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Thymic Epithelial Genotype Influences the Production of Recombinant Leukemogenic Retroviruses in Mice

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By using T_1 oligonucleotide fingerprinting and mapping techniques, we analyzed the genomic structure of retroviruses produced by thymocytes and splenocytes of reciprocal bone marrow- and thymus-grafted chimeras. We found that the genetic factor(s) derived from NZB mice that suppresses the development of thymic leukemia in (AKR × NZB)F_1 mice also prevents the formation of recombinant leukemogenic viruses and the expression of preleukemic changes in the (AKR × NZB)F_1 thymocytes. The NZB mouse gene or genes appeared to exert this suppressive effect by acting on the thymic reticuloepithelial cells and not on the thymic lymphocytes of (AKR × NZB)F_1 hybrids. Prospective studies with thymic epithelial grafts from young mice showed that the AKR thymic epithelium could mediate the formation and expression of leukemogenic recombinant viruses and preleukemic changes in thymocytes that lead to the development of thymic leukemia, whereas the (AKR × NZB)F_1 thymic epithelium was deficient in this regard. Our results also confirmed a previous observation that during in vivo generation of recombinant leukemogenic viruses, the acquisition of polytropic virus-related sequences in the 3′ portion of the p15E gene and the U3 region and in the 5′ part of the gp70 gene can occur independently.

An important step in the development of thymic leukemia in AKR mice is the formation of leukemogenic retroviruses that arise by recombination of non-leukemogenic endogenous precursor viruses (15). In the late preleukemic stage beyond 5 months of age, there is a marked amplification in the expression of retroviral antigens by AKR thymocytes accompanied by the emergence of viruses with polytropic host range, also called dual-tropic or mink cell focus-forming viruses (15, 18). These polytropic leukemogenic viruses are capable of replicating in thymocytes and arise by recombination between the endogenous ecotropic Akv virus and xenotropic-related precursor viruses (3, 4, 9, 13, 22, 24, 28). Genomic analysis of the leukemogenic polytropic viruses has revealed that they differ most from the Akv parents in three regions. Two are in the *env* gene region, located in the 5′ portion of the gp70 gene and the 3′ end of the p15E gene, and the other is in the U3 noncoding region which forms part of the long terminal repeat of the provirus (13, 19, 21, 22). These recombinational changes are shown schematically in Fig. 1. The origin of the non-Akv sequences in polytropic viruses is unknown, but there is evidence suggesting that multiple independent recombinational events of the Akv virus with different endogenous viral sequences may give rise to the full-fledged leukemogenic polytropic viruses (31). Concurrent with these preleukemic changes in the genotype and phenotype of viruses produced by the AKR thymocytes are major shifts in their maturation and differentiation phenotypes (18, 35, 37).

Despite these observations and the fact that thymocytes are the cellular targets for the leukemogenic recombinant viruses, the precise role of the thymus in mediating recombination, selection, and expression of viral sequences remains unclear. We have been investigating the cellular mechanisms in the thymus that are responsible for the phenotypic changes in thymocytes associated with the development of thymic leukemia. We used (AKR × NZB)F_1 (ANF_1) hybrid mice that inherit dominant loci for ecotropic and xenotropic virus expression from their AKR and NZB parents, but fail to develop leukemia. Owing to an NZB-derived genetic influence, these ANF_1 hybrids manifest a thymus-specific restriction of expression of retroviruses and retroviral antigens throughout their life-span which correlates with their leukemia resistance (6). The mechanism of the restricted expression

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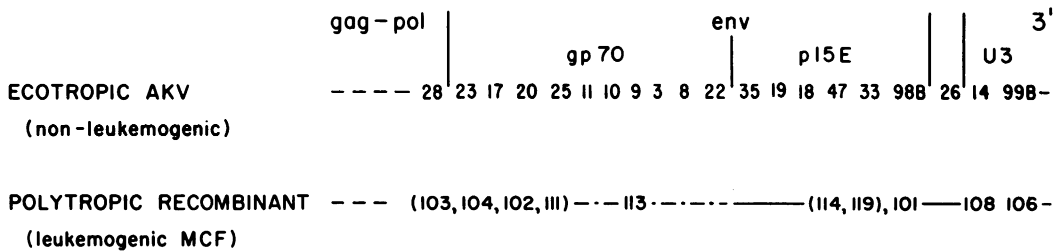


