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# Role of Recombinant Ecotropic and Polytropic Viruses in the Development of Spontaneous Thymic Lymphomas in HRS/J Mice

CHRISTOPHER Y. THOMAS,[1]* RAMAN KHIROYA,[2] ROBERT S. SCHWARTZ,[2] AND JOHN M. COFFIN[3]

*Department of Medicine and Microbiology, University of Virginia Hospital, Charlottesville, Virginia 22908[1]; and Department of Medicine[2] and Department of Molecular Biology and Microbiology,[3] Cancer Research Center, Tufts University, Boston, Massachusetts 02111*

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The biological and genetic characteristics of murine leukemia viruses (MuLV) derived from leukemic and normal HRS/J mice were studied. $T_1$-oligonucleotide fingerprinting and mapping of viral RNAs from unpassaged isolates revealed the presence of complex mixtures of viral genomes. MuLV that were purified by endpoint dilution were genetically heterogeneous. Thus, endogenous retroviral sequences expressed in the tissues of HRS/J mice readily recombined with one another. Furthermore, the regular recovery of recombinant ecotropic MuLV suggested reciprocal in vivo complementation of a genetic defect(s) in each of the endogenous ecotropic proviruses *Emv-1* and *Emv-3*. Some recombinant ecotropic viruses contained sequences in the p15E-U3 region that were not derived from *Emv-1* or *Emv-3* but were found in recombinant polytropic HRS/J viruses. Finally, comparison of the genetic structures of leukemogenic and nonleukemogenic MuLV of this strain implied that the oncogenic phenotype of these MuLV is encoded within *env* or the U3 region of the genome or both. Our results are consistent with a stepwise convergent evolution of recombinant MuLV in vivo in individual HRS/J mice. Ultimately, this process of selection results in formation of leukemogenic polytropic viruses.

HRS/J is an inbred strain of mice that spontaneously develops thymic lymphomas which closely resemble those found in AKR mice (13, 24). HRS/J mice are unique in that they carry the recessive mutation *hr*. Mice homozygous for this allele develop complete alopecia by the age of 6 weeks and have an increased incidence of lymphoma when compared with heterozygotes (13, 24). As in AKR mice, the development of lymphomas in HRS/J mice is linked to the expression of endogenous murine leukemia virus sequences present in the chromosomal DNA of these mice (13, 16). There is variation in the phenotype of the viruses recovered from both HRS and AKR mice, including the ability to infect certain cells in vitro (host range) and the oncogenicity of the viruses in vivo (14, 26). Heterogeneity in the genetic structure of these viruses has also been documented (3, 13, 22, 27, 28, 30, 31, 38). Thus, it is possible to deduce which regions of the viral genome confer oncogenicity by comparing the genetic structures of leukemogenic AKR and HRS viruses with those of nonleukemogenic isolates such as Akv. The latter virus can be isolated from normal young AKR mice, has an ecotropic host range (replicates only in mouse cells), and appeared to be the unmodified viral product of at least two of the endogenous ecotropic proviruses (30, 34, 37). Such comparative studies implicate the gp70 and p15E encoding regions of the envelop gene, *env*, and the noncoding U3 region of the genome as the mediators of the oncogenicity and extended host range (i.e., polytropism) of the leukemogenic viruses (3, 7, 10, 13, 20, 22, 28, 33, 38). Furthermore, the genomes of the leukemogenic viruses apparently arise by genetic recombination between endogenous retroviral sequences (5, 13, 14, 31, 38).

It has been postulated that the expression of the nonleukemogenic endogenous ecotropic viruses, such as Akv, leads to recombination between this virus and other endogenous viral sequences in AKR mice (14, 22, 31). These latter sequences likely represent those of xenotropic or defective proviruses (5, 6, 11, 13, 14, 31). Recombination results in the generation of recombinant thymotropic leukemogenic viruses, many of which have polytropic host range (13, 14, 26, 33).

Although substantial data support a major role for the nonecotropic *env* genes in determining viral oncogenicity, viruses that do not contain recombinant gp70 *env* sequences can induce thymic lymphoma when injected into neonatal mice (3, 10, 20, 25). This finding seems to contradict the importance of recombinant *env* sequences in leukemogenesis, but leukemogenic ecotropic viruses may undergo recombination with endogenous viral *env* sequences in vivo after injection (12, 39). We have postulated that a similar phenomenon occurs during the development of spontaneous thymic tumors in AKR/J mice (38). In this model, the nonleukemogenic endogenous Akv-like viruses are expressed and undergo recombination with other endogenous sequences to form new ecotropic viruses that contain non-Akv p15E-U3 region sequences. These viruses subsequently acquire polytropic (non-Akv) gp70 gene sequences by recombination and are then capable of infecting target thymocytes and inducing a rare cell transformation event. Thus, our model predicts that the p15E-U3 and gp70 genes of the leukemogenic AKR viruses each encode a separate but necessary function that is needed to convey the oncogenic phenotype.

In the current study, we used $T_1$-oligonucleotide fingerprinting and mapping to extend our studies of the genetic structure of murine leukemia viruses isolated from HRS/J mice. Our observations suggest a sequence of molecular events during the evolution of leukemogenic viruses in HRS/J mice that involves both recombinant ecotropic and polytropic viruses and is similar to the scheme we have proposed for the AKR strain (38).

* Corresponding author.

## MATERIALS AND METHODS

**Mice.** HRS/J and CBA/J mice were purchased from Jackson Laboratory and maintained by sibling mating in the Tufts University and the University of Virginia animal care facilities.

**Cells.** NIH 3T3 mouse fibroblasts were obtained from the laboratory of N. Rosenberg, Tufts University, and maintained on Eagle minimal essential medium fortified with 4× amino acids and 10% fetal calf serum. Mink lung fibroblasts (CCL64) were a gift from S. Datta, Tufts University. They were grown in McCoy 5a media with 10% calf serum.

**Viruses. (i) Protocol for unpassaged isolates.** The technique used for the isolation of viruses from mice was similar to that described by Green et al. (13). Briefly, NIH 3T3 and mink cells were plated at $5 \times 10^5$ cells per 60-mm petri dish, respectively, and 24 h later they were exposed for 30 min to serum-free medium containing 25 μg of DEAE-dextran per ml. After the dishes were washed once, 5 ml of culture medium was added to each dish. Mice were selected and sacrificed, and appropriate organs were removed aseptically and transferred briefly to 60-mm dishes containing 5 ml of medium. Tissues were ground between glass slides and washed, and clumps were allowed to settle. The resulting single-cell suspensions were counted and seeded onto the DEAE–dextran-treated fibroblasts at $2 \times 10^6$ to $3 \times 10^6$ cells per 60-mm dish. Adherent cells were subcultured every 3 to 4 days, with loss of most of the lymphoid cells by the second passage. After five passages, the culture supernatants were screened for the presence of virus by reverse transcriptase assay (32). Positive cultures were subsequently used for $^{32}P$ labeling of viral RNA, and negative cultures were subcultured for an additional five passages and then retested for reverse transcriptase activity. CBN-1 was isolated on NIH 3T3 cells from a normal 1-year-old CBA/J mouse as described above, except that an equal mixture of thymocytes and splenocytes was used for cocultivation.

