ecotropic proviral loci that are present in this strain, *Emv-1* and *Emv-3* (21). In addition, recombinant polytropic viruses were isolated from some of the leukemic animals, and recombinant proviruses were present in the tumor DNAs. The genetic structure of the recombinant viruses differed from those of the class I thymotropic leukemogenic polytropic viruses of AKR/J and HRS/J mice (2, 16, 26, 34, 35). The CWD recombinant viruses were of the class II genotype and contained extensive substitutions by nonecotropic virus sequences within the 3′ end of the viral genome. Injection of a mixture of ecotropic and polytropic viruses into neonatal CWD mice accelerated the onset of lymphoma in these animals. Although class II polytropic viruses isolated from other strains are not thought to be pathogenic (5, 33), the results of our studies suggest that the CWD recombinant MuLVs may play a role during the induction of spontaneous nonthymic lymphomas in these mice.

MATERIALS AND METHODS

Mice. Mice were purchased from Jackson Laboratory, Bar Harbor, Maine. The mice were curly whisker stock (*cw + d, a/a* and *cw d/cw d, a/a*). The animals were kept in the University of Virginia vivarium and maintained by brother-sister matings (*cw/+* to *cw/cw*). The mice were inspected for signs of illness or enlarged spleens and sacrificed when preterminal. Those that died in the cages were refrigerated and autopsied within 24 to 48 h.

Cells and viruses. The sources of NIH 3T3 mouse fibroblasts and CCL64 mink cells and methods of virus culture were described previously (16, 34). Endpoint dilutions were performed as reported by Green et al., except that the dilution plates were passaged 10 times and screened for the presence of reverse transcriptase activity in the culture medium (32).

The class I recombinant MCF 69L1 virus was obtained from P. O'Donnell, Memorial Sloan-Kettering Cancer Center (28). The class II recombinants *C58v-2*-C45, *C58v-1*-C48, and *Akv-2*-C34 and the class I intermediate *Akv-1*-C36 were obtained from J. Hartley, National Institutes of Health, Bethesda, Md. (27). Friend ecotropic MuLV was obtained from A. Oliff, Memorial Sloan-Kettering Cancer Center (29).

T_1 oligonucleotide fingerprinting of viral RNAs. Uniformly ^{32}P-labeled viral RNA was prepared as previously described (6, 34). The yield of viral RNAs for analysis was increased by preincubation of the infected cells in P-free medium followed by four harvests of labeled virions at 12-h intervals.

Mapping of oligonucleotides. T_1 oligonucleotide maps of the viral genomes were constructed by comparison to previously published maps and DNA sequences of MuLVs and to unpublished data from earlier studies of viruses from AKR/J and HRS/J mice (16, 18, 20, 23, 30, 31, 34, 35). Oligonucleotide numbers were assigned by the convention of Rommeleare et al. (31), Green et al. (16), and Thomas, Coffin, and co-workers (34, 35). Oligonucleotides 98B and 99B were originally numbered by Pederson and Haseltine (30). Because complex virus mixtures were present in some isolates, the existence of viruses with different genomes was inferred from differences in the molar yield of the oligonucleotides or from the simultaneous presence of known allelic oligonucleotides or from both.

Southern blot analysis of tissue DNAs. Frozen portions of normal or neoplastic tissues or virus-infected mink cells

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TABLE 1. Isolation of viruses from normal and leukemic CWD mice[a]

Condition of mice	Mouse	Age (mo)	Tissue	Virus isolated[b]	
				Ecotropic	Polytropic
Nonleukemic	CW-1	3	Spleen	CWN-S-1	NA[c]
			Thymus	CWN-T-1	NA
	CW-2	8	Spleen[d]	CWN-S-2	NA
			Thymus	CWN-T-2	NA
	CW-3	3	Spleen	NA	NA
			Thymus	NA	NA
Leukemic	CW-5	9	Spleen	CWN-S-5	CWM/N-S-5
	CW-6	10	Spleen	CWN-S-6	CWM/N-S-6
	CW-7	11.5	Spleen	CWN-S-7	NA
	CW-9	8	Spleen	CWN-S-9	NA
	CW-13	15	Spleen	NA	NA
	CW-15	12	Thymoma	CWN-T-15	CWM/N-T-15
	CW-100	13	Spleen	CWN-S-100	NA

[a] Production of viruses was determined by reverse transcriptase assays of supernatants from fibroblast cell lines that had been cocultivated with cells from the tissues indicated.

[b] Nomenclature: CW, strain; N, isolated on NIH 3T3 cells; M/N, isolated on mink cells and replicated in NIH 3T3 cells; S, spleen; T, thymus; the number indicates the mouse from which the virus was isolated.

[c] NA, Absence of reverse transcriptase activity in cell culture supernatants after 5 and 10 passages.

[d] Slightly enlarged spleen at autopsy.

were rapidly thawed and ground in DI buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 10 mM EDTA, 1% sodium dodecyl sulfate) in a homogenizer. After 2 h of incubation at 50°C with 100 μg of proteinase K per ml, the viscous solution was extracted three times with an equal volume of phenol-chloroform (1:1). After one additional extraction with chloroform alone, 2 volumes of ethanol were added to the aqueous phase. The DNA precipitate was removed to a new tube containing 5 ml of Tris-EDTA (TE) buffer (10 and 1 mM, respectively). After dissolving the DNA at 4°C, the solution was treated with 100 μg of pancreatic RNase A (Sigma Chemical Co.) per ml from a stock that had previously been heated to 100°C for 10 min. The phenol-chloroform extractions were repeated, and the DNA was precipitated with 10 ml of ethanol and 0.25 ml of 2 M sodium acetate. The DNA was dissolved in TE buffer, and the concentration was determined by spectrophotometry. A 10-μg sample of DNA from each tumor was digested with ≥50 U of the appropriate restriction enzymes (New England BioLabs, Inc.) for 6 to 8 hours, precipitated with ethanol, dried, and resuspended in 20 μl of TE buffer. The samples were loaded into 0.7% horizontal agarose gels and electrophoresed at 4°C at 2 V/cm. The gels were blotted and hybridized as described by Wahl et al. and Herr and Gilbert (19, 36), except that the acid wash was omitted. Some of the DNAs were blotted to nylon membranes (Zetaprobe; Bio-Rad Laboratories) rather than nitrocellulose filters.

