AKR Ecotropic Murine Leukemia Virus SL3-3 Forms Envelope Gene Recombinants In Vivo

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The ecotropic AKR virus SL3-3 was injected into neonatal mice of the high-leukemia strains HRS/J and CWD/J and the low-leukemia strains CBA/J, SEA/J, and NIH Swiss. SL3-3 was highly leukemogenic in each strain, and 90% of the inoculated animals died by 6 months of age. T₁ oligonucleotide fingerprint analysis of the genomic RNAs of viruses recovered from 9 of 13 leukemic animals revealed the presence of the SL3-3 virus and recombinant viruses with polytropic virus-related envelope gene sequences. Recombinant proviruses were detected by the Southern blot technique in the DNAs of 17 of 18 tumors. The pattern of substitutions within the envelope genes of the SL3-3 recombinant viruses appeared to be dependent on the strain of the animal. These observations indicate that the SL3-3 virus formed envelope gene recombinants in vivo in each of the strains that were studied. However, the role of these recombinants during leukemogenesis remains to be defined.

The AKR SL3-3 virus is a highly leukemogenic ecotropic murine leukemia virus (MuLV) that was originally isolated from a mixture of viruses produced by an AKR thymic leukemia cell line, SL3 (26, 27). Lenz and co-workers (22) have shown that the transcriptional enhancer element located in the U3 region of the SL3-3 viral genome is primarily responsible for the differences in the oncogenic potential of this virus compared with the AKV, a closely related endogenous AKR virus that is only weakly pathogenic. The development of spontaneous leukemia in AKR and other high-leukemia strains, on the other hand, appears to depend on the formation of recombinant viruses that contain non-AKV sequences in the envelope genes, *env*, as well as in the U3 region (6, 12, 15, 25, 28, 32, 33). Many of these recombinants are leukemogenic following inoculation into neonatal mice and express envelope glycoproteins that permit virus infection of both mouse and heterologous cells, such as mink fibroblasts. Viruses that exhibit this host range phenotype are referred to as polytropic, dualtropic, or mink cytopathic focus-forming viruses (13, 14). Despite the known association of viruses with recombinant envelope genes with spontaneous murine lymphomas, the relationship of such viruses to the induction of lymphomas by the ecotropic SL3-3 virus has not been well studied.

In the experiments reported here, the SL3-3 virus readily induced or accelerated the onset of leukemia in mice of both high- and low-leukemia inbred strains. T₁-oligonucleotide fingerprints of viral RNAs and Southern blot analysis of proviral DNAs demonstrated that recombinant viruses were associated with tumor tissues from 14 of the 16 leukemic mice studied. These viruses appeared to be derived from recombination between the SL3-3 viral genome and endogenous viral sequences related to those found in the gp70 genes of polytropic MuLVs. These observations suggest that biologic functions encoded in the polytropic virus-related *env* sequences of the recombinants contributed to the specific association of these viruses with the leukemic tissues. These functions were likely distinct from those encoded in the U3 region of the genome and may have been similar to those mediated by the envelope genes of recombinant MuLVs recovered from spontaneous or other virus-induced murine leukemias (10-13, 17, 18, 28, 31-34; C. Y. Thomas,

B. J. Boykin, N. G. Famulari, and M. A. Coppola, *J. Virol.*, in press).

MATERIALS AND METHODS

Mice. All mice were purchased from Jackson Laboratory, except NIH Swiss, which were obtained from the National Institutes of Health Small Animal Section. They were maintained and bred in the University of Virginia vivarium, Jordan Hall.

Cells. The source and maintenance of NIH 3T3 cells and mink lung fibroblasts have been described previously (33).

Viruses. The SL3-3 virus was kindly supplied by J. Lenz and was derived from the transfection of a proviral clone (21). The particular isolate maintained in our laboratory in chronically infected NIH 3T3 cells was designated SLN-1. Viruses that were used for injection were obtained from filtered culture supernatants. Ten milliliters of supernatant was collected from 100-mm-diameter petri dishes containing confluent infected fibroblasts, passed through 0.22- μ m (pore size) filters, aliquoted, and stored frozen at -70°C.