FIG. 1. Partial oligonucleotide maps of recombinant retroviruses. Since most of the oligonucleotide changes of interest in the recombinant polytropic viruses occur toward the 3' end of the viral genome, only this portion is shown. The viral RNA is represented in the linear form with the 3' end to the right. Oligonucleotides are numbered according to the conventions of Rommelaere et al. (27, 28), Lung et al. (21, 22), Green et al. (13), and Thomas and Coffin (31). The genome of endogenous AKR ecotropic virus (Akv) is drawn on the top. The presence of oligonucleotides in the viruses that are identical to those of Akv virus is indicated by an uninterrupted solid line directly below the corresponding Akv-derived numbered oligonucleotides. Bracketed oligonucleotides have not been mapped precisely and are shown in their approximate locations. Oligonucleotides numbered above 100 are found in the polytropic viruses, but not in Akv virus. Dot-dashed line represents the gp70 genetic region of polytropic viruses where oligonucleotides derived from either Akv or non-Akv parents may be present.

of retroviral genes by thymocytes of ANF_1 mice was investigated by the technique of radiation chimeras. The rationale for these experiments was based on the evidence that thymic epithelial cells resist otherwise lethal doses of total-body radiation and that precursor cells in the bone marrow (prothymocytes) differentiate in the thymic microenvironment under the influence of the thymic epithelium (8, 11, 12, 17, 32, 38). When ANF_1 bone marrow precursor cells are allowed to differentiate to thymic lymphocytes in the thymic environment of lethally irradiated young AKR mice, they manifest a specific amplification in the expression of retroviral antigens and retroviruses, including polytropic viruses, as well as leukemic transformation (7, 36). By contrast, those phenotypic changes do not occur when ANF_1 thymocytes differentiate in leukemia-resistant irradiated hosts, such as ANF_1 and C57BR strains (7, 36). Reciprocal thymic graft chimeras localized the component that mediates these changes to the radiation-resistant thymic reticuloepithelium. Prospective studies with heavily irradiated thymic epithelial grafts from young (2- to 3-month-old) mice showed that AKR thymic epithelium can induce preleukemic changes in thymocytes. These include amplified expression of retroviruses, retroviral antigens, and changes in thymocyte differentiation patterns that precede the development of leukemia. In contrast, ANF_1 thymic epithelium is incapable of mediating such preleukemic changes in thymocytes (36).

We have now extended our studies to structural analysis of the genomes of retroviruses expressed by the thymocytes of the reciprocal bone marrow and thymic epithelial graft chimeras. To minimize loss and selection of viruses that occur during rounds of limiting dilution purification in vitro, we performed RNA T_1 oligonucleotide fingerprinting and mapping of genomes of the mixtures of viruses isolated from the originally infected NIH 3T3 mouse or mink cell lines that were cocultivated with the thymocytes. We found that expression of viruses with recombinant genotypes characteristic of thymic isolates from preleukemic and leukemic AKR mice occurred only in chimeras that retained or had received thymic epithelial cells of AKR origin. Our studies suggest that radiation-resistant cells in the AKR thymus can directly or indirectly select, amplify, or mediate expression of AKR types of recombinant viruses. Our results also establish a strong association between the preleukemic thymocyte phenotype and expression of viruses with the leukemogenic genotype. Finally, we have confirmed an earlier report that viruses recovered from preleukemic thymuses may contain recombinant genomes with polytropic virus-related sequences localized exclusively to the p15E-U3 gene region (31), and have shown that polytropic virus-related sequences in the 5' portion of the gp70 gene can also occur independently of other recombinational changes.

MATERIALS AND METHODS

Mice. AKR/J, C57BR, and NZB mice were obtained from the Jackson Laboratories (Bar Harbor, Maine). (AKR × NZB)F_1 and (NZB × AKR)F_1 hybrids were bred at the Tufts University animal facility. As reported previously, there were no differences in results between the hybrids of the reciprocal crosses (6, 7, 36), and both are therefore referred to as ANF_1.

Bone marrow radiation chimeras. Preparation of bone marrow radiation chimeras and analysis of their virus expression and thymocyte differentiation patterns have been described previously in detail (7, 36). Lethally irradiated (950 to 1,200 rads) recipient mice were reconstituted with donor bone marrow cells that

had been depleted of mature T cells. The ages of the donors and recipients at the time of bone marrow transplantation and at the time of analysis and virus assays have been described previously (7, 36). Chimeras are designated as bone marrow donor into (→) irradiated recipient.

