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Genetic Alterations of RNA Leukemia Viruses Associated with the Development of Spontaneous Thymic Leukemia in AKR/J Mice

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T_1-oligonucleotide fingerprinting and mapping were used to study the expression of RNA leukemia viruses in leukemic and preleukemic AKR/J mice, with techniques designed to minimize the loss or inadvertent selection of viruses in vitro before biochemical analysis. In leukemic animals, complex mixtures of ecotropic and mink-tropic viruses were expressed. Unique but similar polytropic virus-like genomes were present in each tumor isolate. In preleukemic mice, viral isolates from the thymus that were grown on NIH3T3 fibroblasts contained genomes with non-Akv polytropic virus-related oligonucleotides. This phenomenon was not evident in fingerprints of viruses from the spleen and bone marrow of the same animals. Remarkably, the non-Akv oligonucleotides located in the 3′ portion of the P15E gene, the U3 noncoding region, and the 5′ part of the gp70 gene were often expressed independently. Our results suggest the following. (i) Recombinant viruses can be detected in the thymuses of young preleukemic AKR mice and increase in relative abundance with age. (ii) During in vivo generation of the recombinant leukemogenic viruses, the selection of polytropic virus-related sequences in the 3′ part of p15E and the U3 region and the 5′ portion of gp70 occurs independently. (iii) Independent biological properties encoded in the gp70 and p15E regions of *env* of the recombinant viruses may mediate viral selection or leukemogenicity. (iv) The leukemogenic polytropic viruses of AKR/J mice arise via genetic recombination involving at least three endogenous viral sequences.

The development of spontaneous thymic lymphoma in AKR mice is accompanied by complex phenotypic and genetic alterations in the type C retroviruses which these mice produce (8–10, 12, 17, 21). Viruses isolated from neonatal or young AKR mice are ecotropic in the in vitro host range, replicating only in murine cells. These viruses are non-leukemogenic when injected into mice. In the late preleukemic and leukemic periods, these mice produce other viruses with extended host range properties. These viruses are called polytropic, dual-tropic, or MCF (mink cell focus-forming) viruses and can replicate in vitro in both murine and non-murine cells such as mink fibroblasts (9). Many of these isolates are leukemogenic when injected into strains of mice with a low incidence of leukemia and accelerate the development of thymic lymphoma in young AKR mice (4, 22). Xenotropic viruses are a third class of viruses rarely isolated from AKR mice and have an in vitro host range restricted to non-murine cells (9, 12, 14).

Genetic analysis with T_1-oligonucleotide fingerprinting and mapping of viral RNA, restriction enzyme mapping of viral DNA, and animal breeding experiments show that the ecotropic non-leukemogenic virus (Akv) of young AKR mice is the product of one of several closely related endogenous proviral loci present in the DNA of these mice, such as *Akv-1* and *Akv-2* (5, 8, 24, 25). Comparison of Akv with leukemogenic polytropic viruses shows that genomic differences are clustered within the U3 noncoding region and in the *env* gene encoding the virion surface proteins. Within *env*, these differences are located primarily in the 5′ portion of the gp70 gene and in the 3′ end of the p15E gene (3, 15, 21). Presumably, these polytropic sequences are responsible for the novel host range and leukemogenic activity of this class of viruses. Nearly identical genetics are observed in viral isolates from HRS/J and C58 mice; other inbred strains develop spontaneous thymic lymphomas (8, 15; C. Y. Thomas, R. H. Khiroya,

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R. S. Schwartz, and J. M. Coffin, manuscript in preparation).

The generation of the leukemogenic polytropic virus through recombination of the endogenous viral sequences in both AKR/J and HRS/J mice is strongly suggested by studies in which genetically similar but unique viruses were isolated from individual mice (8, 15; Thomas et al., in preparation). In addition, analysis of the polytropic viral envelope glycoprotein reveals the presence of both ecotropic and xenotropic virus-related peptides (7). It has been proposed that these leukemogenic viruses originate by genetic recombination between endogenous ecotropic (Akv) and xenotropic viruses (9, 10). However, the origin and role of the non-Akv viral sequences and the events surrounding such a genetic recombination have not been well studied.

In this report we examine the genomes of viruses which are expressed during the development of spontaneous thymic lymphomas in AKR/J mice. To prevent the alteration or loss of viral RNA sequences by extensive in vitro passage or endpoint dilution purification, we labeled the isolates on their original infected cell line, which was subcultured five to ten times. From our observations of the genetic composition of virus mixtures obtained from tissues of preleukemic and leukemic mice, we propose a model for stepwise recombination during the in vivo generation of leukemogenic viruses. Our model postulates a recombination between three distinct parental endogenous viruses, with independent in vivo selection for the non-Akv sequences found in gp70 and the p15E region.

MATERIALS AND METHODS

Mice. AKR/J mice were purchased from Jackson Laboratory and maintained by sibling mating in the Tufts University animal care facility.

Cells. NIH3T3 mouse fibroblasts were obtained from the laboratory of N. Rosenberg, Tufts University, and maintained in Eagle minimal essential medium fortified with 4× amino acids and 10% fetal calf serum. Mink lung fibroblasts (CCL64) were a gift from R. Schwartz, Tufts University. They were grown in McCoy 5a media with 10% calf serum.

Viruses. The technique used for the isolation of viruses from mice was similar to that described by Green et al. (8). Briefly, NIH3T3 and mink cells were plated at 5×10^5 and 2×10^5 cells per 60-mm petri dish, respectively, and 24 h later 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 2×10^6 to 3×10^6 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 (22). 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.