Leukemogenicity assays. Approximately 0.05 to 0.1 ml of culture supernatant containing SLN-1 (SL3-3) was inoculated into 24- to 48-h-old mice. The mice were observed for signs of illness for a minimum of 8 months, sacrificed when moribund, and autopsied. Mice that died in their cages were removed, refrigerated, and autopsied within 24 h. The diagnosis of malignant lymphoma was made by gross inspection of the thymus, spleen, and lymph nodes. Tissues from selected animals were fixed in 10% Formalin, sectioned, and stained with hematoxylin and eosin. The histopathology of the tumors was reviewed by Donald Innes, Department of Pathology, University of Virginia. Tissues to be used as a source of DNA were placed in vials and snap frozen in a dry ice-methanol bath.

DNA extraction and Southern blot analysis. Extraction of DNA from frozen or fresh tissue or cultured cells and the Southern blot procedure have been described previously (Thomas et al., in press). Briefly, DNA was obtained following homogenization of tumor or control tissues, sodium dodecyl sulfate and proteinase K treatment, serial phenol-chloroform extractions, and ethanol precipitation. DNA from each sample (5 to 10 μ g) was digested with 50 U or

TABLE 1. Leukemogenicity of SLN-1 (SL3-3) virus

Strain	No. leukemic/no. injected ^a	Median latency (wk)
HRS/J	8/8	12 ^b
CWD/J	16/16	12 ^b
SEA/J	8/8	26
CBA/J	9/9	16
NIH Swiss	17/21	17

^a Neonatal mice were injected within 48 h of birth. Animals were observed for a minimum of 8 months.

^b The median latency for the development of spontaneous lymphomas in HRS/J and CWD/J mice is approximately 64 weeks (1, 13; unpublished data).

more of the appropriate restriction enzymes. The resultant fragments were separated by agarose gel electrophoresis and blotted to nylon membranes (Zetaprobe; Bio-Rad Laboratories). The filters were prehybridized and hybridized to ³²P-labeled DNA probes under conditions described by Jenkins et al. (20), and the filters were washed under stringent conditions (0.1× SSC at 65°C for 30 min). Bands that hybridized to the probes were detected by autoradiography at -70°C using Kodak XRP-5 film and intensifier screens.

Probes for Southern blotting. The pAKV-5 probe represents a 167-base-pair subclone of the 5' portion of AKV p15E gene and was a gift of W. Herr and W. Gilbert (16). The

FIG. 1. T₁ oligonucleotide fingerprints of RNAs from control viruses and those isolated from leukemic SL3-3-injected CBA/J mice. The fingerprints of CWN-S-1, an ecotropic virus isolated from the spleen of a normal CWD/J mouse, is shown (A) only for comparison. (B through F) Only those oligonucleotides that were not found in the CWN-S-1 genome are numbered. The absence of oligonucleotide 106 in the SLN-1 virus, the strain of SL3-3 used in these experiments, is indicated by a dash (—). The crosshatches (#) in the CBN-T-6 fingerprint (C) show the locations of faint spots that represented oligonucleotides 10 and 11.

FIG. 2. T_1 oligonucleotide fingerprints of viruses recovered from SL3-3-injected mice. Only those oligonucleotides not found in CWN-S-1 (Fig. 1A) are numbered. The dashes (—) in A and B indicate the absence of oligonucleotide 106, which is found in the endogenous ecotropic viruses of HRS/J and CWD/J mice (19, 20, 33). The crosshatches (#) in C and D mark unidentified oligonucleotides that are not found in the SLN-1 (SL3-3) viral genome.

SX *pol* probe was derived by gel purification of the internal 800-base-pair *SacI-XhoI* fragment from the AKV proviral clone kindly supplied by D. R. Lowy (24). The ecotropic virus-specific gp70 gene probe has been described previously (3).