**(ii) Protocol for isolates derived by endpoint dilution.** Ecotropic and mink-tropic viruses were isolated as described above. These isolates were then subjected to three cycles of limiting dilution in mink CCL64 cells followed by three cycles on NIH 3T3 cells and an additional three cycles on the mink cells. Virus titers were established by XC-plaque or fluorescent focus-forming assay as previously described (13).

**Leukemogenecity assays.** Neonatal mice (24-h old) were injected intraperitoneally with $6 \times 10^3$ to $8 \times 10^3$ focus-forming units of the test virus and returned to their mothers. They were observed for 6 to 12 months and sacrificed when ill. Autopsies and histological sections confirmed the presence of tumor.

**$T_1$-oligonucleotide fingerprinting of the viral RNA.** Uniformly labeled RNA was prepared as previously described for avian viruses (1). The 70S RNA was isolated from peak fractions after the preparation was centrifuged through 5 to 23% sucrose gradients. The RNA was then precipitated, digested with RNase $T_1$, and subjected to two-dimensional polyacrylamide gel electrophoresis as previously described, except that for most fingerprints we used a second-dimension buffer that consisted of 89 mM Tris base, 89 mM boric acid, and 2.5 mM disodium EDTA at pH 8.0. Most of the oligonucleotides could be identified unambiguously by their electrophoretic mobility. In most cases confirmation was obtained by subdigestion with RNase A. Briefly, oligonucleotides were punched from the gels and eluted at 4°C for 48 h into 0.5 ml of sterile water containing 60 μl of yeast RNA per ml. The supernatant was removed to new tubes and lyophilized, and the pellets were redissolved in 10 μl of pancreatic RNase A (150 μg/ml). After 30 min of incubation at 37°C, the resultant products were spotted on DEAE chromatography paper. The nucleotides were separated by high-voltage ionophoresis in pyridine acetate buffer (pH 3.5) as described previously (1, 2). The dried paper was placed at −70°C with Kodak XAR film and intensifier screens to detect the separated digestion products. Published RNase A digestion patterns and those inferred from nucleic acid sequencing data were then used to confirm the identity of specific $T_1$-oligonucleotides (17, 19, 22, 30, 31).

**Mapping of oligonucleotides.** Maps of the viral genomes were constructed by using previously published maps, recent sequencing data, and unpublished data from our studies on the viruses of HRS/J and AKR/J mice. Numbers were assigned by the convention of Rommelaere et al. (30, 31), Green et al. (13), and Thomas and Coffin (38). Oligonucleotides 98B and 99B were originally numbered by Pederson and Haseltine (29). The positions of oligonucleotides P7 and 6L were assigned by their apparent allelic relationship to oligonucleotide 6 and similar RNase A digestion products. Because complex virus mixtures were present in some cases, the existence of different genomes was inferred from the differences in molar yield of the oligonucleotides or from the simultaneous presence of known allelic oligonucleotides.

## RESULTS

**Structure of the ecotropic viruses encoded by the *Emv-1* and *Emv-3* loci of HRS/J mice.** HRS/J mice contain two endogenous proviruses with the potential for encoding infectious murine leukemia viruses (16). Jenkins et al. have named these two loci *Emv-1* and *Emv-3* (16). We sought to identify the viral products of these loci by two different approaches.

In collaboration with Jenkins and co-workers, we performed $T_1$-oligonucleotide fingerprinting of the virus encoded by *Emv-3*. This virus, DBN-4, was derived by transfection into NIH 3T3 cells of the molecularly cloned *Emv-3* provirus from a DBA mouse. The results will be published elsewhere, but they are shown here in Fig. 1 for comparison with Akv, the AKR viruses encoded by *Akv-1* and *Akv-2*, and the HRS/J viruses (8). DBN-4 contains a single additional oligonucleotide relative to Akv, number 106, which maps in the end of the U3 region of the genome (17, 31). Thus, the *Emv-3*-encoded virus is closely related to the viruses encoded by the *Akv-1* and *Akv-2* loci of AKR mice.

The structure of the *Emv-1* virus product was inferred from a fingerprint of the ecotropic virus CBN-1, which was recovered from the spleen of a normal CBA/J mouse. This strain carries only a single endogenous ecotropic provirus, *Emv-1* (16). This virus differed from Akv by the presence of oligonucleotide 106 and from both Akv and the *Emv-3*-encoded virus (DBN-4) by the presence of oligonucleotide H1 and the absence of spots 2, 12, and 30 (Fig. 1). Oligonucleotide H1 is probably allelic to Akv oligonucleotide 2, since elution and treatment of the two oligonucleotides with RNase A yielded an identical pattern of major digestion products. Moreover, the oligonucleotides were never present simultaneously in equal molar yield relative to the other Akv-related oligonucleotides. The difference in migration of oligonucleotides 2 and H1 on our gels is consistent with a single base difference in the nucleotide sequences. A virtually identical observation was made when Akv was compared with the Gross A-NIH AKR virus, and the allelic relationship between the two oligonucleotides was confirmed by RNA sequencing (3). Therefore, we conclude that the *Emv-*