T_1-oligonucleotide fingerprinting of the viral RNA. Uniformly labeled RNA was prepared as previously described for avian viruses (1). 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 the second-dimension buffer consisted of 89 mM Tris base–89 mM boric acid–2.5 mM disodium EDTA at pH 8.0 (19). Most of the oligonucleotides could be identified unambiguously by their electrophoretic mobility. However, in most cases confirmation was obtained by elution of the oligonucleotides from the gels, subdigestion with RNase A, and separation of the products on DEAE paper by high-voltage ionophoresis (2).

Mapping of oligonucleotides. Maps of the viral genomes were constructed by using previously published maps (6, 15, 18), recent sequencing data (N. Hopkins, personal communication), and unpublished data from our studies on the viruses of HRS/J mice. Numbers were assigned by the convention of Rommelaere et al. and Green et al. (8, 20). Oligonucleotides 98B and 99B were originally numbered by Pederson et al. (19). Because complex mixtures were present in some cases, the existence of different viruses was inferred from the differences in molar yield of the oligonucleotides or from the presence of known allelic oligonucleotides.

RESULTS

Isolation of viruses from leukemic mice. AKR/J mice that were developing spontaneous lymphomas were sacrificed, and single-cell suspensions obtained from the thymomas were cocultivated with mink or NIH3T3 cells. To minimize the loss, alteration, or inadvertent selection of viruses that could result from repeated passages or endpoint dilution, we grew the primarily infected adherent cells for five or ten passages, and the culture supernatants were then tested for reverse transcriptase activity. Positive cultures derived from the originally infected cell lines were used to obtain ^{32}P-labeled viral RNA for subsequent analysis.

In all of the leukemic animals tested, virus was detected in the supernatants from both mink and NIH3T3 mouse fibroblasts within two to five passages. For this study, we chose five isolates obtained from four leukemic AKR mice between 10 and 13 months of age (Table 1).

Isolation of viruses from preleukemic animals.

TABLE 1. AKR/J viruses analyzed

Virus[a]	Source	Age (mo)	Cocultivation cells	Figure
Leukemic mice				
AKM-2	Thymoma	10.5	Mink	1B
AKM-3	Thymoma	11.5	Mink	1C
AKM-T-6	Thymoma	10.0	Mink	1D
AKM-15	Thymoma	13.0	Mink	1E
AKN-15	Thymoma	13.0	NIH3T3	1F
Preleukemic mice[b]				
AKN-T-16	Thymus	1.5	NIH3T3	2A
AKN-S-16	Spleen	1.5	NIH3T3	
AKN-T-20	Thymus	2.0	NIH3T3	2B
AKN-S-20	Spleen	2.0	NIH3T3	
AKN-T-11	Thymus	3.0	NIH3T3	2C
AKN-S-11	Spleen	3.0	NIH3T3	1A
AKN-14	Thymus	7.0	NIH3T3	2D
AKM-T-18	Thymus	8.5	Mink	2E
AKN-T-18	Thymus	8.5	NIH3T3	2F
AKN-S-18	Spleen	8.5	NIH3T3	
AKN-B-18	Bone marrow	8.5	NIH3T3	

[a] Conventions for naming the viruses: first two letters indicate the strain of origin (AK, AKR), third letter indicates cell type used to isolate virus, and fourth letter, if present, indicates organ from which virus was isolated. If fourth letter is not present, the isolate was from thymic tissues. Numbers represent individual animals.

[b] These mice had no evidence of gross disease on autopsy.

In this set of experiments, preleukemic AKR/J mice of various ages were selected at random for study. The term preleukemic is used here to indicate the time interval in the life span of the animal from birth until the development of thymic lymphoma. None of these animals had gross evidence of lymphoma when autopsied, although the presence of microscopic foci of malignant cells was not excluded. Spleen, thymus, or bone marrow single-cell suspensions were cocultivated with NIH3T3 or mink cells as described above. Ecotropic viruses were isolated from all of the animals and tissues studied (Table 1). AKM-T-18 was the only mink-tropic virus recovered. Ten passages of the infected mink cells were required before this virus could be detected and subsequently fingerprinted. In other experiments, we recovered mink-tropic viruses from younger preleukemic animals, but again only after prolonged culturing on fibroblasts. Though not quantitated, we found that the difficulty of isolating mink-tropic virus from the thymuses of preleukemic animals as compared with leukemic animals was a reproducible phenomenon. Similar results have been reported by others (4, 12, 14).

Genomes of viruses from leukemic AKR/J mice. Viral RNA was uniformly labeled with ^{32}P, purified, digested with RNase T_1, and subjected to two-dimensional electrophoresis as previously described (1, 8). The resultant T_1-oligonucleotide fingerprints of these viruses and AKN-S-11 (identical to Akv) are shown in Fig. 1. These fingerprints are rather complex, but can be readily interpreted as mixtures of two major RNA components.