Recovery of viruses from leukemic tissues. Viruses were recovered from fresh or cryopreserved tumor cells as described by Thomas et al. (33). In brief, tumor cells were cocultivated with NIH 3T3 cells or mink fibroblasts in the presence of 2 μ g of polybrene per ml. After 5 to 10 passages of the adherent cells, the culture supernatants were tested for production of retroviruses by reverse transcriptase assay (30).

T_1 oligonucleotide fingerprinting of viral RNA. Virus-producing fibroblasts were exposed to phosphate-free medium and then cultured in medium containing 0.5 mCi of 32 P_i (13). Labeled viral RNA was purified from cell culture supernatants and analyzed by T_1 ribonuclease digestion and two-dimensional electrophoresis in polyacrylamide gels as previously described (7, 32). Kodak XRP-5 film was exposed to gels covered with plastic film for 3 to 10 days at 4°C.

Pancreatic ribonuclease A analysis of T_1 -resistant oligonucleotides. The identities of selected T_1 oligonucleotides were confirmed by partial sequence analysis. The portion of the gel containing the oligonucleotide was punched out, and the RNA was eluted for 48 to 72 h into 0.5 ml of sterile water containing 60 μ g of carrier yeast RNA per ml. The eluted oligonucleotides were concentrated by lyophilization and

then digested with 10 μ l of 150- μ g/ml pancreatic ribonuclease A solution. The resultant products were spotted onto DEAE paper and separated by high-voltage ionophoresis as described previously (7, 32). The products were visualized by autoradiography at -70°C using Kodak XAR-5 film and intensifier screens.

RESULTS

Leukemogenicity of the SL3-4 (SLN-1) virus. Culture supernatants from NIH 3T3 cells chronically infected with the SL3-3 virus were inoculated into neonatal mice of the high-leukemia strains HRS/J and CWD/J and the low-leukemia strains CBA/J, SEA/J, and NIH Swiss. These mice were chosen because they were representative of inbred strains that may respond differently to murine leukemia virus infection. For instance, HRS/J mice express two endogenous ecotropic proviruses early in life, *Emv-1* and *Emv-3*, frequently develop thymic leukemia, and produce leukemogenic class I recombinant polytropic viruses (13, 20, 33). CWD/J is another high-leukemia strain that expresses the *Emv-1* and *Emv-3* viruses (1, 19). However, these mice develop splenic lymphoma and express recombinant polytropic viruses of the class II genotype (M). SEA/J mice, like HRS/J and CWD/J mice, express the *Emv-1* and *Emv-3* viruses but have a relatively low incidence of leukemia (unpublished data). CBA/J is a low-leukemia strain that may express the *Emv-1* virus late in life (20, 23, 33). Finally, NIH

FIG. 3. Major RNase A digestion products of selected T_1 oligonucleotides from fingerprints of HRN-T-12 and from control viruses. The products listed correspond to those previously reported or predicted from the sequence of the appropriate oligonucleotides (29). Marker RNA is shown at the left. Lanes: A, oligonucleotide 1 from HRN-T-12 (A_2C , A_3C); B, oligonucleotide 379 from HRN-T-12 (A_2U , A_3G); C, oligonucleotide 5 from control viral RNA (A_2U , A_3C); D, mixture of oligonucleotides 5 and 102 from HRN-T-12 (A_2C , A_2U , A_3C); E, oligonucleotides 38 and 38a from control (A_2U); F, oligonucleotide 104 from HRN-T-12 (A_2U , A_2G). The dashes (—) in lanes D and F indicate digestion products from polytropic virus-related oligonucleotides 102 and 104, respectively.

Swiss is a low-leukemia strain that lacks infectious endogenous ecotropic proviruses (20). These five strains were also selected for these studies because the genomic structures of the endogenous ecotropic MuLV and some of the polytropic MuLVs they produce were known (5, 8, 13, 25, 33; Thomas et al., in press).

The SL3-3 virus was highly leukemogenic or dramatically accelerated the onset of leukemogenesis in each of these strains (Table 1). Autopsies of all ill or dead mice revealed the presence of gross thymic enlargement, splenomegaly, or

both, usually associated with lymphadenopathy. Histologic sections of 12 selected tumors confirmed the presence of lymphoblastic, immunoblastic, large-cell, mixed-cell, or undifferentiated lymphomas (data not shown).