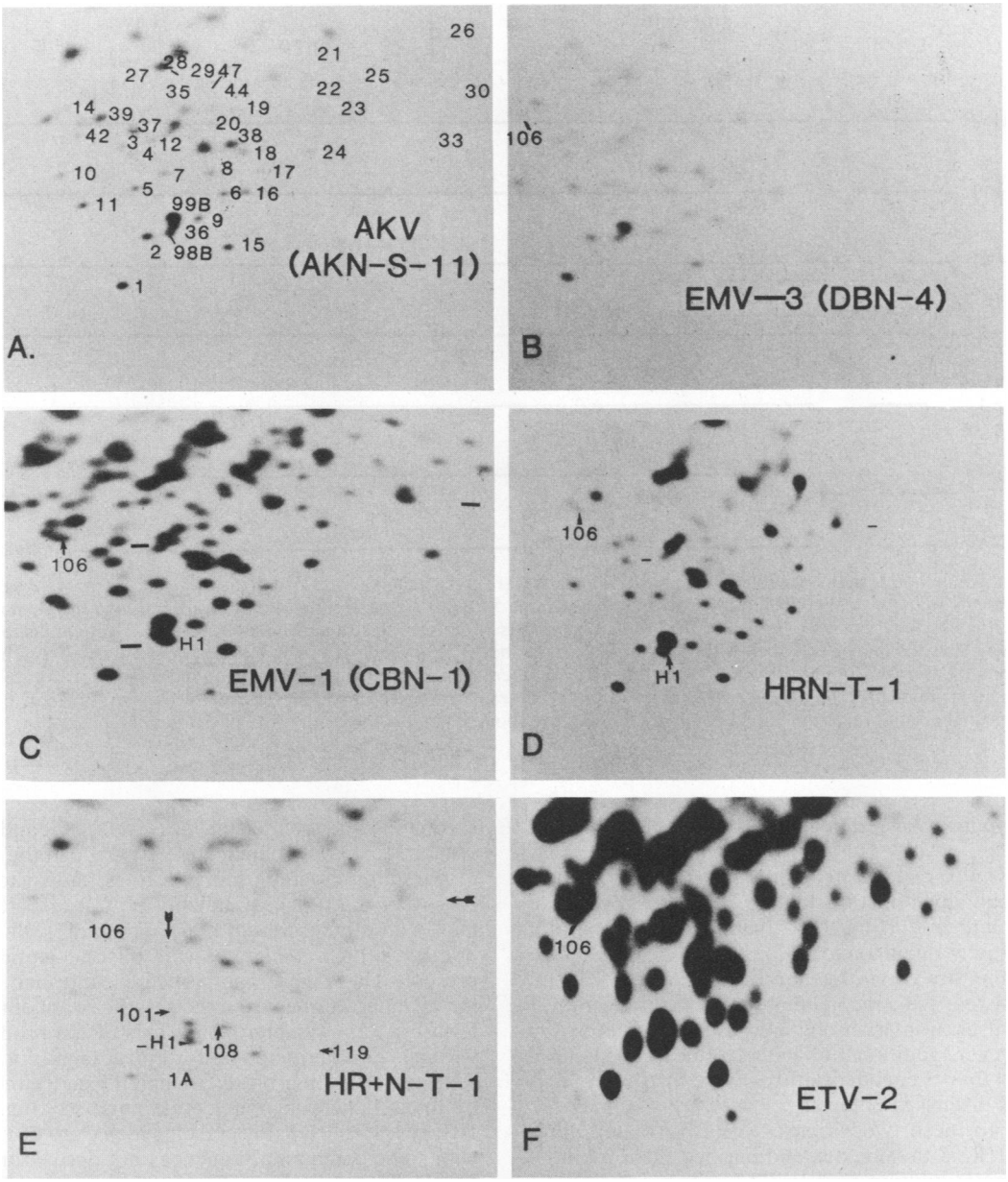


FIG. 1. $T_1$-oligonucleotide fingerprints of ecotropic viruses. The fingerprint of Akv (AKN-S-11) is displayed in (A) for comparison. In (B through F), only oligonucleotides not found in Akv are numbered. The position of Akv oligonucleotides that are missing in each fingerprint are indicated by a dash (—). Unlabeled arrows in (E) show the location of faint Akv oligonucleotides 12 and 30. The identity of the faint non-Akv oligonucleotides was confirmed as shown in Fig. 3. (A) AKV (AKN-S-11); (B) EMV-3 (DBN-4); (C) EMV-1 (CBN-1); (D) HRN-T-1; (E) HR+N-T-1; (F) ETV-2.

*1*-encoded virus is closely related to Akv and the *Emv-3* product. However, the fingerprint of the virus derived from *Emv-1* was unique in that it did not contain oligonucleotides 2, 12, or 30, but it did display oligonucleotide H1. All of these oligonucleotides map within the *gag* region of the viral genome (30). The oligonucleotide maps of the genomes of the *Emv-1* and *Emv-3* viruses are compared with that of Akv. (Fig. 2).

**Expression of *Emv-1* and *Emv-3* in HRS/J mice.** To determine whether both the *Emv-1* and *Emv-3* proviruses are expressed in HRS/J mice, we cocultivated thymocytes from individual animals with NIH 3T3 cells (see above). To retain as many viral sequences as possible and reduce inadvertent selection in vitro, we exposed the primarily infected NIH 3T3 cells to $^{32}P_i$ to label the viral RNA. The fingerprint of a virus mixture recovered in this manner, HRN-T-1, demonstrated the simultaneous presence of the allelic spots 2 and H1, although H1 was present in low molar yield relative to other oligonucleotides (Fig. 1D). Thus, a mixture of viral genomes was present. The absence of oligonucleotides 12 and 30 indicated that the principal component of this virus mixture was a recombinant between the *Emv-1* and *Emv-3* proviruses; oligonucleotide 2 was donated by *Emv-3*, and the loss of spots 12 and 30 was likely an inherited trait from *Emv-*

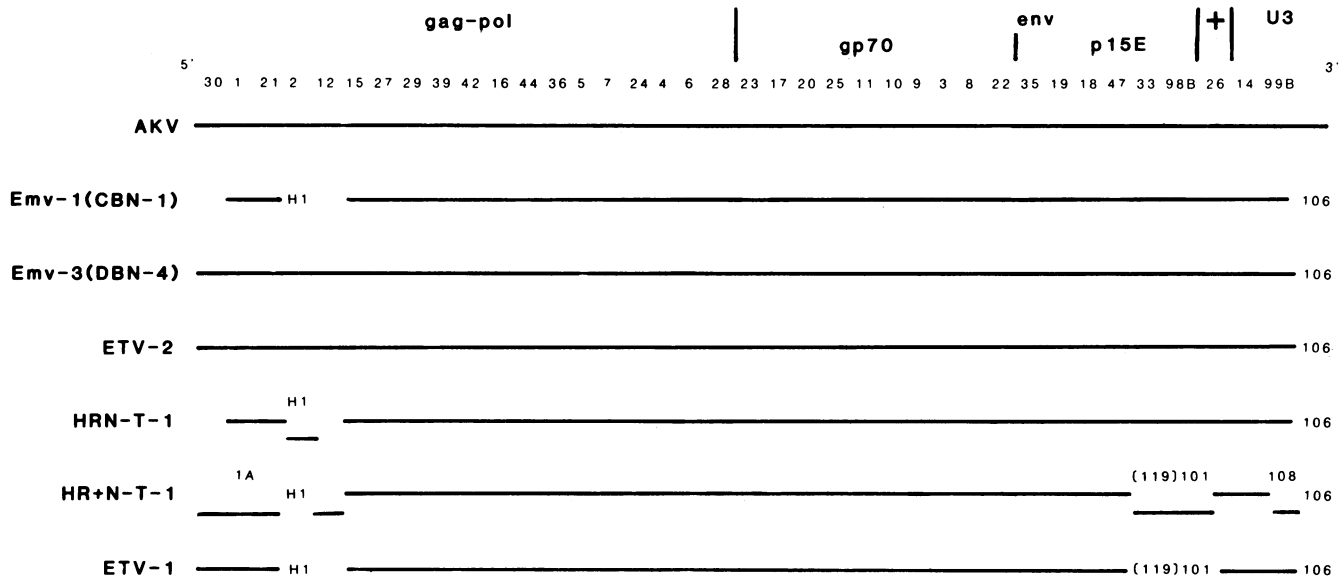


FIG. 2. $T_1$-oligonucleotide maps of ecotropic viruses. The maps were constructed as outlined in the text. The linear RNA genome is shown with the 3′ end to the right. Akv oligonucleotides are ordered from the 5′ to the 3′ end. Boundaries of viral genes and the U3 noncoding region are noted by vertical bars, whereas (+) indicates the origin of synthesis of the second strand. 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 of non-Akv oligonucleotides are shown by numbered oligonucleotides in the genomic map. Non-Akv oligonucleotides whose map position is 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.

*1*. A small proportion of genomes retained the H1 marker of *Emv-1*.

Another thymic isolate from a 2-month-old HRS/J mouse, HR+N-T-1, contained oligonucleotide H1 in the absence of allelic spot number 2 (Fig. 1E). Faint representation of submolar amounts of oligonucleotides 12 and 30 indicated that another mixture of viral genomes was present. Again, this was consistent with recombination between the *Emv-1* and *Emv-3* viruses, as marker oligonucleotides for both were present. However, additional oligonucleotides not found in the *Emv-1*- or *Emv-3*-encoded viruses were also seen. This observation is discussed below.