Thymic epithelium-grafted chimeras. Preparation of thymic epithelium-grafted chimeras and their analysis have also been described previously (36). ANF_1 recipient mice were thymectomized and 2 weeks later were irradiated with 1,000 rads. On the same day they were transplanted with irradiated (1,200 to 2,000 rads in vitro) thymuses of AKR or ANF_1 mice. Simultaneously, they received an intravenous injection of ANF_1 bone marrow cells that had been depleted of mature T cells (36). Chimeras are designated as thymic epithelial graft donors (TE→) into ANF_1 recipients.

Viruses. Thymocytes and splenocytes from proven bone marrow chimeras and thymocytes that had differentiated in the thymic epithelial grafts were prepared into single cell suspensions (7, 36). Samples were taken at the time of the other analysis of these cells (7, 36), and seeded on the DEAE-dextran-treated NIH 3T3 mouse and mink fibroblasts as described previously (7, 31). Adherent cells were subcultured every 3 to 4 days with loss of most of the lymphoid cells by the second passage. After 5 to 10 passages, the culture supernatants were screened for the presence of retroviruses by reverse transcriptase (31) or fluorescent focus assays (7, 36). Positive cultures were subsequently used for ^{32}P labeling of viral RNA, and negative cultures were subcultured for additional passages and retested for virus production.

T_1 oligonucleotide fingerprinting and mapping of viral RNA. ^{32}P-labeled 70S viral RNA was isolated, digested with RNase T_1, and subjected to two-dimensional polyacrylamide gel electrophoresis exactly as described previously (13, 31). The oligonucleotides were identified by their electrophoretic mobility, and their identity was further confirmed by elution of the oligonucleotides from the gels, subdigestion with RNase A, and separation of the products on DEAE paper by high-voltage ionophoresis (13, 31). Because complex mixtures were present, the existence of different viruses was inferred from the differences in molar yield of the oligonucleotides or from the presence of known allelic oligonucleotides. Numbers were assigned by the conventions of Rommelaere et al. (27, 28), Green et al. (13), and Thomas and Coffin (31). Maps of viral genomes were constructed by using previously published data on the order of T_1 oligonucleotides in the viral RNA (13, 21, 28, 31), recent nucleic acid sequencing data from cloned viral DNA (19, 20), and unpublished data (C. Thomas and J. Coffin).

RESULTS

Genomes of viruses isolated from bone marrow chimeras. (i) ANF_1→ANF_1 chimeras. Retrovirus expression and thymocyte differentiation patterns in 44 low-leukemia ANF_1 mice ranging in age from 1.5 to 22 months have been described previously (6, 7, 35, 37). Although the spleen and bone marrow cells of all of these mice produce high levels of ecotropic and xenotropic viruses, only eight of these animals had barely detectable levels of ecotropic virus in their thymocytes. ANF_1→ANF_1 bone marrow chimeras were constructed by repopulating lethally irradiated 2- to 3-month-old ANF_1 mice with bone marrow cells (mature T lymphocyte depleted) from 3- to 4-month-old ANF_1 mice. The chimeras were analyzed 2 months later for virus expression (7). These chimeras resemble intact ANF_1 mice; expression of retroviral antigens and ecotropic (NIH 3T3-tropic) and mink-tropic viruses by their thymocytes are severely restricted, whereas their spleen cells express those viruses in high levels (7). ANF_1 thymocytes that differentiate in a syngeneic ANF_1 thymic environment, i.e., the thymocytes of these ANF_1→ANF_1 chimeras, do not undergo any amplified retrovirus or retroviral antigen expression or changes in thymocyte differentiation patterns that are characteristic of late preleukemic AKR thymocytes (7, 35–37). For the present study, samples of thymocytes and splenocytes from three of these chimeras and from three intact ANF_1 mice that had barely detectable virus expression in their thymuses (6, 7) were cocultivated with mink or NIH 3T3 mouse cells. Twenty passages of mink and NIH 3T3 mouse cells that were inoculated with thymocytes of these animals were required before viruses could be detected and subsequently fingerprinted. T_1 oligonucleotide fingerprints of viruses from splenocytes and thymocytes of these animals were identical, and representative examples are shown in Fig. 2A and Fig. 3A. NIH 3T3 mouse cells cocultivated with splenocytes and thymocytes of these animals (Fig. 2A) produced viruses with oligonucleotide patterns identical to the endogenous ecotropic Akv virus. No oligonucleotides indicative of leukemogenic polytropic viruses were seen. Upon cocultivation of mink cells with samples of thymocytes and splenocytes of the same animals (Fig. 3A), we obtained viruses with oligonucleotide patterns (designated by an "x" prefix) identical to those of xenotropic viruses isolated from mink or rabbit SIRC cells cocultivated with splenocytes of young NZB mice (31; C. Thomas, S. Datta, and J. Coffin, unpublished data). Note that viruses grown in NIH 3T3 mouse cells cocultivated with the same samples of cells lacked these oligonucleotides (Fig. 2A). Nearly identical oligonucleotides have been previously seen among mink-tropic virus mixtures from spontaneous thymomas of HRS/J (13) and AKR mice (31). This virus seems to be unrelated to the induction of leukemia since it does not grow on mouse cells, it shares no oligonucleotides either with Akv or with polytropic leukemogenic viruses, and its presence does not seem necessary for the induction or acceleration of leukemia in mice