Recovery of viruses from leukemic mice. Tumor cell suspensions from 13 selected leukemic HRS/J, CWD/J, CBA/J, and NIH Swiss mice were cocultivated with NIH 3T3 cells or CCL64 mink lung fibroblasts. Reverse transcriptase activity was detected in the culture medium from each of the NIH 3T3 cell coculture plates. None of the mink cell cultures produced detectable retroviruses, even after 15 to 20 passages, with one exception. Culture supernatants from mink cells cocultivated with lymphoma cells from CWD/J mouse no. 66 reproducibly contained low levels of reverse transcriptase activity. Attempts to infect NIH 3T3 cells with these supernatants were unsuccessful, suggesting that this mink-tropic virus was a xenotropic virus of CWD/J mice (1).

T_1 oligonucleotide fingerprint analysis of viral RNAs. The RNAs of the unpassaged viruses were labeled by incubating the primarily infected NIH 3T3 cells in phosphate-free medium and then adding 0.5 mCi of $^{32}P_i$ per ml. Virions were recovered from the cell culture supernatants, and labeled viral RNAs were extracted. Following digestion of the RNAs with T_1 ribonuclease, the resultant oligonucleotides were separated by two-dimensional polyacrylamide gel electrophoresis and visualized by autoradiography (Fig. 1 and 2).

The fingerprint of SLN-1, the designation given to the strain of SL3-3 used in these experiments, was identical to one that had been published previously (21, 27). Although this virus shares many oligonucleotides with the endogenous ecotropic viruses of AKR/J (AKV) and CWD/J mice, such as CWN-S-1 (Fig. 1A), oligonucleotides 379 and 208B are unique to the SL3-3 genome (21, 27). The fingerprints of viruses recovered from 11 of 13 leukemic mice revealed the presence of the SL3-3 genome. The genomes of two of the CWD/J viruses, one recovered from a lymphomatous spleen and the other from a thymoma, did not contain the SL3-3 marker oligonucleotides. The genomic structures of both of these isolates closely resembled those of the endogenous ecotropic viruses found in this strain [20; Thomas et al., in press; data not shown].

The fingerprints of viruses from four leukemia CBA/J mice contained oligonucleotides not present in the SL3-3 viral genome, including those numbered 102, 104, 111, 113, and B1 (Fig. 1C–F). These oligonucleotides could be identified

TABLE 2. Summary of fingerprint and Southern blot assays of viruses and tumor DNAs from SL3-3^a-injected mice

Strain	Presence of specific T_1 oligonucleotides in recovered viruses ^b			Southern blot analysis of tumor DNAs ^b	
	SL3-3 ^c	gp70 gene recombinant ^d	Endogenous ecotropic virus ^e	Class I recombinant provirus ^f	Class I or II recombinant provirus ^g
HRS/J	2/2	2/2	0/2	4/4	3/3
CWD/J	3/5	1/5	2/5	0/5	5/5
SEA/J	ND ^h	ND	ND	2/2	2/2
CBA/J	4/4	4/4	0/4	3/3	ND
NIH Swiss	2/2	2/2	NA ⁱ	1/4	2/3

^a Strain designated SLN-1.

^b Number of animals positive over the number studied.

^c Oligonucleotides 379 and 208B.

^d Oligonucleotides 102, 104, 111, and 113.

^e Oligonucleotides H1 and 106.

^f The 1.4-kilobase *EcoRI*-*PstI* proviral fragment detected by the AKV 5' p15E probe.

^g The 4-kilobase *HindIII*-*EcoRI* proviral fragment detected by the ADKV *pol* probe.

^h ND, Not done.