The probes were kindly supplied by D. Lowy, National Cancer Institute, Bethesda, Md., and by W. Herr and W. Gilbert, Harvard University, Cambridge, Mass. The ecotropic virus-specific Np15E probe (pAKV-5) represents a 167-base-pair fragment from the 5′ portion of the p15E gene of the Akv virus of AKR mice (19). The SX *pol* probe was obtained by excising the 800-base-pair *Sac*I-*Xho*I restriction fragment from intact cloned Akv provirus (25). The ecotropic virus envelope probe pBENV represents a 400-base-pair fragment from the 5′ portion of the gp70 gene of Akv and hybridizes only to viruses with an ecotropic host range (2, 21). The cloned viral fragments that were used as probes were separated from the vector DNA and other viral sequences by purification from low-melting-point agarose gels. The DNAs were labeled (2×10^8 to 5×10^8 cpm/μg) by nick translation with [^{32}P]dATP and -dCTP (8). After hybridization, the nitrocellulose or nylon filters were repeatedly washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1× SSC, with the final wash in 0.1× SSC at 65°C. XRP-5 film (Eastman Kodak Co.) was exposed to the dried blot at 70°C with the aid of intensifier screens for 3 to 10 days and then developed.

Analysis of MuLV envelope proteins. Spleen and mesenteric lymph node cell suspensions were prepared with a tissue grinder. Cultures of 10^7 cells per ml were radiolabeled for 2 h with 350 Ci of [^{3}H]leucine (New England Nuclear Corp.) per ml of Eagle minimal essential medium supplemented with 5% fetal calf serum and lacking leucine. Cytoplasmic extracts were prepared and precleared with normal rabbit serum as previously described (11). The extracts were reacted with goat anti-Rauscher MuLV gp70 serum, rabbit anti-Rauscher MuLV p15E serum, or normal goat serum. The anti-gp70 and anti-p15E sera are group-specific reagents obtained, respectively, from the Office of Program Resources and Logistics, Viral Oncology, National Cancer Institute, and E. Fleissiner, Memorial Sloan-Kettering Cancer Center.

Analysis of immunoprecipitates was carried out on 7.5% acrylamide slab gels as previously described (13). Peptide mapping by limited proteolysis with *Staphylococcus aureus* V8 protease was done by the method of Cleveland et al. (4). For details of the procedure, see reference 11.

Leukemogenicity assays. Neonatal CWD mice (*cw*/*cw* and *cw*/+) 24 to 48 h old were injected intraperitoneally with 0.1 ml of tissue culture supernatants from cells infected with individual viruses. The supernatants had been harvested from confluent growth on 100-mm plates, filtered, divided into 10 1-ml portions, and stored frozen at −70°C. Before injection, individual portions were rapidly thawed and kept on ice. Injected mice were observed for signs of illness or death. Ill animals were sacrificed, and autopsies were per-

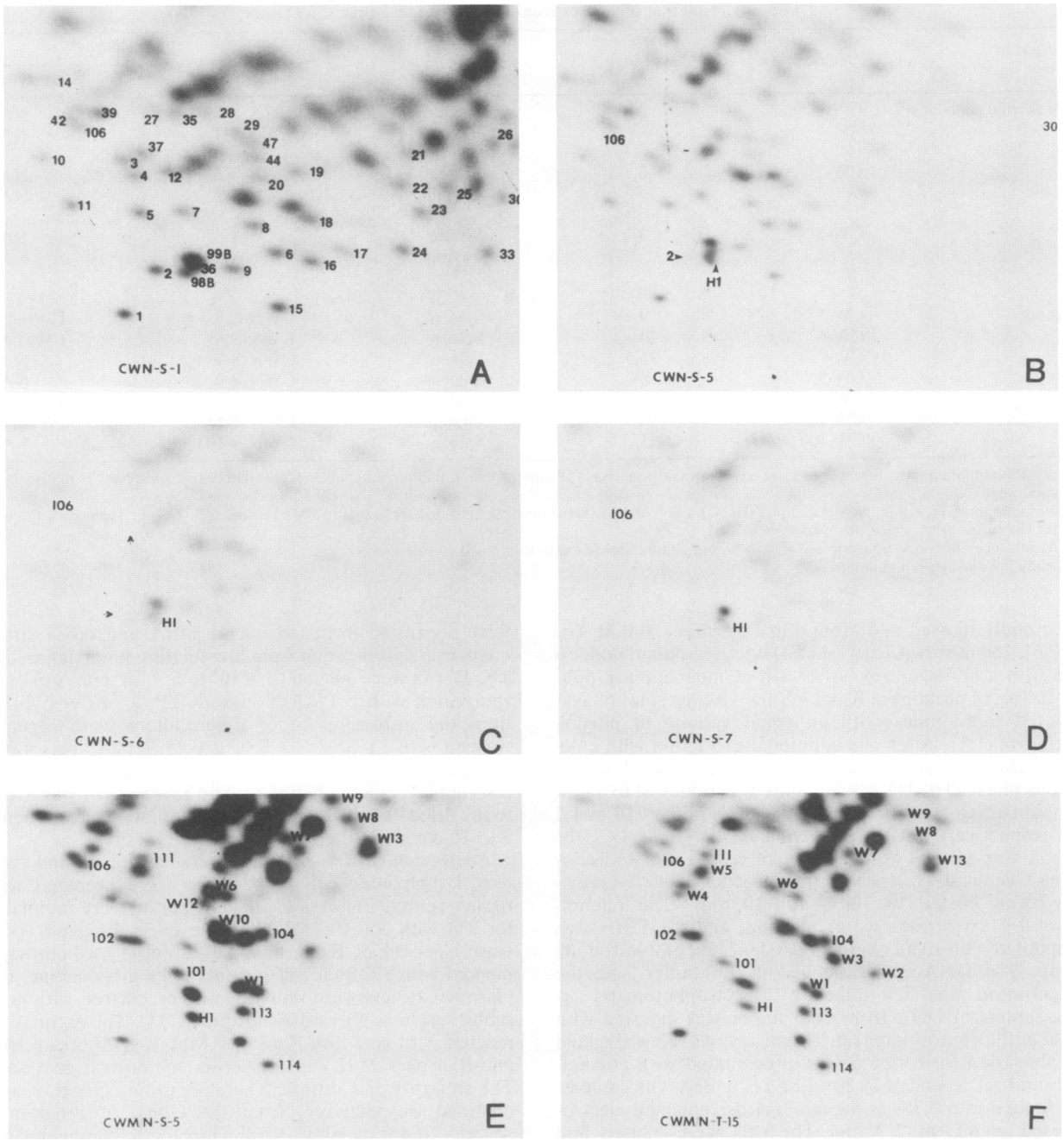

FIG 1. T_1 oligonucleotide fingerprints of CWD viruses. (A) CWN-S-1; (B) CWN-S-5; (C) CWN-S-6; (D) CWN-S-7; (E) CWM/N-S-5; (F) CWM/N-T-15. The Akv-related oligonucleotides and oligonucleotide 106 are numbered in panel A. In panels B through F, only oligonucleotides not found in Akv are numbered. In some instances, arrows or numbers are used for emphasis of faint Akv-related oligonucleotides that were visible in the original fingerprints. In panels B through D, the positions of specific oligonucleotides that were missing relative to CWN-S-1 are indicated by dashes. Oligonucleotides W-1 through W-13 were unique to the CWD polytropic viruses.