In contrast to these two viruses, we also isolated other viruses from HRS/J thymocytes and purified them by three cycles of endpoint dilution on NIH 3T3 cells before genetic analysis to obtain pure stocks of virus from the original complex mixture. The fingerprint of ETV-2, a virus which was isolated by this procedure, is shown in Fig. 1F. Because its genomic structure appeared to be identical to that of DBN-4, it may represent the intact viral product of *Emv-3* or possibly a recombinant genome without specific oligonucleotide markers.

We conclude that HRS/J mice express both *Emv-1*- and *Emv-3*-encoded viruses, as judged by the recovery of marker oligonucleotides. Moreover, frequent recombination between *Emv-1* and *Emv-3* sequences was observed. The oligonucleotide maps of HRN-T-1, HR+N-T-1, and ETV-2 are included in Fig. 2.

**Some HRS/J mice release ecotropic viruses with genomes derived by recombination between *Emv-1*, *Emv-3*, and other endogenous sequences.** As described above, the fingerprint of the HR+N-T-1 virus mixture contained four additional oligonucleotides not found in *Emv-1* or *Emv-3*. The four oligonucleotides are present in some previously described recombinant leukemogenic viruses of AKR and HRS/J viruses (13, 31, 38). Oligonucleotide 1A, seen in the lower portion of the autoradiograph in Fig. 1E, is known to be allelic to Akv spot 1 (3). Oligonucleotides 119 and 101 were seen faintly and therefore did not photograph well. Oligonucleotide 108, which comigrates with oligonucleotide 9, was also present. These observations were confirmed by elution of the oligonucleotides from the gel, digestion of the RNA with RNase A, and qualitative analysis of the products by high-voltage ionophoresis and autoradiography. The major RNase A digestion products match exactly those predicted by the assigned oligonucleotide numbers (Fig. 3 and Table 1). Thus, HR+N-T-1 represents a mixture of viral genomes, with some containing sequences not derived from *Emv-1* or *Emv-3*. These sequences map within the 3′ end of p15E gene (101 and 114), the U3 region (108), and *gag* (1A) and presumably are encoded by other endogenous proviruses. The oligonucleotide maps of the viruses represented in the fingerprint of HR+N-T-1 are also shown (Fig. 2). A previously described HRS/J ecotropic virus, ETV-1 (13), is shown for comparison, as it has a structure similar to a virus present in the HR+N-T-1 virus population.

**HRS/J polytropic viruses are derived by genetic recombination between *Emv-1*, *Emv-3*, and other endogenous viral sequences.** We studied eight viruses that were isolated by cocultivation of HRS/J thymocytes on mink cells. The viruses were purified by three cycles of endpoint dilution on mink cells, followed by three more endpoint dilutions on NIH 3T3 cells and reculturing on mink cells. Seven of the HRS polytropic viruses were derived from older nonleukemic animals; the eighth virus was cultured from a 13-month-old mouse with lymphoma (Table 2). The genetic composition of five of these viruses, as assayed by $T_1$-oligonucleotide fingerprinting, has been previously reported (13). We char-

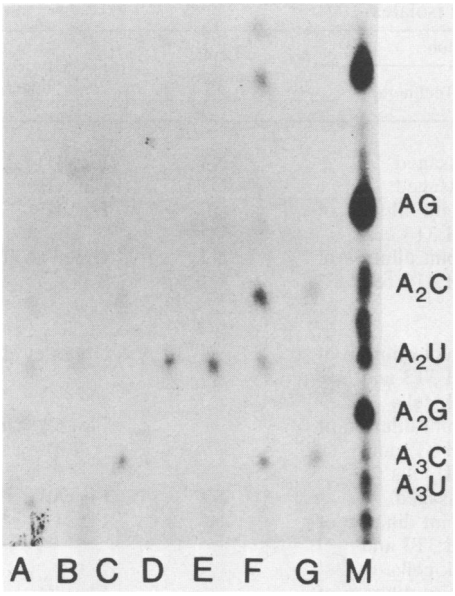


FIG. 3. Major RNase A digestion products of selected $T_1$-oligonucleotides found in HR+N-T-1. Products were separated by high-voltage ionophoresis and detected by autoradiography as described in the text. The results were compared with published patterns of specific oligonucleotides (30, 31). In the case of lane F, the pattern is clearly not that of the single Akv oligonucleotide 9, but is compatible with a comigration of oligonucleotides 9 and 108 on the original fingerprint. For contents of other lanes, see Table 1. Marker oligonucleotides were run in lane M.

TABLE 1. Major RNase A digestion products of selected $T_1$-oligonucleotides found in HR+N-T-1

| Lane | Assigned oligonucleotide no. | Major RNase A products |
|---|---|---|
| A | 101 | $A_2C$, $A_2U$, $A_3U$, AG |
| B | 114 | $A_2U$ |
| C | 1A[a] | $A_2C$, $A_3C$ |
| D | 30 | $A_2U$ |
| E | 33 | $A_2U$ |
| F | 9 + 108 | $A_2C$, $A_2U$, $A_3C$, AG |
| | (9 alone) | $A_2C$ |
| G | 1 | $A_2C$, $A_3C$ |

[a] Cytosine to uracil change of oligonucleotide 1.

acterized the genomes of three additional viruses (PTV-3, PTV-4, and PTV-5), restudied and reclassified three other isolates (XTV-1, XTV-2, and XTV-3), and updated and improved our oligonucleotide maps.

Five of the isolates, referred to as the PTV viruses, clearly demonstrated polytropic host range by high-titer replication on mink and NIH 3T3 cells (13; unpublished data). The viruses were labeled and fingerprinted, and the results for PTV-3, PTV-4, and PTV-5 are shown in Fig. 4. The genomic maps, along with those of PTV-1 and PTV-2, are displayed in Fig. 5. All of these isolates were genetically unique but had similar patterns of substition of oligonucleotides when compared with *Emv-1* or *Emv-3*, which is consistent with their origin by recombination.

The substitutions occurred throughout the polytropic virus genomes but were grouped within the 5′ end of the gp70 gene, the 3′ end of the p15E gene, and the U3 region. This pattern closely resembles that seen in leukemogenic AKR isolates, sometimes referred to as class I mink cell focus-forming viruses (7, 22, 31, 33).

*Emv-1* and *Emv-3* were very likely the source of the *gag* sequences in our recombinants because of the presence of identical *gag* oligonucleotides, the frequent representation of oligonucleotide H1, and the absence of spots 2, 12, and 30. However, recombination with other endogenous viruses within the *gag* region must also have occurred, as evidenced by the recovery of oligonucleotide 1A and the loss of *Emv-1* and *Emv-3* oligonucleotide 44 as shown for PTV-2.

The *Emv-1*- and *Emv-3*-encoded viruses may have donated the middle sequences of *env* and portions of the U3 region as well. The exact origin of the other oligonucleotides that map within *env* and the U3 region of these viruses could not be determined from our experiments. However, the same p15E and U3 region markers were present in the ecotropic viruses described earlier, HR+N-T-1 and ETV-1. Thus, viruses with this type of genome would have been capable of donating these 3′ end sequences in a recombination event with polytropic gp70 gene sequences to generate the PTV viruses.