ⁱ NA, Not applicable.

because of their existence in the gp70 genes of recombinant polytropic viruses of AKR/J, C58, HRS/J, and CWD/J mice (13, 25, 28, 33; Thomas et al., in press). Although these oligonucleotides were sometimes faint, they were clearly visible on the original autoradiographs, and their identities were confirmed by analysis of the pancreatic ribonuclease A products of the oligonucleotides (data not shown). Similarly, the fingerprints of five of nine viruses from SL3-3-injected HRS/J, CWD/J, and NIH Swiss mice contained polytropic virus-related oligonucleotides. The autoradiographs from four of these fingerprints are displayed in Fig. 2. Oligonucleotides 102 and 104 were very faint in the fingerprint of HRN-T-12, but pancreatic ribonuclease digestion of these RNAs yielded the predicted fragments (Fig. 3). For example, the presence of the trinucleotide AAC in Fig. 3, lane D, was consistent with the comigration of oligonucleotide 102 with oligonucleotide 5, and the predicted AAU and AAG residues of oligonucleotide 104 were seen in lane F (28).

The results of the fingerprint experiments are summarized in Table 2. The SL3-3 virus was recovered from 11 of 13 leukemic animals, but the genomes of other viruses with polytropic virus-related gp70 gene oligonucleotides were detectable in nine of the isolates. The most likely explanation for this latter observation is that a proportion of the recovered viruses represented recombinants between the SL3-3 virus and endogenous envelope gene sequences present in the chromosomal DNAs of these strains (2). This view is supported by the fact that the allelic *env* oligonucleotides 9, from SL3-3, and 113, which is derived from endogenous viral sequences, were both present in the fingerprints of CBN-T-10, CWN-T-25, and SWN-T-5 but in lower yield relative to other nonenvelope oligonucleotides. Such a pattern would be expected if a virus mixture included two viruses whose genomes were identical except in the gp70 genes. The absence of oligonucleotides 106 and H1 in the fingerprints of the CBA/J, CWD/J, and HRS/J viruses provided evidence that the endogenous ecotropic viruses of these strains were not involved in the generation of the recombinants (8, 33; Thomas et al., in press). Since NIH Swiss mice lack endogenous ecotropic viruses (20), the only infectious virus that could have recombined with the endogenous polytropic virus-related *env* sequences was the injected SL3-3 isolate.

The failure to recover the injected virus from two of the leukemic CWD/J mice remains unexplained. Several possibilities exist, however. For instance, normal or leukemic cells that were cocultivated with NIH 3T3 cells may have produced substantial quantities of infectious endogenous ecotropic viruses that may have overgrown the SL3-3 isolate. Alternatively, the tumor cells may not have been infected with the SL3-3 virus, or a defective SL3-3 provirus may have been present in the cells.

Southern blot assays of tumor DNAs. Southern blot assays were performed in an attempt to detect the presence of class I or class II recombinant proviruses in the tumor tissue DNAs. Class I proviruses contain the 5' sequence of the ecotropic p15E gene and the recognition sequence for the restriction endonuclease *EcoRI* in the gp70 gene (5). Simultaneous cleavage of class I proviral DNA with *EcoRI* and *PstI* endonucleases produces a 1.4-kilobase fragment that hybridizes to the ecotropic provirus-specific p15E gene sequences (5, 16). Intact SL3-3 and endogenous ecotropic proviruses do not contain *EcoRI* recognition sites but are cleaved in the U3 regions of the long terminal repeats by *PstI* to yield 8.2-kilobase fragments (20, 21).

The results of Southern blot assays of tumor DNAs for

FIG. 4. Detection of class I recombinant MuLVs in leukemic tissues of SLN-1 (SL3-3)-injected CBA/J mice. Control and tumor DNAs were digested simultaneously with *PstI* (P) and *EcoRI* (E) and then analyzed by the Southern blot technique (top). The predicted restriction sites in the SLN-1, endogenous ecotropic, and class I recombinant proviruses are shown and compared with those of class I AKR recombinants (5, 16, 20, 21). The relative location of the p15E gene probe (■) within the viral genomes is also displayed. Lanes 2 to 4 contain DNAs from tumors of SL3-3-injected CBA/J mice. Lanes: 1, normal control DNA, CBA/J liver; 2, thymoma, CB-6; 3, thymoma, CB-7; 4, thymoma, CB-9; 5, control DNA from spontaneous AKR/J thymoma, AK-44. The numbers on the left indicate size in kilobases (kb).