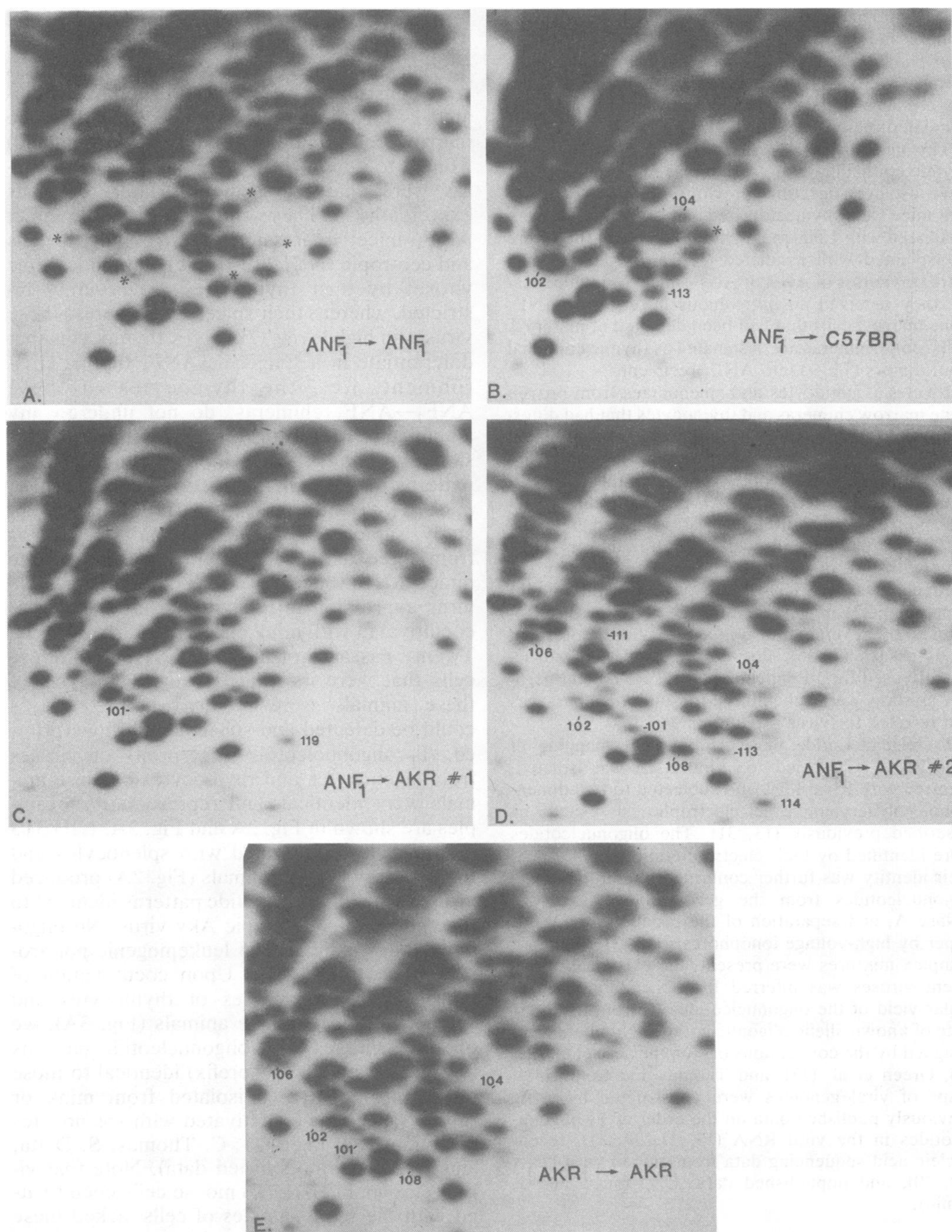


FIG. 2. Viruses from thymocytes of bone marrow chimeras isolated on NIH 3T3 mouse cells. Fingerprints of the genomes of viruses isolated from thymocytes of the respective bone marrow chimeras (A to E) are shown. The (A) fingerprint pattern is identical to that of endogenous ecotropic Akv virus (27, 31). Spleen cells of intact ANF_1 mice and all types of chimeras studied here yielded viruses with identical fingerprints on NIH 3T3 mouse cells (not shown). Asterisks (*) in (A) indicate unidentified oligonucleotides that may be nonviral in origin. These nonviral oligonucleotides are also present in the other panels, but they have not been marked. The polytropic non-Akv oligonucleotides are numbered in (B) through (E) as described in the text and in the legend to Fig. 1.

(13, 31). In addition to the oligonucleotides of this virus, the mink-tropic virus mixtures from $ANF_1 \rightarrow ANF_1$ chimeras also contained another RNA species with oligonucleotides (designated by arrows in Fig. 3A) similar to those of xenotropic viruses isolated from dog or human cells cocultivated with NZB tissues (C. Thomas, J. Levy, and J. Coffin, unpublished data). We believe that both of these RNA species (Fig. 3A) are associated with endogenous xenotropic viral genomes; however, we cannot exclude the possibility that these oligonucleotides represent VL-30 sequences or other nonviral RNA (1). Therefore, the fingerprints of isolates recovered on mink cells contain a complex pattern of oligonucleotides, probably representing xenotropic viral genomes, but not related to Akv or leukemogenic viruses of AKR/J mice.

(ii) $ANF_1 \rightarrow$C57BR chimeras. C57BR mice are H-2^K, similar to AKR mice (therefore histocompatible with the ANF_1 mice), but are a low-leukemia-, low-virus-expressing strain (7). C57BR mice (2 to 3 months old) were lethally irradiated and reconstituted with bone marrow cells from ANF_1 mice (7, 36). A total of 16 proven chimeras were analyzed for retrovirus expression and thymocyte differentiation patterns between 1 and 5 months after bone marrow transplantation; the results were described previously (7, 36). Thymocytes of the $ANF_1 \rightarrow$C57BR chimeras express moderate amounts of ecotropic virus but barely detectable mink-tropic