We also restudied three thymic isolates that we initially reported as having xenotropic host range (13). We subsequently found that these viruses are weakly polytropic and replicate in NIH 3T3 cells at a low titer, as measured by assays for reverse transcriptase activity (data not shown). These three viruses were originally obtained from different animals and were subjected to endpoint dilution on mink cells as described for the PTV isolates (Table 2). On reinfection onto NIH 3T3 cells for the second series of dilution, very low infectivity was seen. The three viruses with this phenotype (XTV-1, XTV-2, and XTV-3) were infected again onto NIH 3T3 cells, and the cells were passaged. Although yields were low, labeled viral RNA could be obtained for the fingerprints of XTV-2 and XTV-3 (Fig. 4D and E). The genomic maps of these viruses are displayed in Fig. 5, along with the genomic map of XTV-1 (13).

The genomes of these XTV viruses resembled those of the PTV viruses. First, as each genome was unique in structure, they were apparently derived by recombination and contained nonecotropic sequences in *env* and the U3 region. Second, the *gag* gene markers suggest participation of *Emv-1* and *Emv-3* viruses in the generation of these recombinants. However, significant differences between the XTV and PTV isolates were evident in the *env* region. Specifically, all *env* oligonucleotides found in *Emv-1* and *Emv-3* were absent in the XTV viruses, including those retained by the PTV viruses. Also, the PTV-related oligonucleotides 102, 111, and 113 that map within *env* were also missing. These observations strongly suggest that the variation in the in vitro host range of the XTV viruses relative to the PTV viruses was due to differences in the structure of the envelope proteins.

Thus, HRS/J mice produced at least two types of polytropic viruses, both of which were isolated from thymic tissues. These viruses differed in phenotype and genotype and were generated by recombination between *Emv-1*, *Emv-3*, and other endogenous viral sequences.

**PTV-type viruses accelerated leukemogenesis in HRS/J mice.** Various HRS/J isolates were tested for their ability to accelerate the development of lymphoma after injection into neonatal HRS/J mice. All five of the PTV viruses accelerated the onset of leukemia, whereas XTV-3 was inactive (Table 3). Prior tests of ETV-1 and XTV-1 were similarly negative (13). PTV-1, PTV-2, and PTV-4 also produced tumors in the CBA/J strain which has a low incidence of leukemia (13; data not shown).

TABLE 2. HRS/J viruses (thymic isolates)

| Virus | Mouse | | Virus isolation | | Leukemogenicity[a] | Source of RNA fingerprint |
|---|---|---|---|---|---|---|
| | Age (mo) | Genotype | Cocultivation cell | Technique | | |
| Ecotropic viruses | | | | | | |
| HRN-T-1 | 2 | *hr/hr* | NIH 3T3 | Unpassaged | NT | Fig. 1D |
| HR+N-T-1 | 2 | *hr/+* | NIH 3T3 | Unpassaged | NT | Fig. 1E |
| ETV-1 | 12 | *hr/hr* | NIH 3T3 | Endpoint dilution of NIH 3T3 cells | 0 | Fig. 1F |
| ETV-2 | 8 | *hr/hr* | NIH 3T3 | Endpoint dilution of NIH 3T3 cells | NT | Green et al. (13) |
| Polytropic viruses | | | | | | |
| PTV-1 | 13 | *hr/hr* (leukemic) | Mink | Endpoint dilution of NIH 3T3 and mink cells | + | Green et al. (13) |
| PTV-2 | 9 | *hr/+* | Mink | Endpoint dilution of NIH 3T3 and mink cells | + | Fig. 6A; Green et al. (13) |
| PTV-HR-2[b] | 5 | *hr/+* (leukemic) | Mink | Unpassaged | NT | Fig. 6B |
| PTV-3 | 9 | *hr/hr* | Mink | Endpoint dilution of NIH 3T3 and mink cells | + | Fig. 4A |
| PTV-4 | 12 | *hr/+* | Mink | Endpoint dilution of NIH 3T3 and mink cells | + | Fig. 4B |
| PTV-5 | 12 | *hr/hr* | Mink | Endpoint dilution of NIH 3T3 and mink cells | + | Fig. 4C |
| XTV-1[c] | 8 | *hr/hr* | Mink | Endpoint dilution of NIH 3T3 and mink cells | 0 | Green et al. (13) |
| XTV-2[c] | 14 | *hr/+* | Mink | Endpoint dilution of NIH 3T3 and mink cells | NT | Fig. 4E |
| XTV-3[c] | 14 | *hr/+* | Mink | Endpoint dilution of NIH 3T3 and mink cells | 0 | Fig. 4D |

[a] NT, Not tested, 0, no leukemogenicity; +, positive leukemogenicity.
[b] Virus from mouse injected with PTV-2.
[c] Virus replicates poorly on NIH 3T3 cells.

To confirm that these induced tumors produced the same virus that was injected, viruses were recovered from leukemic cells and subjected to RNA fingerprinting. For each of the PTV isolates, the fingerprint of the recovered virus was clearly recognizable as that of the injected virus (data not shown). However, occasional single oligonucleotide changes were seen. For instance, the fingerprints of viruses isolated from two different leukemic mice that were injected with PTV-2 are shown in Fig. 6. The first was identical to PTV-2 itself, but PTV-HR-2 did not contain the gp70 oligonucleotide 102. Instead, an oligonucleotide migrating just to the left of position 102 was observed. This oligonucleotide, designated 102L, was most likely derived from a point mutation in the nucleotide sequence of PTV-2 oligonucleotide 2. The difference in migration of 102L, relative to 102 on our gels, was consistent with a base substitution of a uridine for a cytosine, and the RNase A digestion products of these two oligonucleotides were identical (data not shown). This sequence change is unlikely the result of recombination in vivo, as the 102L oligonucleotide has never been observed in any other HRS/J or AKR isolates (13, 22, 27, 31, 38; unpublished data). Thus, all our PTV viruses were leukemogenic and differed genetically from the nonleukemogenic XTV and ETV viruses most consistently within *env*. In some cases, in vitro and in vivo passaging of these viruses resulted in point mutations that were detected by fingerprinting.