class I proviruses are shown in Fig. 4 and 5. All three CBA/J tumors contained a 1.4-kilobase band that hybridized to the ecotropic p15E probe (Fig. 4). These bands comigrated with the 3'-end proviral fragment that was seen in the DNA from a spontaneous AKR/J thymoma (lane 5). The 8.2-kilobase bands in the tumor DNAs represented the endogenous *Emv-2* and, presumably, intact SL3-3 proviruses. Class I proviruses were also detected in the DNAs of SL3-3-induced tumors from SEA/J mice (Fig. 5, lanes l and m), in all three tumors from HRS/J mice (lanes q to s), and in 1 of 4 tumors from NIH Swiss mice (lane y). In contrast, the absence of the 1.4-kilobase band in lanes e through i of Fig. 5 indicated that class I recombinant proviruses were not present in the DNAs of tumors from SL3-3-injected CWD/J mice. The 8.2-kilobase band seen in the control DNAs from normal tissues in lanes b, j, and f represented the endogenous *Emv-1* and *Emv-3* proviruses of CWD/J, SEA/J, and HRS/J mice.

FIG. 5. Assays for class I recombinant proviruses in tumor DNAs from SL3-3-injected CWD/J, SEA/J, HRS/J, and NIH Swiss mice. Lanes: b to i, DNAs from CWD/J mice; j to m, DNAs from SEA/J mice; n to s, DNAs from HRS/J mice; t to aa, DNAs from NIH Swiss mice. Tumor DNAs listed below from SL3-3-injected mice are followed by an asterisk (*). Dashes (—) on the autoradiogram mark the presence of the 3'-end 1.4-kilobase (kb) *EcoRI-PstI* fragment of class I proviruses. Lanes: a, positive control, CBA/J thymoma CB-6*; b, negative control, CWD/J embryo; c, positive control, CWD/J thymoma CW-18 induced by class I HRS/J virus; d, spontaneous thymic lymphoma, CW-15; e, splenic lymphoma CW-11*; f, thymic lymphoma CW-25*; g, thymic lymphoma CW-69*; h, thymic lymphoma CW-70*; i, thymic lymphoma CW-75*; j, negative control, normal SEA/J liver; k, spontaneous SEA/J splenic lymphoma, SE-8; l, thymic lymphoma, SE-16*; m, thymic lymphoma SE-9*; n, negative control, normal HRS/J liver; o, spontaneous HRS/J thymic lymphoma, HR-20; p, thymic lymphoma, HR-4*; q, thymic lymphoma, HR-7*; r, splenic lymphoma, HR-8*; s, thymic lymphoma, HR-10*; t, negative control, normal NIH Swiss spleen; u, negative control, normal spleen of NIH mouse injected with AKV virus; v, splenic lymphoma, SW-2*; w, splenic lymphoma, SW-3*; x, thymic lymphoma, SW-3*; y, thymic lymphoma, SW-5*; z, thymic lymphoma, SW-6*; aa, thymic lymphoma from an NIH mouse injected with AKV, SW-11.

A second set of Southern blot experiments was performed to detect acquired recombinant proviruses of the class II as well as class I genotypes. This assay was originally devised to identify class II proviruses in the DNAs of spontaneous lymphomas in CWD/J mice (Thomas et al., in press). Briefly, the assay relied on the prediction that the genomes of most class I and class II recombinant viruses should have acquired the *HindIII* restriction endonuclease recognition site in the *pol* gene from the SL3-3 or endogenous ecotropic parent, and the *EcoRI* recognition sequences in the *gp70* gene from the nonecotropic *env* sequences. The 3.9- to 4.0-kilobase internal *HindIII-EcoRI* restriction fragment carrying *pol* and *env* sequences from the recombinant proviruses would then be detectable in Southern blot assays by hybridization to a labeled 800-base-pair AKV *pol* probe. This probe represented the *SacI-XhoI* fragment from the middle of the Akv provirus, but it was not specific for ecotropic proviruses, since it hybridized to endogenous xenotropic sequences.