One RNA component contained oligonucleotides known to be present in the previously described ecotropic and polytropic viruses of AKR/J and HRS/J mice (8, 21; Thomas et al., in preparation). Oligonucleotide maps representative of the linear viral genomes were then constructed as described above (Fig. 3A). Although similar in structure, the genomes differed slightly from one another. Non-Akv sequences were concentrated in the 5′ portion of gp70 and 3′ part of p15E, consistent with previously published genomic maps of polytropic viruses (8, 15, 21). In addition, most of the viruses also contained polytropic virus-related sequences clustered in the U3 region. However, AKM-6 retained the Akv U3 region oligonucleotides 14 and 99B and did not contain the polytropic-related oligonucleotide 106. In the following discussion, the polytropic virus-related oligonucleotides in the gp70-coding portion of *env* which are not found in Akv (i.e., oligonucleotides 102, 103, 104, 111, 113, and B1) will be referred to collectively as "gp70 oligonucleotides." Similarly, the non-Akv oligonucleotides found in the 3′ portion of the p15E-coding region and the U3 region (oligonucleotides 114, 119, 101, 108, and 106) will be referred to as "3′ oligonucleotides."

The other RNA species, with oligonucleotides indicated by the "x" prefix (Fig. 1B), was present in varying molar yields. We have seen nearly identical sequences in mink-tropic virus mixtures from HRS/J mice (8). Several observations indicated that this RNA was not directly involved in leukemogenesis. First, these oligonucleotides were not present in known leukemogenic viruses. Second, nearly identical sequences have been found in xenotropic viruses isolated from mink cells cocultivated with normal splenocytes from NZB mice (C. Thomas, S. Datta, and J. Coffin, unpublished data). Third, this RNA has been seen only in viruses released from mink and rabbit SIRC cells and not in those from murine NIH3T3 or SC-1 cells (Thomas et al, unpublished data). Therefore, we believe this RNA represents the genome of an endogenous xenotropic virus or possibly sequences of nonmurine origin.

Of interest is the fingerprint of AKN-15 (Fig. 1F). This virus was derived from the same tumor

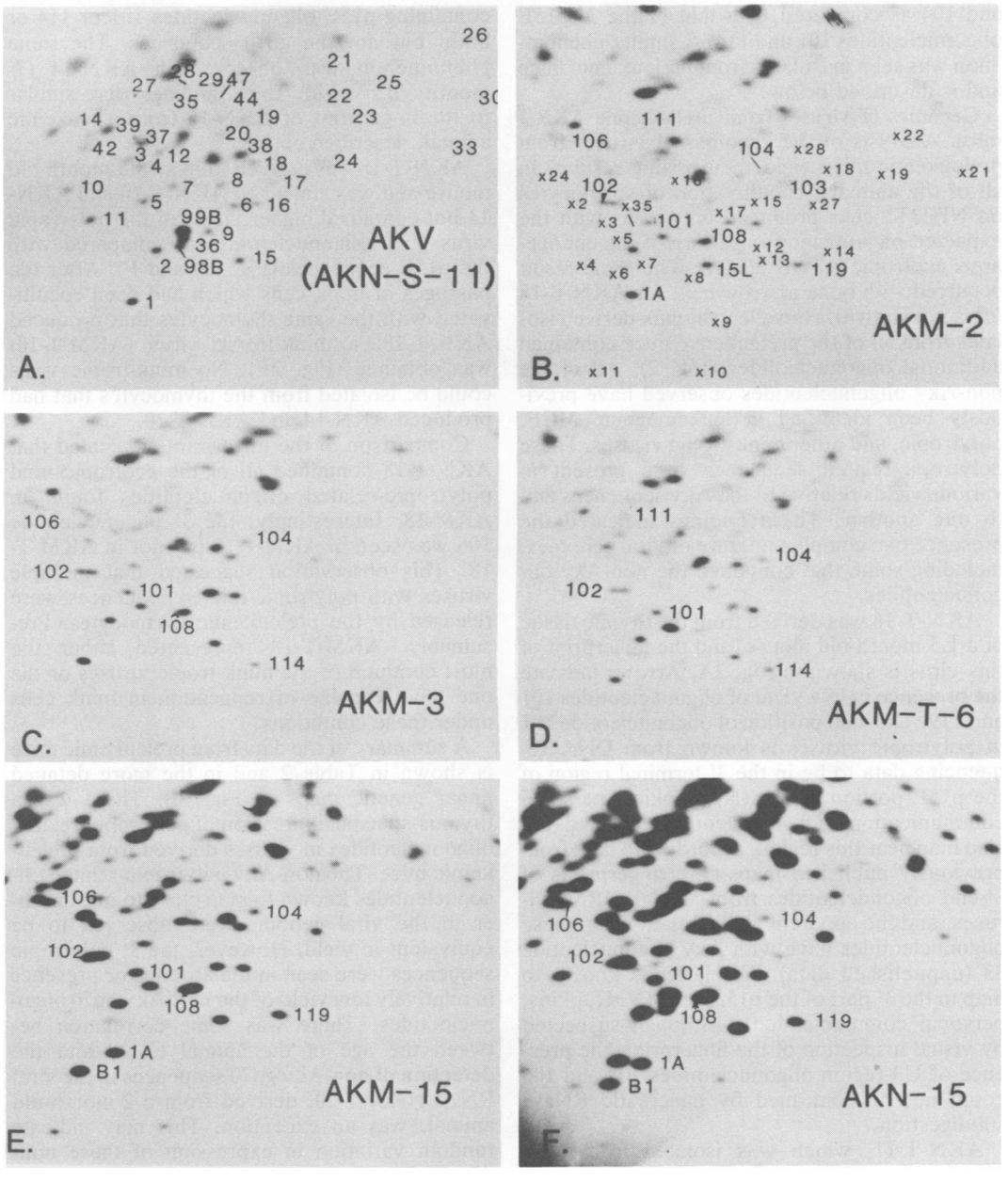

FIG. 1. Viruses isolated from leukemic AKR/J mice. The fingerprint of the genome AKN-S-11 grown on NIH3T3 cells is shown for comparison in panel A. The pattern is identical to that of endogenous ecotropic Akv (20). Oligonucleotides were numbered as described in the text. In panels B through F, only oligonucleotides not found in Akv are numbered. The x-oligonucleotides are numbered in panel B only, although they were present in panels C through F. (A) AKN-S-11 (Akv); (B) AKM-2; (C) AKM-3; (D) AKM-T-6; (E) AKM-15; (F) AKN-15.