## DISCUSSION

The studies of the genetic structure of HRS/J viruses reported here suggest a specific sequence of events in the molecular evolution of leukemogenic recombinant viruses within individual mice of this strain. We showed that both endogenous ecotropic proviral loci present in HRS/J mice, *Emv-1* and *Emv-3*, were expressed. In addition, genetic recombination between these viral RNAs was clearly demonstrated in $T_1$-oligonucleotide fingerprints of unpassaged HRS/J viruses. We observed a similar phenomenon in ecotropic viruses recovered from two other strains, curly-whisker (*cw*) mice and SEA/J, that also carry the *Emv-1* and *Emv-3* proviruses (unpublished data). The frequent recovery of hybrid genomes suggests that recombination confers a selective advantage for viruses with this type of structure. Alternatively, such observations may be due to random recombination events either in vivo or in vitro. The poor infectivity of a molecularly cloned *Emv-3* provirus and lack of infectivity of a cloned *Emv-1* locus in DNA transfection assays support the former interpretation (8; N. Jenkins, personal communication). Furthermore, cotransfection of

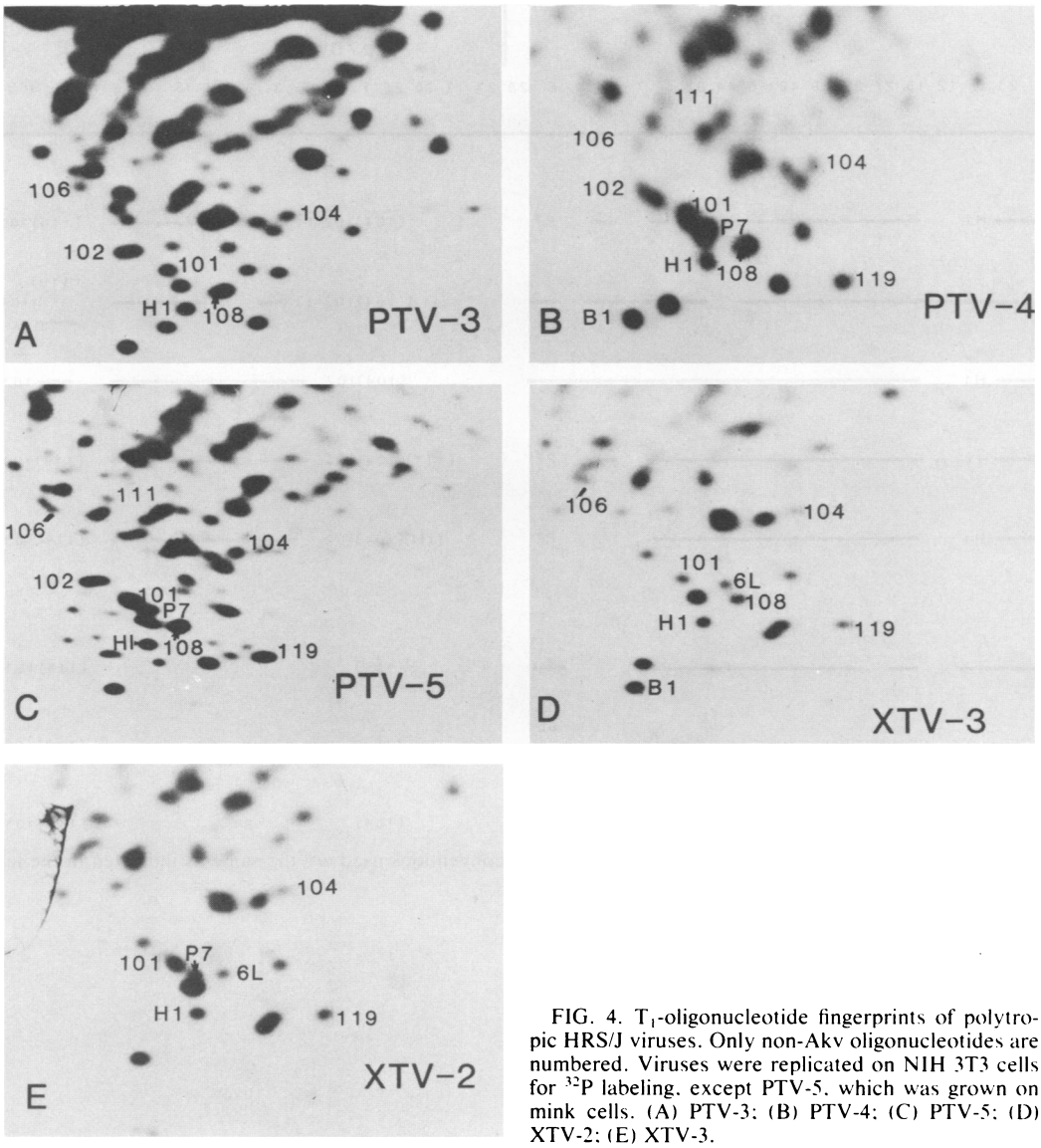


FIG. 4. $T_1$-oligonucleotide fingerprints of polytropic HRS/J viruses. Only non-Akv oligonucleotides are numbered. Viruses were replicated on NIH 3T3 cells for $^{32}P$ labeling, except PTV-5, which was grown on mink cells. (A) PTV-3; (B) PTV-4; (C) PTV-5; (D) XTV-2; (E) XTV-3.

*Emv-1* with *Emv-3* DNA dramatically increases infectivity, and marker rescue experiments indicate that the defect in the *Emv-3* provirus maps within *gag*. Therefore, the studies of the molecularly cloned *Emv-1* and *Emv-3* proviruses and our studies of the genomic structure of the corresponding HRS/J viruses suggest that these proviruses may be completely or partially defective. These defects may be overcome by reciprocal genetic complementation between these two viral sequences. Similar conclusions about the interaction of endogenous ecotropic proviruses were formed by McCubrey and Risser (23) from studies of the F1 progeny of mice carrying *Emv-1* or *Emv-2*. The ability to induce ecotropic viruses from tissues of the hybrid mice was markedly enhanced when compared with cells from either parental strain, suggesting that complementation for virus expression occurred in the hybrids. Moreover, crosses between BALB/Mo mice that carried different defective endogenous Moloney proviruses yielded viremic animals (36). Defects in the structure of *Emv-1* and *Emv-3* proviruses may partially explain the reason that certain strains of mice that carry only one of them, such as CBA/J and DBA/J, are low virus producers and have a low incidence of leukemia (16, 35).

We also documented that the progeny of *Emv-1* and *Emv-3* can recombine with other endogenous viral sequences in HRS/J mice. The genomes of the ecotropic viruses ETV-1 and a minor component of the HR+N-T-1 mixture possessed $T_1$-oligonucleotides that were not derived from either *Emv-1* or *Emv-3*. These non-*Emv* oligonucleotides were recognizable because of their presence in the p15E gene and the U3 region of many leukemogenic and polytropic viruses of HRS/J and AKR/J mice (13, 31, 38). We have proposed that ecotropic viruses with recombinant sequences of this type in the p15E-U3 regions of the genome play an important role in the evolution of leukemogenic polytropic viruses in individual AKR mice (38). Several lines of evidence support this hypothesis. (i) Viruses with similar structures occur in young preleukemic AKR mice, but not in control mice or (AKR × NZB) F1 mice that do not develop leukemia, despite expression of the Akv virus (9, 38). (ii) The structures of integrated proviruses and gp85 precursor envelope polypeptides in