formed on all of them to confirm the presence of lymphoma. Tissues from selected animals were fixed in Formalin for sectioning and histopathologic analysis, snap-frozen for DNA extractions, or cryopreserved in 10% dimethyl sulfoxide in liquid nitrogen. There was no correction made for the relative concentrations of the individual viruses in the supernatants because each virus tested had yielded 1×10^5 to 2×10^5 cpm/ml in reverse transcriptase assays performed under identical conditions (32).

RESULTS

Isolation of viruses from normal and leukemic CWD mice. Ecotropic MuLVs were readily isolated from most of the CWD mice tested (Table 1). However, infectious viruses

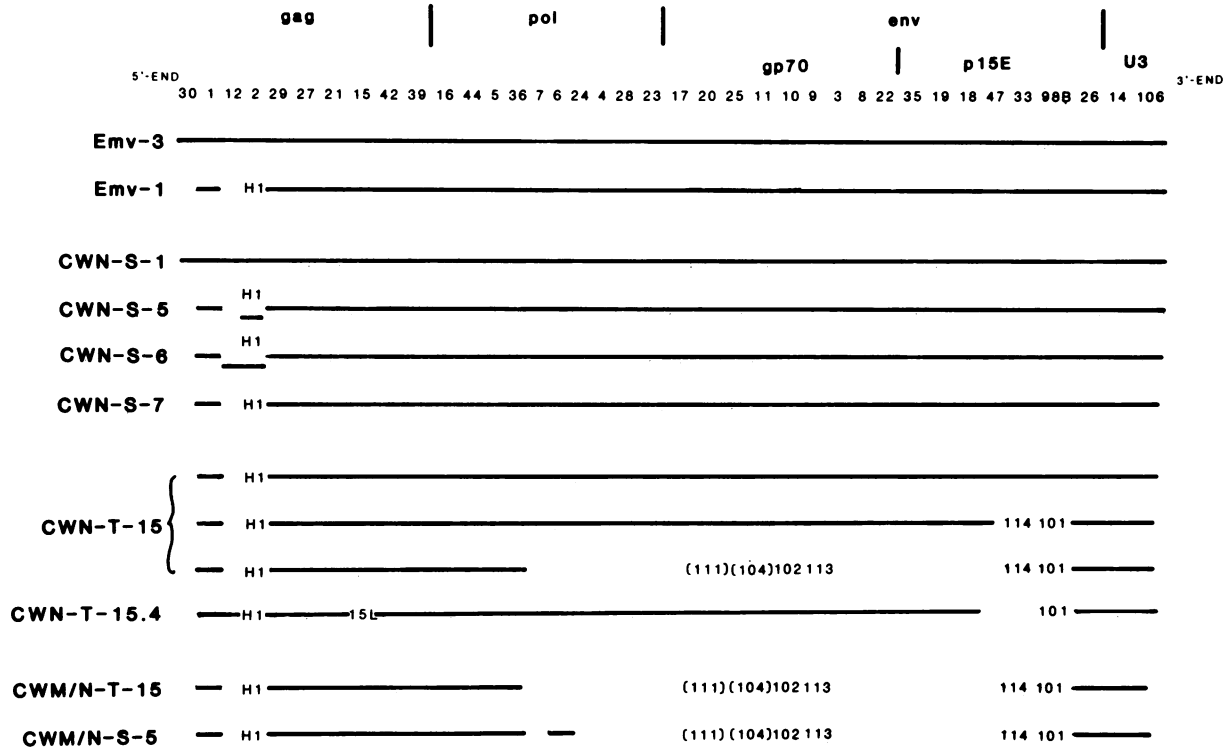

FIG. 2. T_1 oligonucleotide maps of CWD viruses. The maps were constructed as outlined in the text. The linear RNA genome is shown with the 3' end to the right. Akv oligonucleotides are ordered from the 5' to the 3' end, with oligonucleotide 106 added to the U3 region. The boundaries of the viral genes and the U3 noncoding region are noted by vertical bars. The presence of each oligonucleotide in the viral genome is shown by a solid line directly below the corresponding number. The absence of an oligonucleotide is indicated by a blank space. Allelic substitutions by non-Akv oligonucleotides are shown as numbered oligonucleotides in the genomic map. Non-Akv oligonucleotides whose map positions are not precisely known are enclosed in parentheses. In cases in which viral mixtures were present, oligonucleotides mapping in or near the same region of the genome are shown just above or below the center line of the genetic map. The genomes of the three major viruses in the CWN-T-15 mixture were deduced from the presence of allelic oligonucleotides and from the genetic composition of the other CWD viruses.

were not recovered from the normal tissues of one young mouse or from the splenocytes of one mouse with an abnormal spleen. Polytropic viruses were recovered from three of six tumors by cocultivation of lymphoma cells with CCL64 mink cells. Two of the viruses were isolated from abnormal spleens and one from a thymic mass (mouse no. 15). The polytropic viruses were separated from any xenotropic viruses by using the culture supernatants from infected mink cells to infect NIH 3T3 fibroblasts. Polytropic viruses were not recovered from any normal tissues in these or later experiments.