cells as AKM-15 but was isolated on NIH3T3 fibroblasts. As can be seen, AKN-15 was also a mixture of viruses, except that it appeared to contain polytropic and the endogenous ecotropic Akv viruses. There was no evidence of the putative xenotropic virus seen in the mink isolates. The polytropic virus-related oligonucleotides were identical to those found in AKM-15 (Fig. 1E and F). Note, however, the lower relative yield of the gp70 oligonucleotides 102

and 104 as compared with that of the 3′ p15E oligonucleotides 101 and 114. A similar phenomenon was seen in isolates from preleukemic mice and is described below.

Genomes of viruses from preleukemic AKR/J mice. Analysis of the genomes of viruses from preleukemic mice yielded surprising results. In all of the animals, cocultivation of splenocytes on NIH3T3 cells produced only virus with the expected oligonucleotide pattern of the endogenous ecotropic Akv (Fig. 1A). The same result occurred with bone marrow cells for AKN-B-18 (data not shown). However, thymus-derived isolates from all of the preleukemic mice contained additional oligonucleotides (Fig. 2). All of the non-Akv oligonucleotides observed have previously been identified in leukemogenic MCF, polytropic, and other mink-tropic viruses. These polytropic-related sequences were present in various yields relative to the Akv sequences and to one another. These findings indicated the presence of a complex mixture of viral genomes, including some that contained the non-Akv oligonucleotides.

AKN-T-16 was derived from the thymic tissue of a 1.5-month-old mouse, and the fingerprint of this virus is shown in Fig. 2A. Arrows indicate the presence in low yield of oligonucleotides 101 and 119. The map position of oligonucleotide 101 in polytropic viruses is known from DNA sequencing data to be in the 3′-terminal region of the p15E portion of *env* (N. Hopkins, personal communication). Oligonucleotides 114 and 119 also map near this region, according to data from previously published maps (15), fingerprints of 3′-end oligonucleotides from similar HRS/J viruses, and the apparent allelic relationship these oligonucleotides have with Akv oligonucleotide 33 (unpublished data). The latter is known to map in the 3′ part of the p15E gene (N. Hopkins, personal communication). Although suspected by visual inspection of the fingerprint, the presence of U3-region oligonucleotides 108 and 106 could not be confirmed by pancreatic RNase subdigestion.

AKN-T-11, which was isolated from a 3-month-old mouse, demonstrated clearly the presence of the 3′ p15E oligonucleotides 119 and 101 and the U3 region oligonucleotides 108 and 106 (Fig. 2B). Notable was the absence of any of the gp70 oligonucleotides. AKN-T-20 from a 2-month-old animal produced a fingerprint pattern with both the 3′ and gp70 oligonucleotides (Fig. 2C). Although the p15E oligonucleotides 101 and 114 were clearly visible, the gp70 oligonucleotides 113 and 102 were observed in low yield. Their existence was confirmed by pancreatic RNase digestion and electrophoresis (data not shown). The relative yield of these oligonucleotides indicated the presence of viral sequences containing p15E oligonucleotides 101 or 114 or both, but not the gp70 sequences. The same phenomenon was observed in AKN-14 (7-month-old mouse). These findings were similar to the fingerprint of AKN-15 from a leukemic animal, described above.

AKN-T-18 was derived from an 8.5-month-old mouse and was similar to AKN-T-20 and AKN-14 but contained higher yields of the polytropic virus gp70 oligonucleotides as compared with the p15E sequences (Fig. 2D and F). After ten passages of mink cells which had been cocultivated with the same thymocytes that produced AKN-T-18, a mink-tropic virus (AKM-T-18) was obtained (Fig. 2E). No mink-tropic virus could be isolated from the thymocytes that had produced AKN-14 and AKN-T-20.

Comparison of the fingerprints indicated that AKN-T-18 contained all of the ecotropic- and polytropic-related oligonucleotides found in AKM-18. Interestingly, the 3′ oligonucleotide 106 was seen in AKN-T-18 but not in AKM-T-18. This observation suggested that multiple viruses with polytropic-related sequences were released by the preleukemic thymocytes. Presumably, AKM-T-18 represented either the most common of the mink-tropic viruses or the one most capable of replication in mink cells under these conditions.

A summary of the data from preleukemic mice is shown in Table 2 and in the more detailed linear genetic maps in Fig. 3B. There was a thymus-specific expression of polytropic-related oligonucleotides in viruses derived from preleukemic mice. The non-Akv polytropic-related oligonucleotides known to map close to one another in the viral genome were observed to be equivalent in yield. However, the 3′ polytropic sequences were seen in the absence or presence in relatively low yield of the non-Akv gp70 oligonucleotides. There was some correlation between the age of the animal tested and the detection of non-Akv gp70 sequences in the viral RNA. AKN-T-20, derived from a 2-month-old animal, was an exception. This may indicate random variation in expression of these non-Akv gp70 sequences within the AKR population. Alternatively, these sequences may have been present in all of the thymic viral isolates but occasionally undetectable under our experimental conditions.