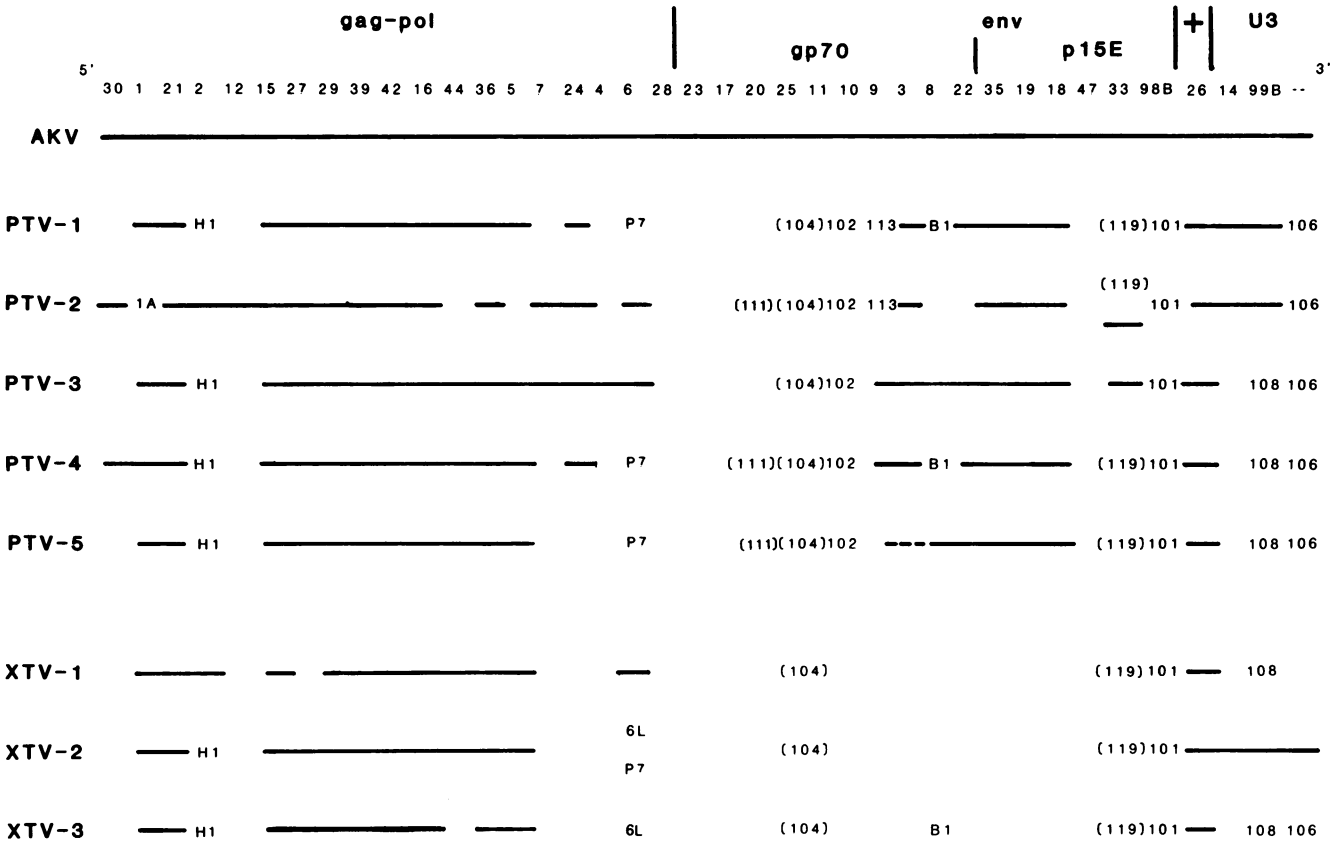


FIG. 5. $T_1$-oligonucleotide maps of polytropic HRS/J viruses. The conventions used are the same as indicated in the legend to Fig. 2.

some AKR tumor cells are consistent with infection of the cells by viruses of this genotype (15; N. G. Famulari and D. Cieplenski, Virology, in press). (iii) Sequencing data of endogenous proviral DNAs are consistent with independent origin of the recombinant U3 and the gp70 gene sequences in leukemogenic polytropic AKR viruses (18). (iv) The 3′ end of *env* or the U3 region or both appear to carry the oncogenic potential of leukemogenic ecotropic viruses derived from other strains or passaged AKR isolates (3, 4, 10, 20). (v) Hybrid genomes between Akv and the leukemogenic recombinant AKR MCF 247 virus have been constructed. One of these viruses, which contains p15E-U3 sequences derived from MCF 247, closely resembles the recombinant ecotropic viruses that we isolated from both AKR/J and HRS/J mice

TABLE 3. Leukemia acceleration assay of PTV viruses in HRS/J mice[a]

| Virus | Incidence of leukemia/ total (%) |
|---|---|
| PTV-1 | 49/75 (65.3) |
| PTV-2 | 24/43 (55.8) |
| PTV-3 | 29/63 (46.0) |
| PTV-4 | 21/39 (53.8) |
| PTV-5 | 40/58 (68.9) |
| XTV-3 | 1/11 (9) |
| Control | 7/45 (15.5) |

[a] Neonatal mice were injected with $6 \times 10^3$ to $8 \times 10^3$ PFU of purified virus and observed for 6 months.

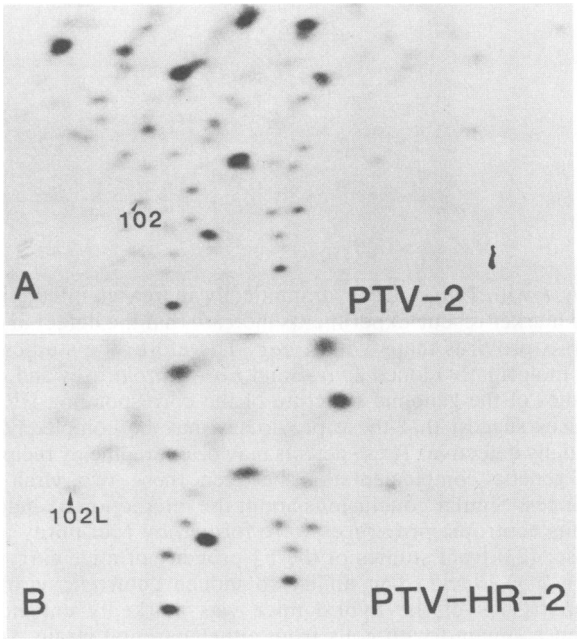


FIG. 6. $T_1$-oligonucleotide fingerprints of PTV-2 (A) and PTV-HR-2 (B). The latter was recovered from an HRS/J mouse that developed thymoma after injection of PTV-2. Note the change in migration of PTV-2 oligonucleotide 102, referred to as 102L, in PTV-HR-2. Background oligonucleotides in the PTV-2 fingerprint represent an additional unidentified RNA species present in the isolate.