Expression of endogenous *Emv-1* and *Emv-3* proviruses in CWD mice. T_1 oligonucleotide fingerprints of viral RNAs of MuLVs isolated from normal or leukemic animals are shown in Fig. 1. Many of the oligonucleotides in the fingerprints could be readily identified because of their presence in HRS/J, CBA/J, BALB/c, DBA, and AKR/J viruses (7, 10, 16, 26, 31, 34, 35). The genetic structure of the CWD ecotropic viruses closely resembled that of the endogenous ecotropic virus of AKR mice, Akv (7, 26, 31). For instance, the only detectable difference in the fingerprints of CWN-S-1 and Akv was the presence of oligonucleotide 106 in the 3' portion of the U3 region. The genome of CWN-S-1, as determined by this technique, appeared to be identical to that of the virus encoded by *Emv-3*, an endogenous provirus that is present in the chromosomal DNA of CWD and other strains of mice (7, 21, 22). The major virus species in the CWN-S-5, CWN-S-6, and CWN-S-7 isolates contained oligonucleotide H1 and lacked the Akv- and *Emv-3*-related oligonucleotides 2 and 12. These genetic features are characteristic of the RNAs of ecotropic viruses isolated from other strains of mice that carry the *Emv-1* endogenous virus (10, 35). Two of the CWD isolates, CWN-S-5 and CWN-S-6, were clearly mixtures of viral genomes because the allelic oligonucleotides 2 and H1 were both present in the fingerprints. The genetic structure of these viruses and those encoded by *Emv-1* and *Emv-3* are shown in Fig. 2. The fingerprints of thymic and splenic isolates from the same mouse were identical for the two nonleukemic animals that were studied (data not shown). These observations strongly suggested that *Emv-1*, *Emv-3*, or both could be expressed in individual CWD mice and that recombination between their viral progeny may have occurred. This phenomenon was previously observed in other strains that carry the *Emv-1* and *Emv-3* proviruses, including HRS/J and SEA/J (35; unpublished observations).

Recovery of recombinant MuLVs from leukemic mice. The fingerprints of the CWD polytropic viruses CWM/N-S-5 and CWM/N-T-15 (Fig. 1E and F) revealed the presence of recombinant viral genomes. A number of oligonucleotides that are known to exist in the 5' portion of ecotropic viruses and that are encoded by *Emv-1* or *Emv-3* were visible in each fingerprint. The genomes of the recombinant polytropic CWD viruses also contained the gp70 gene oligonucleotides

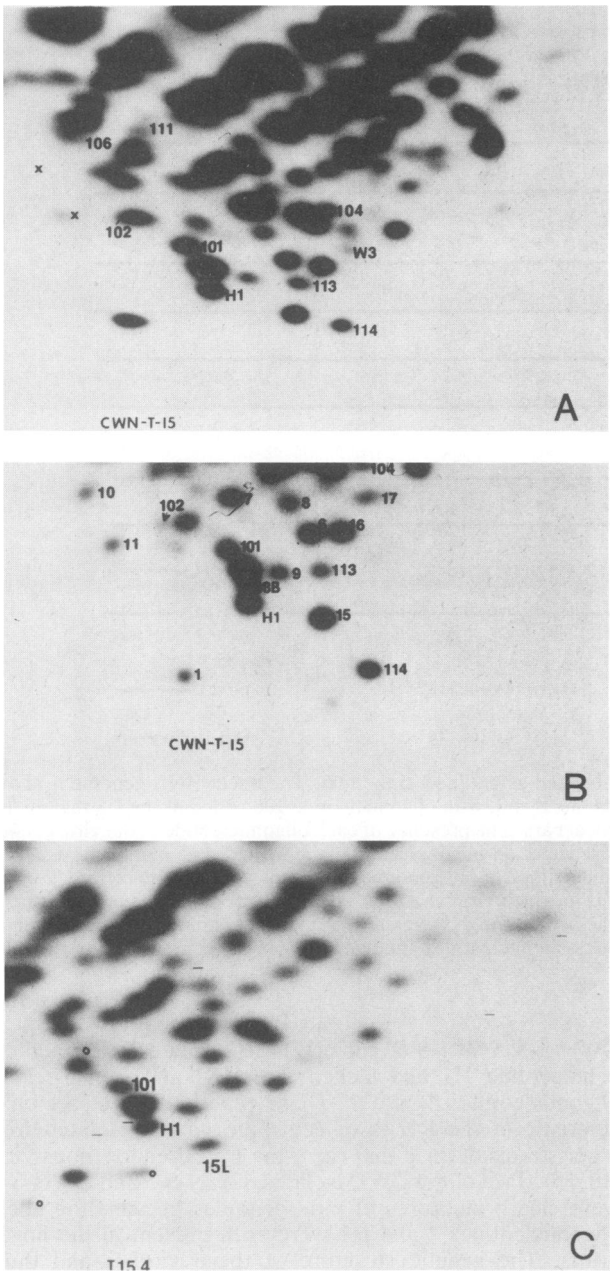

FIG. 3. Fingerprints of CWN-T-15 and T15.4 viruses. (A) CWN-T-15. Oligonucleotides unrelated to those in Akv and CWN-S-1 (Fig. 1A) are numbered. Oligonucleotides 10 and 11 that were present at very low yields relative to the other oligonucleotides in this fingerprint are indicated by an ×. (B) Large T_1 oligonucleotides of the CWN-T-15 viruses. This fingerprint was run longer to illustrate the relative yields of specific oligonucleotides. Note the differences in the intensities of the polytropic virus-related p15E gene oligonucleotides 101 and 114 and those from the gp70 gene, 102, 104, and 113. (C) T15.4. Oligonucleotides that were unrelated to those in CWN-S-1 are numbered, and the absence of specific CWN-S-1 oligonucleotides is indicated by a dash. Circles indicate fainter and unidentified oligonucleotides.

102 and 104, which are found in the fingerprints of most other polytropic MuLV RNAs (16, 26, 31, 34, 35). There were no detectable differences between the U3 regions of the recombinants and those of the endogenous ecotropic viruses. The oligonucleotides W-1 through W-13 appeared to be unique to CWD isolates, and their precise positions within the viral genomes have not yet been determined.

The genomes of the CWM/N-S-5 and CWM/N-T-15 viruses were similar, but not identical, and contained non-*Emv* sequences within the 3′ end of *pol* and within the entire *env* gene. The overall genetic structure of the recombinant CWD viruses is reminiscent of recombinant polytropic viruses that have been isolated from NFS mice congenic for the *Akv-1* and *Akv-2* proviruses (26, 27, 31, 33). These and other related viruses are referred to as class II recombinants to distinguish them from another group of polytropic viruses that have been recovered from AKR, HRS, and C58 mice (26, 27, 31, 35). This latter group, termed class I viruses, have less extensive substitution of *Emv*-related sequences in the *env* gene than do the class II viruses and usually contain different U3 regions (2, 26, 27, 31, 34, 35).