DISCUSSION

The most surprising result in our experiments was the independent expression of non-Akv p15E sequences in viruses isolated from the thymuses of young, preleukemic AKR/J mice. All of the known leukemogenic AKR/J and HRS/J polytropic viruses contain non-Akv sequences in the 3′ p15E and 5′ gp70 regions of the

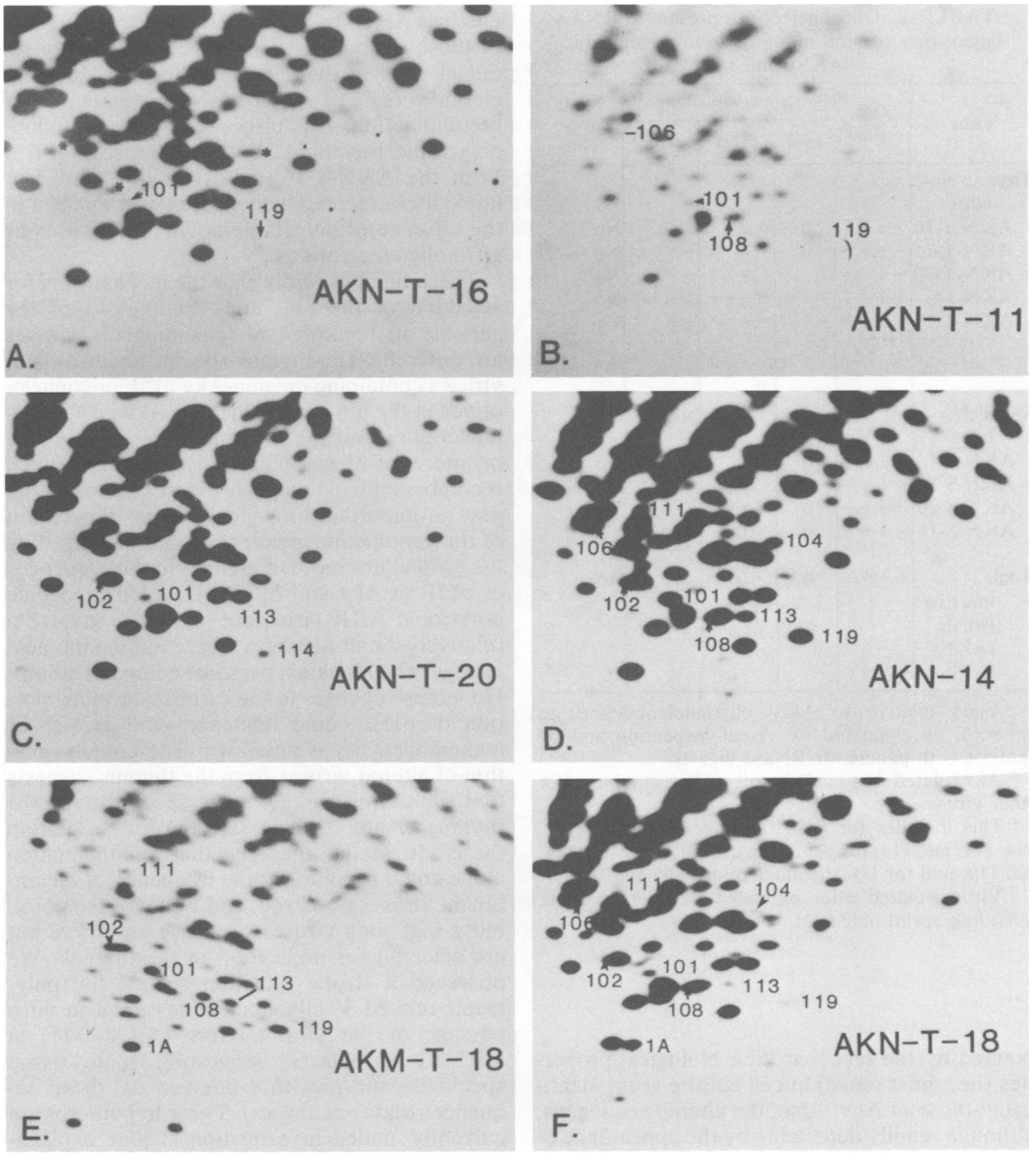

FIG. 2. Genomes of viral isolates from thymuses of preleukemic AKR/J mice. In all panels, only non-Akv oligonucleotides are numbered. Asterisks indicate unidentified oligonucleotides which may be nonviral in origin. Fingerprints can be compared to AKN-S-11 (Fig. 1A), which was identical to endogenous Akv, to all of the splenic isolates from preleukemic mice, and to the bone marrow-derived AKN-B-18 (data not shown). (A) AKN-T-16; (B) AKN-T-11; (C) AKN-T-20; (D) AKN-14; (E) AKM-T-18; (F) AKN-T-18.

genomes (8, 15, 21). Remarkably, the non-Akv 3′ oligonucleotides were often present in excess of the gp70 oligonucleotides. Therefore, it is impossible for the former to be associated exclusively with polytropic viral genomes. These data are most consistent with the presence of recombinant viruses that do not have a polytropic host range. The genomes of such viruses would be identical to that of Akv except for sequences in the 3′ portion of p15E or the U3 region or both. A virus of this type has been isolated from the thymus of an HRS/J mouse (8).