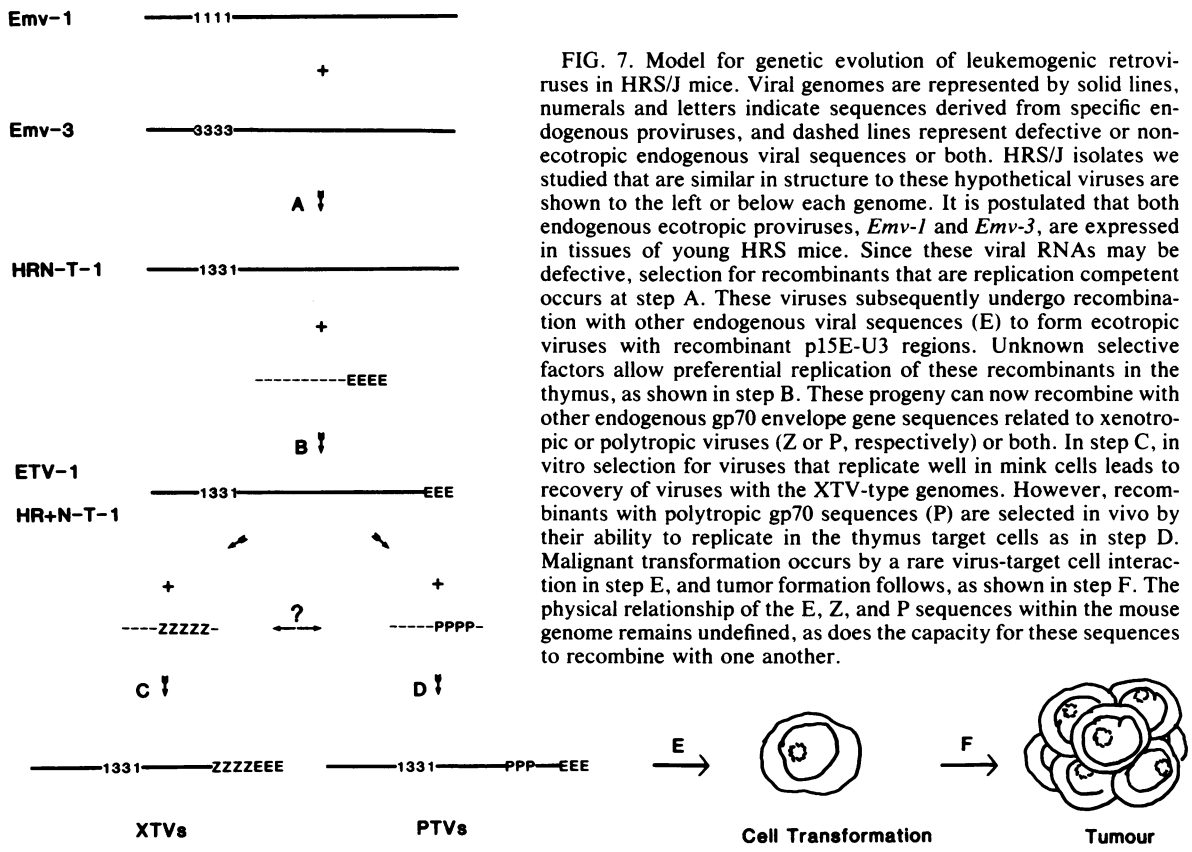


FIG. 7. Model for genetic evolution of leukemogenic retroviruses in HRS/J mice. Viral genomes are represented by solid lines, numerals and letters indicate sequences derived from specific endogenous proviruses, and dashed lines represent defective or nonecotropic endogenous viral sequences or both. HRS/J isolates we studied that are similar in structure to these hypothetical viruses are shown to the left or below each genome. It is postulated that both endogenous ecotropic proviruses, *Emv-1* and *Emv-3*, are expressed in tissues of young HRS mice. Since these viral RNAs may be defective, selection for recombinants that are replication competent occurs at step A. These viruses subsequently undergo recombination with other endogenous viral sequences (E) to form ecotropic viruses with recombinant p15E-U3 regions. Unknown selective factors allow preferential replication of these recombinants in the thymus, as shown in step B. These progeny can now recombine with other endogenous gp70 envelope gene sequences related to xenotropic or polytropic viruses (Z or P, respectively) or both. In step C, in vitro selection for viruses that replicate well in mink cells leads to recovery of viruses with the XTV-type genomes. However, recombinants with polytropic gp70 sequences (P) are selected in vivo by their ability to replicate in the thymus target cells as in step D. Malignant transformation occurs by a rare virus-target cell interaction in step E, and tumor formation follows, as shown in step F. The physical relationship of the E, Z, and P sequences within the mouse genome remains undefined, as does the capacity for these sequences to recombine with one another.

(C. Holland, personal communication). This new virus is moderately active in accelerating the development of leukemia after injection into neonatal AKR mice, although it is not as active as the leukemogenic parent MCF 247. Our ETV-1 virus, a genetically similar virus, gave negative results in similar experiments (13). It is possible that the potential for leukemia acceleration by ecotropic viruses of this type varies, as has been noted with polytropic recombinant AKR viruses (7, 25, 26, 33).

We also found that recombination between *Emv-1*, *Emv-3*, and other endogenous viral sequences that were expressed generated at least two types of polytropic viruses. The PTV group of viruses accelerated leukemogenesis after injection into neonatal HRS mice, whereas viruses of the XTV group did not. Comparison of the genomes of the leukemogenic PTV viruses with those of the nonleukemogenic Akv, ETV-1, XTV-1, and XTV-3 viruses suggested that the oncogenic phenotype is mediated by the *env* genes and possibly the U3 region. Similar conclusions were reached by Lung et al. after analysis of the genomic structures of leukemogenic and nonleukemogenic AKR viruses (22). They noted that the non-Akv oligonucleotides 108 and 106 were invariably present in the U3 region of the genome of the leukemogenic viruses. Oligonucleotide 106 was also present in all our PTV viruses, probably because it is also contained in the *Emv-1* and *Emv-3* virus, but oligonucleotide 108 was found in only three of them. PTV-1 and PTV-2 were leukemogenic, and yet their genomes did not contain this particular oligonucleotide. Using only our fingerprint data, we could not exclude the possibility that other recombinant non-*Emv* sequences were present within the U3 region. On the other hand, we concluded that the presence of oligonucleotide 108 itself within the viral genome was not required for leukemogenesis by these two recombinant HRS/J viruses.

The XTV isolates we examined replicated poorly on NIH 3T3 mouse cells, and the two XTV viruses tested were nonleukemogenic. They differed from the PTV isolates by the extent and type of substituted non-*Emv* sequences, particularly in the *env* region. We believe that the XTV viruses represented a minority of the recombinant viruses that were present in the original virus mixture derived by cocultivation of thymocytes on mink cells. Selection in vitro for these viruses occurred during the endpoint dilution procedure and the multiple rounds of replication of the viruses in mink cells. The XTV viruses described here are probably not involved in leukemogenesis, as they are nonleukemogenic and replicate poorly on mouse cells, implying a limited ability to multiply in vivo. Also, we never observed oligonucleotides unique to the XTV viruses in unpassaged virus mixtures recovered from either HRS/J or AKR/J mice (unpublished data). However, it is possible that the XTV viruses may have represented intermediates in the formation of the leukemogenic recombinant PTV viruses.

In summary, we propose a model for in vivo generation of leukemogenic recombinant viruses in individual HRS/J mice (Fig. 7). Portions of this scheme are similar to that of Hartley et al. (14), which postulates that the formation of leukemogenic AKR viruses is the result of recombination between endogenous ecotropic and xenotropic viruses and to a model we have previously proposed to explain viral leukemogenesis in the AKR strain (38). We believe that leukemogenic HRS/J viruses arise in vivo by a process of stepwise conver-

gent evolution of recombinant ecotropic and polytropic viruses. These events are dependent on the presence, expression, replication, recombination of certain endogenous viral sequences, on other genetic factors inherited by HRS/J mice that control selection of the leukemogenic viruses and transformation of the target thymocytes.

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