The genomes of recombinant viruses were also visible in the fingerprint of the CWN-T-15 isolate. This mixture of viruses was obtained by cocultivating the same tumor cells that produced the CWM/N-T-15 polytropic virus with NIH 3T3 cells instead of mink fibroblasts. Close examination of the fingerprint of CWN-T-15 for the relative yield of specific and allelic oligonucleotides suggested that at least three major viral species were present (Fig. 3A and B). Genomes that were essentially identical in structure to the CWM/N-T-15 polytropic virus and endogenous ecotropic viruses were represented, because both polytropic and ecotropic virus-specific envelope gene oligonucleotides were visible. A third viral species appeared to be an ecotropic virus that carried substitutions within the 3′ end of the p15E gene. This was deduced from the observation that the relative yields of the p15E oligonucleotides 101 and 114 were in excess of those of other polytropic virus-related gp70 gene oligonucleotides such as 102 and 104 (Fig. 3B).

To confirm that a recombinant ecotropic virus of this type was present, components of the CWN-T-15 mixture were separated from one another by using the end-point dilution technique. The fingerprint of one of these viruses, T15.4, is shown in Fig. 3C. The dominant viral genome in this fingerprint closely resembled that predicted for the recombinant ecotropic virus. Oligonucleotides 33, 47, and 98B, which are encoded in the 3′ end of the p15E gene of the *Emv-1* and *Emv-3* ecotropic proviruses, were absent. Oligonucleotide 101, which is allelic to 98B and is found in the p15E gene of many polytropic recombinant MuLVs, including CWM/N-T-15, was visible in the T15.4 fingerprint. Interestingly, the T15.4 genome carried a marker oligonucleotide, 15L, which was not seen in the original CWN-T-15 virus mixture. A single base change in the nucleic acid sequence of the *gag* oligonuclotide 15 found in the genomes of the other CW viruses may have resulted in the appearance of the 15L oligonucleotide in the T15.4 fingerprint (data not shown). Presumably, the 15L oligonucleotide may have been present in a minor subset of the genomes in the original CWN-T-15 virus mixture, or it may have arisen as a spontaneous mutation during the endpoint dilution procedure.

Tumor DNAs contained recombinant proviruses. The recovery of polytropic viruses from the leukemic tissue of CWD mice suggested that there may be an association between the expression of these viruses and the development of lymphoma. Chromosomal DNA was extracted from the tissues of normal and leukemic animals and from mink cells infected with the CWM-T-15 virus. The absence of class I recombinant viruses was confirmed by Southern blots of control and CWD tumor DNAs digested with *Pst*I and

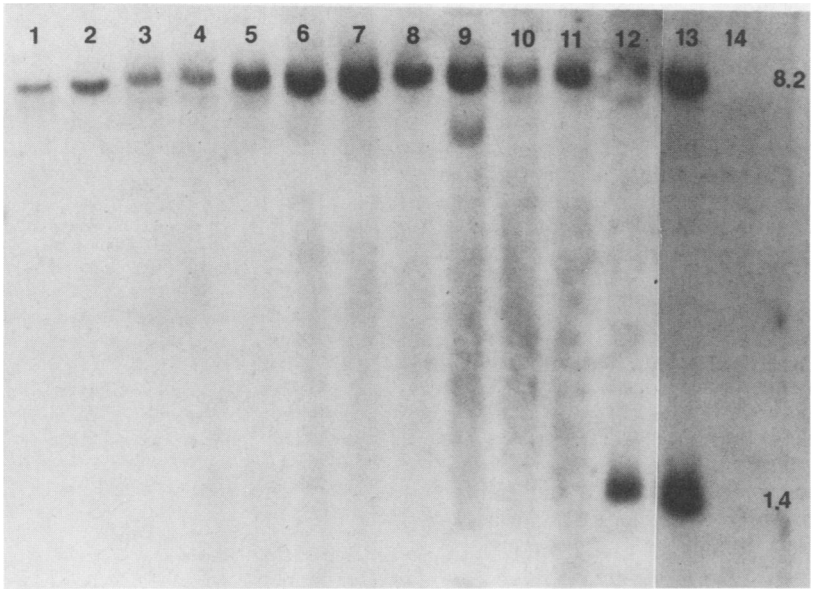

FIG. 4. Southern blot analysis of normal and tumor DNAs for class I recombinant proviruses. The DNAs were digested with *Pst*I and *Eco*RI and hybridized to a nick-transplanted pAKV-5 probe that contained a small segment of the Akv p15E gene sequences (19). Lanes 1 to 5 (negative control DNAs): 1, CWD embryo; 2, CWD brain; 3, SEA/J liver; 4, HRS/J liver; 5, AKR/J liver. Lanes 6 to 11 (CWD tumor DNAs): 6, spleen, CW-5; 7, thymoma, CW-15; 8, spleen, CW-16; 9, spleen, CW-9; 10, spleen, CW-14; (11) spleen, CW-25. Lanes 12 and 13 (positive control tumor DNAs): 12, spleen from mouse injected with the class I recombinant HRS/J virus; 13, spontaneous AKR thymoma AK-44. Lane 14, Mink cells infected with the class II CWD virus CWM/N-T-15. The numbers in the margin represent fragment sizes in kilobases.

*Eco*RI restriction enzymes. The blots were hybridized to a labeled probe from the 5′ portion of the p15E gene of Akv. These sequences are found only in the proviruses of exogenous and endogenous ecotropic MuLVs and in class I recombinant polytropic viruses. Class II polytropic proviruses and endogenous xenotropic or polytropic virus-related sequences do not hybridize to this probe (19). Southern blot assays of this type reliably detect class I recombinant proviruses in lymphomatous tissues of AKR, HRS, and several other mouse strains (19; unpublished observations).

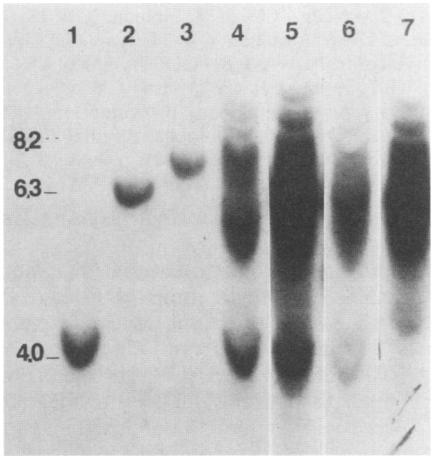

FIG. 5. Southern blot analysis of proviruses in DNAs of CWM/N-T-15-infected cells and in normal and leukemic tissues. The DNAs were cleaved with the indicated restriction enzymes and then hybridized to labeled *Sac*I-*Xho*I *pol* sequences from the Akv MuLV. DNAs from mink cells infected with CWM/N-T-15 were digested with *Hin*dIII and *Eco*RI (lane 1), *Pst*I and *Eco*RI (lane 2), or *Pst*I alone (lane 3). The other lanes contained DNAs digested with *Hin*dIII and *Eco*RI from AKR thymoma (lane 4); CWD lymphomatous spleen, CW-5 (lane 5); CWD lymphomatous spleen, CW-15 (lane 6); and CWD embryo (lane 7). The numbers in the margin represent fragment sizes in kilobases.