We detected viruses with recombinant 3′ sequences in the youngest AKR mice (1.5 months) we examined, but they have apparently escaped notice in previous studies. This may be ex-

TABLE 2. Oligonucleotides present in RNA fingerprints of viral mixtures from preleukemic AKR/J mice[a]

Virus	Akv-related[b]	Polytropic virus-related[c]			Mink-tropic virus
		gp70	p15E	U3	
Thymic isolates					
AKN-T-16	++++	0	+	0	No
AKN-T-11	++++	0	++	++	No
AKN-T-20	++++	+	++	++	No
AKN-14	++++	+++	++++	++++	No
AKN-T-18	++++	++	++	++	Yes (AKM-T-18)
Splenic isolates					
AKN-S-16	++++	0	0	0	No
AKN-S-11	++++	0	0	0	No
AKN-S-20	++++	0	0	0	No
AKN-S-18	++++	0	0	0	Yes[d]
Bone marrow isolate (AKN-B-18)	++++	0	0	0	No

[a] Yield relative to AKV oligonucleotides (0 to ++++), as estimated by visual inspection and by analysis with pancreatic RNase digests.

[b] Akv related, but may be present in recombinant or other viruses.

[c] This includes for gp70 the oligonucleotides 102, 104, 111, and 113; for p15E, oligonucleotides 101, 114, and 119; and for U3, digonucleotides 106 and 108.

[d] Virus isolated after extended passage on mink cells; fingerprint indistinct.

plained by the fact that their biological properties (i.e., host range) in cell culture seem identical to those of Akv. Also, the altered sequences, although readily detectable by the appearance of four new oligonucleotides, do not alter the pattern of cleavage sites for the most commonly used restriction enzymes (5). Finally, such viruses seem to have a reduced replication ability on fibroblasts, as judged by their disappearance from the population after repeated passage in cell culture (unpublished data). Thus, viruses isolated after purification by endpoint dilution, as is the common practice, would have lost this species.

Other interpretations of our data are possible. We cannot firmly exclude the possibility that some of the non-Akv 3′ oligonucleotides seen were present in incomplete genomes otherwise so diverse that other specific oligonucleotides were not detectable. However, mixtures of virus (such as AKN-T-14; Fig. 2D) with large amounts of these oligonucleotides have a noticeably reduced yield of the corresponding Akv oligonucleotides (eg., 14 and 30), which is indicative of recombination. It is also unlikely that the non-polytropic recombinant viruses arose in vitro. With the AKN-T-16 and AKN-T-11 viral isolates, these recombinant viruses were present in the absence of detectable polytropic viruses or gp70 oligonucleotides.

These findings imply that the mechanisms for selection of the two non-Akv regions of the genome in recombinant leukemogenic viruses are different. The thymus-specific acquisition of viruses containing the non-Akv p15E oligonucleotides in the absence of the non-Akv gp70 oligonucleotides and the invariable presence of one or more of these oligonucleotides in known recombinant leukemogenic viruses strongly suggest an important biological role for this region of the genome independent of that conferred by the gp70 alteration. Differences in the sequences of p15E in Akv and MCF 247, a leukemogenic polytropic AKR virus, are known to involve a relatively small number of base and amino acid changes (N. Hopkins, personal communication). How these changes in the carboxy-terminal portion of p15E could influence viral growth or leukemogenicity is unknown. The unique isolation of altered viruses from the thymus suggests that such viruses either arose specifically in the thymus or are strongly selected for replication there. It seems unlikely that recombination alone could account for the frequency of recombinant viruses observed, and we favor the possibility that such viruses can arise anywhere but are selected for replication in the thymus. We observed a strong selection against the polytropic-related 3′ oligonucleotides upon in vitro passage of the viral mixture (AKM-T-11) in NIH3T3 fibroblasts, suggesting some tissue specificity for growth conferred by these sequences (data not shown). These hypotheses are currently under investigation. Other explanations such as immune selection, interaction of the gene product with a lymphocyte receptor controlling cell proliferation, or a noncoding fuction of the p15E sequences are possible. The data presented here and in previous reports (5, 6, 8, 15, 21) indicate that the gp70 sequences are also important in mediating leukemogenesis of AKR polytropic viruses. Differences in the gp70 genes of Akv and the polytropic viruses account for the different in vitro host range properties of these viruses. However, the biological role of this region of the genome in leukemogenesis is also unknown. Postulated functions have included those listed above for p15E (16, 17). From our data, we would predict that the biological activity of gp70 and p15E of the polytropic viruses is