Tumor and control DNAs from CWD/J, SEA/J, HRS/J, and NIH Swiss mice were digested simultaneously with *HindIII* and *EcoRI* enzymes, and the resultant fragments were separated by gel electrophoresis, transferred to nylon membranes, and then hybridized to the ³²P-labeled AKV *pol* sequences. Numerous endogenous and acquired virus-related sequences hybridized to this probe (Fig. 6 and 7). However, the DNAs from all CWD/J, SEA/J, HRS/J, and two of three NIH Swiss mouse tumors contained a 3.9- to 4.0-kilobase band that was not present or only barely visible in DNAs from control tissues. These bands comigrated with the internal *HindIII-EcoRI* fragments found in the proviruses of the class I HRS/J recombinant PTV-1 and the class II CWD/J recombinant CWM/N-T-15 (Fig. 7, lanes h and i). In some cases, the tumor DNAs contained other novel *pol*-related sequences that may have represented other re-

FIG. 6. Detection of acquired proviral fragments in DNAs of SLN-1-induced lymphoma in CWD/J, SEA/J, and HRS/J mice. Tumor and control DNAs were digested with restriction enzymes *EcoRI* and *HindIII* prior to Southern blot analysis. The DNAs were hybridized to a probe from the *pol* region of the AKV provirus (see Materials and Methods). Bands that were present in tumor DNAs and not control DNAs are indicated by a dash (—). The majority of these bands migrated with 3.9- to 4.0-kilobase DNA fragments. DNAs from tumors of SL3-3-injected mice are listed below with an asterisk (*). Lanes: 1, negative control DNA, CWD/J embryo; 2, spontaneous CWD/J thymic lymphoma, CW-15; 3, positive control, CWD/J thymic lymphoma induced by class I HRS/J virus, CW-18; 4, splenic lymphoma, CW-11*; 5, thymic lymphoma, CW-25*; 6, thymic lymphoma, CW-69*; 7, thymic lymphoma, CW-70*; 8, thymic lymphoma, CW-75*; 9, negative control, normal SEA/J liver; 10, splenic lymphoma, SE-16*; 11, thymic lymphoma, SE-4*; 12, thymic lymphoma, SE-9*; 13, negative control, normal HRS/J liver; 14, spontaneous thymic lymphoma, HR-20; 15, thymic lymphoma, HR-4*; 16, thymic lymphoma, HR-7*; 17, thymic lymphoma, HR-10*.

combinants, defective proviruses, or both. A summary of the results of both Southern blot experiments is included in Table 2.

DISCUSSION

This study demonstrated that the ecotropic AKR virus SL3-3 induced or accelerated the onset of leukemia in a variety of high- and low-leukemia strains and that the development of disease was accompanied by the formation of MuLV with recombinant envelope genes. The genomes of the recombinant viruses were detected in T₁ oligonucleotide fingerprints of RNAs of virus mixtures isolated from leukemic animals and by Southern blot analysis of DNAs from tumor tissues. The SL3-3 recombinants contained gp70 gene sequences closely related to those found in the genomes of leukemogenic recombinant polytropic viruses of AKR, C58, and HRS/J mice and nonleukemogenic isolates from NFS.Ak_v and NFS.C58_v mice (13, 25, 26, 33). Despite the presence of these envelope gene sequences, the SL3-3 recombinant viruses did not replicate well in mink cells. Recombinant AKR viruses with similar phenotypes have been previously described (27; M. Cloyd, personal communication). It is uncertain whether the SL3-3 recombinants failed to penetrate mink cells or whether their growth was inhibited at subsequent steps in the replication cycle.

The SL3-3 recombinants fell into two groups as defined by the pattern of substitution of the envelope gene sequences within the viral genome. The envelope gene structure of the HRS/J, CBA/J, and SEA/J viruses resembled those of the class I recombinant polytropic viruses, whereas the genomes of recombinants detected in CWD and some NIH Swiss mice were similar to those of the class II viruses (5, 6, 25, 28). The original definition, however, also included a distinction between the U3 regions of the viral genomes and the biologic phenotypes of the class I and class II recombinants (6, 25). Although not firmly established during the course of this study, the genomes from both groups of SL3-3 recombinants appeared to contain SL3-3 sequences within the U3 region. Thus, some of the SL3-3 recombinants may represent a new class of recombinant viruses, perhaps related to those recovered from mice injected with Moloney or Friend MuLV (10, 12). More detailed analyses of the genetic structure and biologic characteristics of the SL3-3 recombinants are necessary to confirm this possibility.