The 8.2-kilobase *Pst*I fragment that carried the endogenous or acquired ecotropic proviruses was seen in all samples tested, except in the DNA of mink cells infected with CWM-T-15 (Fig. 4). This verified that the probe was specific for the ecotropic viruses and would not hybridize to the class II CWD recombinant viruses. The increased intensity of the 8.2-kilobase band in some tumor DNAs probably resulted from the acquisition of additional ecotropic proviruses. In another experiment, hybridization of ecotropic-specific envelope gene sequences to tumor DNAs cleaved with *Eco*RI or *Hin*dIII and *Eco*RI confirmed the presence of newly integrated ecotropic proviruses in four of the five DNAs that were analyzed (data not shown). The 1.4-kilobase *Eco*RI-*Pst*I fragment corresponding to the 3′ end of class I recombinant viruses was seen only in the control DNA from an AKR thymoma (Fig. 4, lane 13) and in a tumor induced by the injection of an HRS/J class I virus (lane 12). None of the DNAs from spontaneous CWD tumors showed evidence of class I proviruses; this was consistent with results previously reported (1).

To detect the presence of recombinants of the class II genotype in the tumor DNAs, an assay was designed based on the results of the T_1 oligonucleotide mapping of the CWD viral RNAs. It was predicted that the recombinant proviruses would contain the *Hin*dIII restriction enzyme site in the *pol* region that was derived from *Emv-1* or *Emv-3* sequences and an *Eco*RI site in the non-*Emv* sequences within the gp70 gene. *Eco*RI cleaves within the gp70 gene of nearly all polytropic and xenotropic MuLV proviruses, but does not recognize sequences within the envelope genes of

ecotropic proviruses (2). Therefore, cleavage of the recombinant proviral DNAs with *Hin*dIII and *Eco*RI should produce a specific internal 3.9- to 4.0-kilobase DNA fragment that would hybridize to a *pol* gene probe. Such a fragment would be absent in ecotropic proviral genomes (2).

The presence of *Eco*RI, *Hin*dIII, *Pst*I, and other restriction enzyme sites in the CWM/N-T-15 provirus was first confirmed by Southern blot assays of DNAs extracted from infected mink fibroblasts. The CWM/N-T-15 provirus contained the expected 4.0-kilobase fragment which hybridized to a *pol* region probe derived from the Akv provirus (Fig. 5). In related experiments, a *Pvu*II restriction site in the *gag* gene and PstI restriction sites in the long terminal repeat sequences and a p15E gene were also found. A similar distribution of restriction enzyme sites have been observed in other class II recombinant proviruses (2).

Southern blot analysis of normal CWD embryo DNA showed that numerous endogenous viral sequences also hybridized to the *pol* gene probe. However, the embryo DNAs digested with *Hin*dIII-*Eco*RI did not contain a 4.0-kilobase fragment that was related to the *pol* gene sequences. This confirmed that the genome of CWM/N-T-15 was not encoded by an intact endogenous provirus, but must have been generated by recombination between endogenous viral sequences or, less likely, acquired by exogenous infection. A 4.0-kilobase band that hybridized to the *pol* probe was seen in Southern-blot assays of *Hin*dIII-*Eco*RI fragments from each of the CWD tumor DNAs, including those from the same tumor that had produced CWM/N-T-15 (Fig. 5 and 6). The bands observed in the autoradiograms comigrated with the internal *Hin*dIII-*Eco*RI proviral fragment from CWM/N-T-15. These bands most likely represented other class II recombinant proviruses because they could not be explained by reintegration of the endogenous ecotropic proviruses and class I recombinant proviruses were not detected in the DNAs of the same tumors. The additional novel bands that were detected in the tumor DNAs probably represented other types of recombinant or defective proviruses. The results of these experiments were consistent with those from the fingerprint analyses of CWD viruses and provided further evidence for an association between class II and other recombinant viruses and the development of lymphomas in the CWD strain.

Analysis of *env* gene proteins in tumor tissue. Expression of polytropic MuLV was assessed in tumor tissue by assaying for synthesis of the viral *env* gene polyprotein (PrENV protein). Polytropic MuLV can be distinguished from ecotropic MuLV on the basis of different molecular weights for this molecule (12); this difference forms the basis for a convenient assay for virus gene expression in primary tissue. Cell suspensions from the tumor tissues of two 1-year-old CWD mice were pulse-labeled for 2 h with [^{3}H]leucine, and prENV protein expression was compared with that of normal spleen cells from a 3-month-old animal. Cytoplasmic extracts of pulse-labeled cells were reacted with either goat anti-Rauscher gp70 serum or rabbit anti-Rauscher p15E serum to identify the PrENV protein which carried antigenic determinants to both *env* gene products. The extracts were reacted with normal goat serum as a negative control. The splenic tumor of one mouse exhibited high expression of a PrENV protein which comigrated with that of MCF 69L1 virus and was distinguishable from that of Friend ecotropic virus (Fig. 7A). Virus *env* gene expression was not detected in tumor tissue from a mesenteric lymph node or in the spleen of the second mouse. No virus proteins were detected in the spleen from the normal animal. Preimmune goat serum did not precipitate any radiolabeled proteins from these extracts (data not shown).