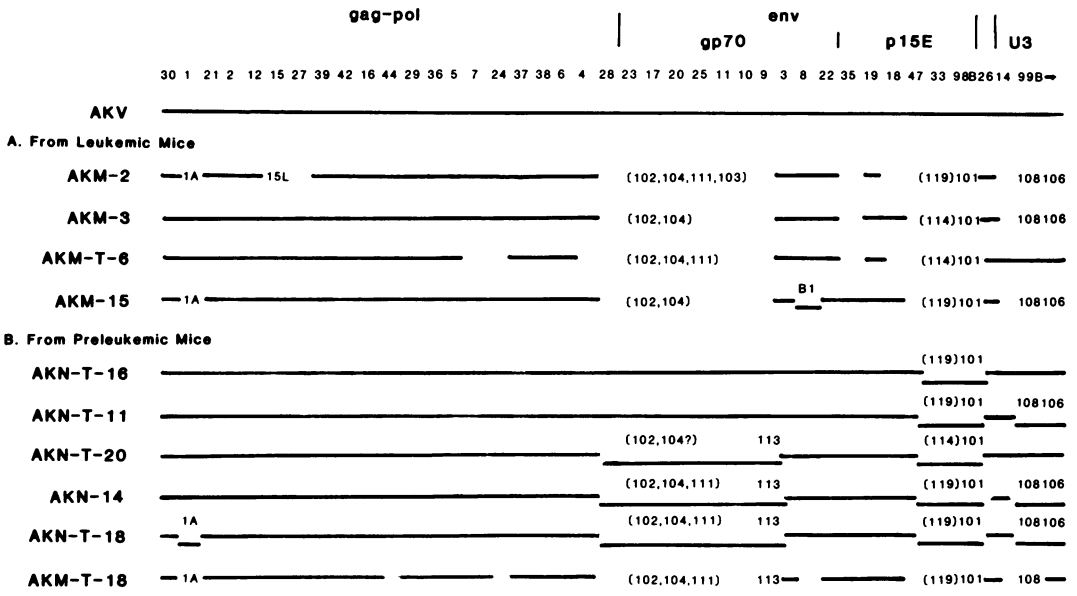

FIG. 3. T_1-oligonucleotide maps of viral isolates from AKR/J mice. Oligonucleotide maps were constructed as outlined in the text. The linear RNA genome is shown with the 3′ end on the right. Akv oligonucleotides are ordered from the 5′ to the 3′ end. The presence of each oligonucleotide in the viral 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 where 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. (A) Genomes of viruses isolated from leukemic mice; (B) genomes of viruses isolated from preleukemic mice.

independent, but that each is necessary for viral selection or leukemogenicity.

The presence of non-Akv sequences in the U3 region of polytropic leukemogenic viruses has been documented previously. A role for this region in the leukemogenesis of avian RNA tumor viruses has been proposed (26). However, AKM-T-6 was isolated from a leukemic AKR mouse and retained all of the known Akv markers in this part of the genome. This result implies that the U3 sequences represented by these non-Akv polytropic virus-related oligonucleotides may not be important in mediating viral recombination, leukemogenesis, or host range. It should be mentioned that non-Akv sequences could be present within the U3 region but not detectable by T_1-oligonucleotide analysis and that AKM-T-6 has not yet been tested for leukemogenicity upon injection into mice.

A corollary of the postulate that the non-Akv p15E and gp70 sequences are selected independently is that the recombination process which generates the leukemogenic viruses probably involves at least three different parental endogenous virus genomes. Although Akv viral genomes are known to exist intact in AKR mice, endogenous viruses containing the non-Akv sequences found in the leukemogenic polytropic viruses have not been identified. We were unable to find any xenotropic virus or combinations of such viruses whose genome(s) contains the full complement of oligonucleotides found in the polytropic virus gp70. On this basis, we have suggested that the parental virus for gp70 may be a defective endogenous virus whose *env* gene already has a polytropic host range (8). Chattopadhyay et al. (5) reached a similar conclusion by analysis of restriction endonuclease cleavage sites. In a recent report, he and his co-workers identified endogenous sequences in AKR mouse embryos that appear to contain the intact polytropic gp70 but not the polytropic virus-related p15E sequence (4). Thus, such a locus may be the parent of the polytropic virus-related gp70 oligonucleotides, but whether it is capable of donating the non-Akv portion of the p15E/U3 region is unknown (S. Chattopadhyay, personal communication).

We believe the latter sequences are derived from a third endogenous virus. This belief is based on two observations. First, a pattern of non-Akv oligonucleotides virtually identical to that predicted for the donor of the 3′ sequences has been found in the genomes of xenotropic