The generation of recombinant viruses of the class I genotype by the SL3-3 virus appeared to be strain specific, as they were detected in all leukemic CBA/J and HRS/J but no CWD/J mice. This finding correlated with the previous observation that spontaneous expression of class I or class II recombinant viruses occurs in HRS/J and CWD/J mice, respectively (13, 33; Thomas et al., in press). A class I recombinant virus was also found in one of three NIH Swiss mouse tumors, whereas the two others contained class II-like proviruses. NIH Swiss mice injected with Friend MuLV also produce recombinant viruses with a spectrum of envelope gene substitutions (10).

Although recombinant viruses are also associated with a variety of spontaneous and other MuLV-induced hematopoietic cell neoplasms in inbred mice (10-13, 17, 18, 28, 31, 34), the role of the recombinants during induction of lymphoma by the SL3-3 virus is unclear. Detection of these viruses in 14 of 16 SL3-3-injected leukemic mice suggests that the recombinants expressed certain biologic characteristics that resulted in their close association with the tumor tissues. Since the tissue tropism of the SL3-3 and other ecotropic

FIG. 7. Detection of acquired proviral fragments in DNAs of lymphomas from SL3-3-injected NIH Swiss mice. Tumor and control tissue DNAs were assayed for the presence of *pol*-related sequences as described in the legend to Fig. 6 and in the text. The arrow indicates the 4.0-kilobase internal *Hind*III-*Eco*RI fragments of the class II CWM-T-15 and the class I PTV-1 recombinant proviruses (13, 33; Thomas et al., in press). The dashes (—) mark acquired bands in the tumor DNAs that comigrated with those found in CWM-T-15 and PTV-1. Tumors listed below which were obtained from SL3-3-injected mice are indicated by an asterisk (*). Lanes: a, negative control, normal NIH Swiss spleen; b, negative control, normal spleen from NIH Swiss mouse injected with AKV; c, splenic lymphoma, SW-2*; d, splenic lymphoma, SW-3*; e, thymic lymphoma, SW-3*; f, thymic lymphoma, SW-5*; g, splenic lymphoma, SW-12, from NIH mouse injected with AKV; h, mink cells infected with class II CWD/J virus, CWM-T-15; i, mink cells infected with class I HRS/J virus, PTV-1.

MuLVs is determined by the U3-p15E region of the viral genome (4, 9, 29, 32, 35), it is unlikely that the function of the envelope genes during leukemogenesis was to promote virus replication of the SL3-3 recombinant viruses in specific organs. Instead, expression of the recombinant gp70 gene sequences may have directly facilitated malignant transformation of the infected cells. Alternatively, one could argue that the SL3-3 recombinant viruses may have been merely passenger viruses that had no active role during leukemogenesis or that they represented the byproducts of the transformation of lymphoid cells by replication-competent ecotropic viruses. The issue cannot be satisfactorily resolved given the limitations of this and previous studies.

The results reported here have important implications for future studies of the pathogenicity of MuLVs. Since SL3-3 and other viruses may recombine with endogenous virus sequences in vivo, it is possible that the pathologic effects of a given isolate may be mediated by secondary recombinant viruses. Moreover, failure to recover mink-tropic viruses or inability to detect class I proviral DNAs by using ecotropic virus-related p15E probes is insufficient evidence to conclude that viruses with recombinant envelope genes are not present. Although the methods we employed may have been more sensitive in detecting recombinant MuLVs, it is conceivable that some types of recombinants escaped detection. Thus, reliable confirmation of the presence or absence of

recombinant MuLVs in mouse tissues awaits the development of more sensitive and specific techniques.

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