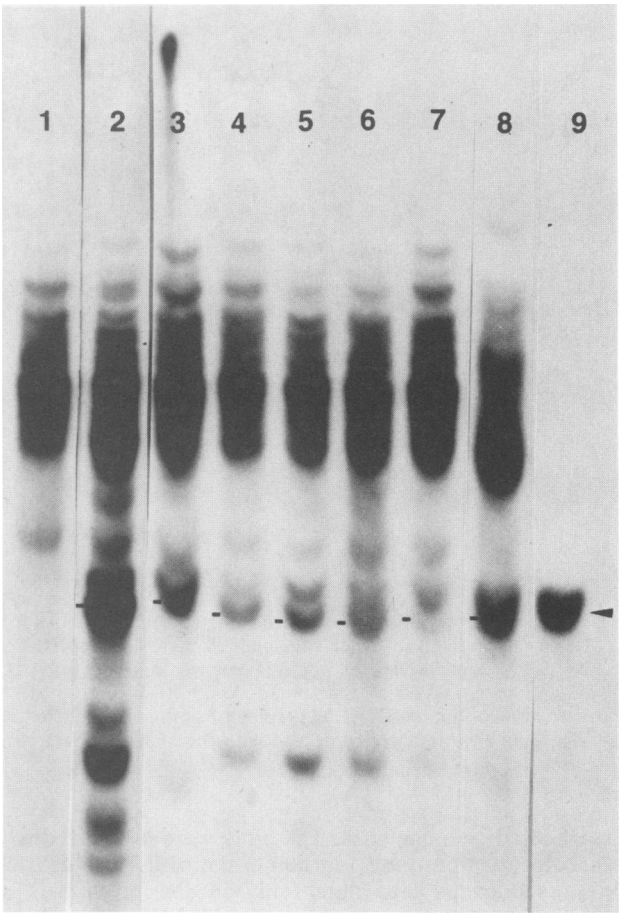

FIG. 6. Recombinant proviruses in CWD control and tumor DNAs. The DNAs were digested with *Hin*dIII and *Eco*RI and hybridized to the *Sac*I-*Xho*I *pol* region probe from Akv MuLV. Lane 1 (negative control DNA), CWD embryo. Lanes 2 to 7 (CWD tumor DNAs): 2, spleen, CW-5; 3, spleen, CW-15; 4, spleen, CW-16; 5, spleen, CW-9; 6, spleen, CW-14; 7, spleen, CW-25. Lanes 8 and 9 (positive control DNAs): 8, AKR thymoma AK-44; 9, mink cells infected with the class II CWD virus CWM/N-T-15. Dashes indicate acquired proviral sequences in tumor DNAs that comigrated with the internal 3.9- to 4.0-kilobase *Hin*dIII-*Eco*RI fragment of the CWM/N-T-15 provirus (arrowhead).

Peptide mapping by partial proteolytic digestion with *S. aureus* protease V8 was used to compare the PrENV protein isolated from the CWD splenic tumor with those encoded by class I, class I intermediate, and class II recombinant MuLVs (Fig. 7B). Maps of the PrENV proteins encoded by class I (MCF 69L1 virus) and class I intermediate (*Akv-1*-C36) MuLVs were very similar to one another and to those of a large number of class I recombinants (11, 12, 14). Two class II recombinants, *C58v-2*-C45 and *C58v-1*-C48, although sharing PrENV protein proteolytic fragments with MCF 69L1 and *Akv-1*-C36, were clearly distinguishable from the class I isolates and from each other. The PrENV protein of a third class II isolate, *Akv-2*-C34, generated a map indistinguishable from that of *C58v-2*-C45 (data not shown). The map of the PrENV protein isolated from the CWD splenic tumor is shown in Fig. 7B, lane 3. This molecule had proteolytic fragments in common with the PrENV proteins of both classes of recombinant MuLVs but was distinct from

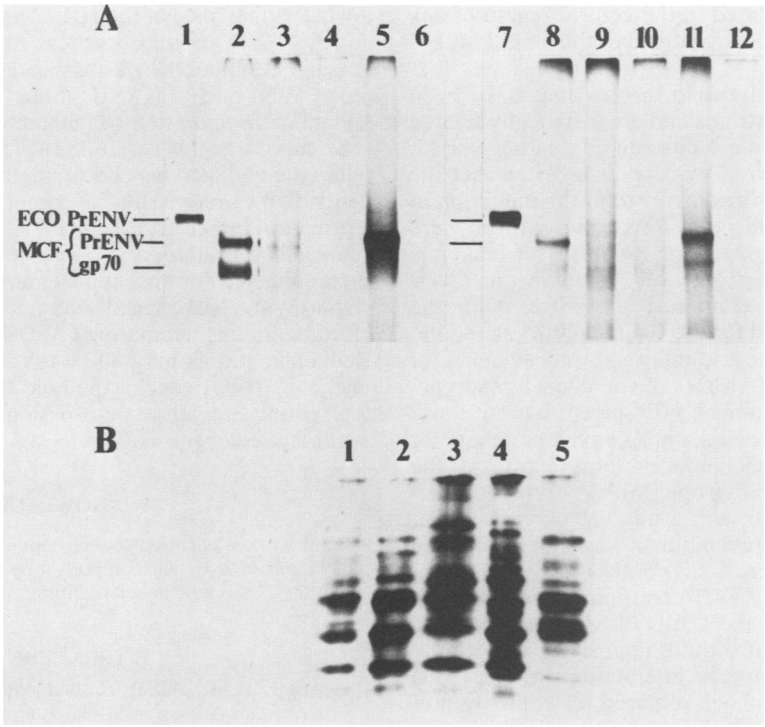

FIG. 7. Analysis of MuLV *env* gene products expressed in CWD tumors. CWD tissues were pulse-labeled for 2 h with [^{3}H]leucine, reacted with anti-viral serum, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Virus marker proteins were prepared from infected fibroblasts. (A) Goat anti-gp70 (lanes 1 to 6) and rabbit anti-p15E (lanes 7 to 12) immunoprecipitates. The precipitates were electrolyzed through 7.5% acrylamide gels. Lanes: 1 and 7, Friend-ecotropic-MuLV-infected NIH 3T3 cells (pulse-labeled for 15 min); 2 and 8, MCF 69L1-infected CCL64 cells (pulse-labeled for 2 h); 3, 4, 9, and 10, leukemic spleen (lanes 3 and 9) and mesenteric lymph node (lanes 4 and 10) from 1-year-old mouse (no. 1); 5 and 11, leukemic spleen from 1-year-old mouse (no. 2); 6 and 12, normal spleen from 3-month-old mouse. (B) *S. aureus* protease V8 partial-digest maps of PrENV proteins analyzed in 15% acrylamide slab gels. Lanes: 1, MCF 69L1; 2, *Akv-1*-C36; 3, CWD mouse no. 2; 4, *C58v-1*-C48; 5, *C58v-2*-C45.

the other PrENV proteins examined. These results were consistent with the fact that the genomes of the CWD recombinant viruses shared some, but not all, envelope gene sequences with the genomes of other recombinant MuLVs.