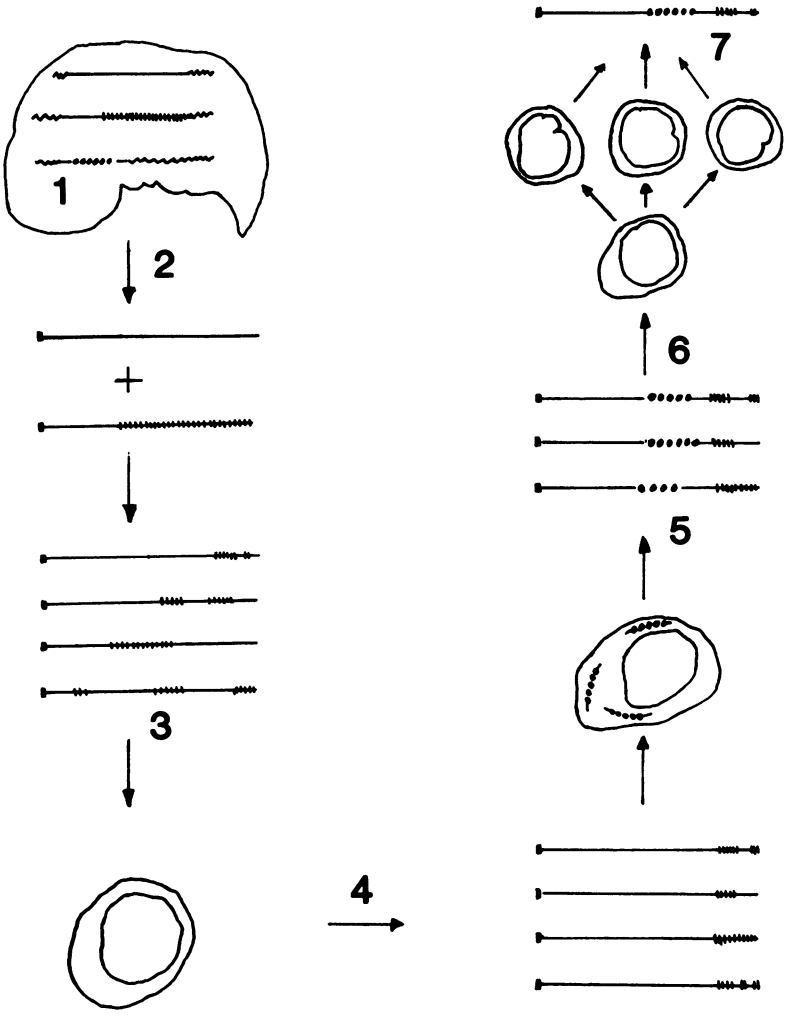

FIG. 4. Proposed model for the in vivo generation of leukemogenic polytropic viruses in AKR/J mice. 1. Embryonic DNA containing ecotropic (——) and xenotropic (HHH) proviral sequences. Also present are polytropic gp70 sequences, probably as part of an incomplete viral genome (· · · · ·). 2. Viral RNAs are released. 3. Genetic recombination results in a family of xenotropic/ecotropic recombinants. 4. After infection, recombinants with hybrid p15E genes with or without non-Akv U3 region sequences are selectively replicated, probably in the thymus. 5. A second recombination event with polytropic envelope sequences in the cell generates a family of polytropic viruses. 6. Infection of the target thymocyte with leukemogenic polytropic virus. 7. Transformation occurs, and there is clonal expansion of the tumor cell population with high levels of production of the polytropic virus.

viruses isolated from a C57L mouse and from AKR mouse fibroblasts (C. Thomas, J. Levy, J. Coffin, unpublished data; B. Crowthers, personal communication). Second, as described in this report, we have documented the independent expression of the non-Akv gp70 and p15E/U3 region oligonucleotides. Therefore, we propose that the three parental genomes include (i) Akv, (ii) the donor for the polytropic virus-related gp70 sequences, and (iii) another genome (probably that of an endogenous xenotropic virus) that donates the 3′ sequences.

Several other findings in this study deserve comment. First, we have shown that single-passaged viral isolates from both leukemic and preleukemic animals are often composed of complex viral mixtures and multiple recombinant genomes. Therefore, careful interpretation of the previous biological and biochemical studies of AKR leukemias and viruses is necessary.

Second, we found the isolation of xenotropic viruses from preleukemic mice to be difficult, a finding supported by two recent studies (4, 14). Although xenotropic-related viral antigens have

been reported in the thymuses of preleukemic AKR or HRS mice, isolation of this class of virus appears to be unusual (6, 10, 13). This may relate to its low expression in vivo, its poor growth characteristics in vitro, or to inhibitory factors known to be present in mouse serum (11). We have observed the x-oligonucleotides and a virus resembling the C57L xenotropic viruses in a mink-tropic virus mixture derived from the spleen of a 3-month-old AKR mouse (C. Thomas, R. Khiroya, S. Datta, and J. Coffin, unpublished data).

Third, virtually all of the isolates of mink-tropic virus seen in this study were found to contain a set of oligonucleotides provisionally identified as belonging to a xenotropic virus much like the predominant virus isolated from NZB mice (C. Thomas, S. Datta, and J. Coffin, unpublished data). This virus seems to be unrelated to the induction of leukemia since it does not grow on mouse cells, it shares no oligonucleotides with either Akv or polytropic viruses, and its presence does not seem to be necessary for the induction or acceleration of leukemia in mice (18, 21). Its frequent isolation from spontaneous lymphomas in both AKR and HRS/J mice and from tumors induced in HRS/J and CBA/J mice by injection with polytropic virus (8; Thomas et al., in preparation) would suggest that it has some relationship to the disease, although we have not definitively shown it to be of mouse origin. Perhaps this virus tends to be induced by the transformation of thymocytes, is readily rescued by polytropic virus, or represents VL30-like sequences.

Fourth, we note some similarities between our data and those of Pederson et al. (18), who analyzed the genomes of a set of ecotropic leukemogenic viruses derived from AKR mice, including Gross passage A (3), and viruses derived from AKR tumor cell lines. These viruses differed consistently from Akv only in the 3′ p15E and U3 region, in a manner very similar to our early thymus isolates. However, the specific non-Akv oligonucleotides found in these viruses are different from those described by us and by Hopkins and co-workers (15, 21). Furthermore, we have been so far unable to induce lymphomas with viruses similar to those found in the early preleukemic thymus (such as ETV-1 [8]), although the tests are incomplete. These disparities remain to be resolved.

In summary, we propose a scheme (Fig. 4) for the in vivo generation of polytropic leukemogenic viruses in AKR/J mice. Three or more independent viral loci are present in the DNA of the embryo. High levels of ecotropic viral genomes are expressed early in the life span of these mice, whereas the xenotropic proviruses are expressed at low levels. Nevertheless, recombination between the genomes occurs, generating a family of recombinants. Recombinants with hybrid p15E sequences are selected and amplified, probably in the thymus. These viruses then interact with defective viral genomes in the cell. This generates a second family of recombinant viruses that now contain polytropic virus gp70 envelope sequences. Finally, the target cell is infected with a polytropic virus, resulting in transformation and clonal outgrowth of the cells and tumor formation. Since each tumor cell is derived from a single cell-virus interaction, the tumor produces high levels of that particular viral genome as well as releasing other endogenous viruses. The mechanism of biological selection of the recombinant viruses and the molecular interaction between the virus and the target cell remain to be clarified.

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