Acceleration of leukemogenesis by CWD viruses. To test the potential of CWD viruses to accelerate the onset of lymphoma, cell culture supernatants containing the CWN-S-5, CWN-T-15, and CWM/N-T-15 isolates were injected into neonatal CWD mice. The time to the onset of clinical disease or death from lymphoma in the injected animals was then compared with that observed in the controls (Table 2). Although the small number of mice tested limited the interpretation of the results, only the CWN-T-15 isolate was clearly capable of accelerating the onset of disease.

As discussed above, the CWN-T-15 isolate was a complex mixture of viruses with at least three genetically distinct components. The polytropic virus found in this mixture, CWM/N-T-15, had only marginal or no ability to accelerate leukemogenesis when tested in the absence of the other viruses. Therefore, it remains uncertain whether any one of the viruses in the CWN-T-15 mixture or the interaction among them was responsible for the biologic activity that was observed. The inactivity of CWN-S-5 in this assay was consistent with the results of similar studies with other endogenous ecotropic MuLVs (5, 33).

TABLE 2. Acceleration of leukemogenesis by CW viruses

Virus injected	No. of animals	No. (%) of animals diseased or dead by 1 yr	Median survival (mo)
None	20	5 (25)	16
CWN-S-5	6	2 (33)	13.5
CWM/N-T-15	14	5 (35)[a]	12
CWN-T-15	16	15 (93)[b]	7.5

[a] Four additional animals died between 12 and 13 months.
[b] One animal died at 8 months without lymphoma.

DISCUSSION

CWD mice were found to be unique among high-leukemia strains in that they developed nonthymic lymphomas and often expressed recombinant polytropic viruses with genomes that structurally resembled those of class II recombinant MuLVs (2, 26). T_1 oligonucleotide fingerprints of the viral RNAs revealed endogenous ecotropic virus-related sequences in *gag*, in most of *pol*, and in the U3 regions. In the remainder of the genome, including the entire *env* gene, there were substitutions by other endogenous viral sequences. Some of the sequences were unique to the CWD recombinant virus, but others have been observed in the genomes of polytropic viruses isolated from other inbred strains of mice (10, 16, 26, 31, 34, 35). For instance, the T_1-resistant oligonucleotides 101, 102, 104, and 113 are also found in the gp70 and p15E genes of the leukemogenic class I polytropic viruses. The genomes of the polytropic CWD viruses, however, lacked the U3 markers and the ecotropic

virus-related sequences located in the central region of *env* that are the other characteristic features of the class I MuLV genomes (26).

Class II recombinant polytropic viruses that have been isolated from other mouse strains and are genetically related to the CWD/J recombinants are considered apathogenic (5, 33). However, several lines of evidence suggested that the CWD/J polytropic viruses were involved in the induction of lymphomas in the CWD mice. (i) Polytropic viruses were recovered only from the spleens or thymuses of leukemic CWD mice. (ii) The DNAs from every spontaneous CWD tumor that was analyzed contained proviruses with the structural features predicted for the CWD polytropic recombinant viruses. None of these tumors showed evidence of integration by recombinant viruses of the class I genotype. (iii) Cells from a lymphomatous CWD spleen, but not those from a normal spleen, expressed a gp85 envelope precursor protein with the immunologic and structural features of the product of a recombinant polytropic MuLV. (iv) The injection of neonatal CWD mice with a mixture of viruses that contained a CWD class II recombinant virus and ecotropic viruses accelerated the onset of nonthymic lymphomas, whereas the injection of a CWD ecotropic virus alone did not. Although injection of the CWD class II recombinant virus CWM/N-T-15 by itself did not appear to significantly accelerate the onset of disease, its interaction with the CWD ecotropic viruses may have been required for expression of its oncogenic potential. The enhancement of the leukemogenicity of recombinant MuLVs after phenotypic mixing with ecotropic viruses has been observed in other systems (3, 17).

Despite the evidence reviewed above, it is not possible to unequivocally assign a pathogenic role for the recombinant viruses during leukemogenesis in CWD mice. For example, infectious recombinant polytropic viruses were not recovered from the tumors of every diseased animal, although the proviral DNA was apparently present in all tumor DNAs. Although this may have resulted from the inability of some of the recombinants to replicate efficiently in mink cells, it is formally possible that leukemic transformation might have occurred in the absence of infectious recombinant viruses. Secondly, the methods that were employed to detect the proviral DNAs of the class II genotype did not distinguish between the genomes that may have been present in the original tumor cell and those that may have resulted from secondary infection of the tumor cell population. Therefore, it could be proposed that the recombinant viruses were merely passenger viruses. Finally, the acceleration of leukemogenesis by the CWN-T-15 virus mixture may have been dependent on the presence of a leukemogenic ecotropic virus rather than the class II recombinant.

Although the CWN-S-5 ecotropic virus was unable to accelerate the onset of leukemia, caution must be exercised in generalizing the tests results and applying them to other ecotropic CWD viruses. T_1 oligonucleotide fingerprinting of the CWN-T-15 mixture and the other ecotropic viruses revealed considerable heterogeneity in their genetic structures. In other mouse leukemia systems, subtle differences in the p15E or U3 region or both, particularly in the gene enhancer element, have dramatic effects on the leukemogenic potential (9, 24). Leukemia acceleration tests with T15.4 and other individual viruses will be required to determine the relative contributions of the polytropic and ecotropic MuLVs to the biologic activity of CWN-T-15 and to determine whether CWD mice produce leukemogenic ecotropic MuLVs.

Individual mice of the HRS/J and SEA/J strains, like those of the CWD strain, coexpress the *Emv-1* and *Emv-3* proviruses (35; unpublished observations). However, in contrast to CWD mice, HRS/J mice usually develop thymic lymphomas and produce leukemogenic polytropic viruses of the class I genotype (16, 35). Therefore, host genes other than the endogenous ecotropic proviruses must regulate the phenotypic expression of spontaneous leukemia and the formation or selection or both of recombinant viruses. This conclusion is supported by earlier studies of NFS mice that are congenic for the endogenous ecotropic viruses of the high-thymic leukemia strains AKR and C58 (15, 26, 27, 31). Future studies comparing CWD/J mice with the other high-leukemia strains may allow the identification of the phenotypic characteristics of the host that regulate the formation, selection, and genotype of recombinant MuLVs associated with leukemogenesis.

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