viruses; however, their splenocytes, similar to those of ANF_1 mice, express high levels of both viruses (7). These ANF_1 thymocytes that had differentiated in the C57BR thymic environment ($ANF_1 \rightarrow$C57BR chimeras), similar to the $ANF_1 \rightarrow ANF_1$ thymocytes, do not undergo any amplified retroviral antigen expression or changes in thymocyte differentiation patterns that are characteristic of late preleukemic AKR thymocytes (7, 36). In this study, T_1 oligonucleotide fingerprinting analyses were done on mixtures of viruses grown on mink or NIH 3T3 mouse cells that had originally been cocultivated with samples of thymocytes and splenocytes of three such chimeras. In all except one case, the NIH 3T3-grown viruses had fingerprint patterns identical to that of the endogenous Akv virus (Fig. 2A). Surprisingly, thymocytes from one of these $ANF_1 \rightarrow$C57BR chimeras not only expressed the Akv-like virus but also produced viruses with non-Akv oligonucleotides that had been identified previously in leukemogenic, polytropic viruses (Fig. 2B). These polytropic virus-related oligonucleotides were restricted to the 5′ portion of the gp70 gene (oligonucleotides 102, 104, and 113) (Fig. 1) (13, 21, 28, 31) and were present in lesser molar yields than the Akv gp70 oligonucleotides. p15E and U3 region polytropic-related oligonucleotides of recombinant viruses (13, 21, 28, 31) (Fig. 1) were conspicuously absent. When the thymocytes and splenocytes of these $ANF_1 \rightarrow$C57BR chimeras, including the one shown on Fig. 2B, were cocultivated with mink cells, the latter yielded viruses with fingerprints identical to that shown in Fig. 3A, i.e., similar to the xenotropic viruses isolated from normal NZB and ANF_1 mice. No polytropic virus-related oligonucleotides were detected in these mink-grown viruses.

In summary, except for one isolate, these chimeric animals did not express viruses whose genomes contained the oligonucleotide markers associated with leukemogenic AKR viruses. The one exception was a novel complex of viral genomes containing Akv and polytropic virus-related oligonucleotide markers limited to the gp70 gene, a pattern not observed in AKR viral mixtures (21, 31).

(iii) $ANF_1 \rightarrow$AKR chimeras. By contrast to the previous two chimeras, thymocytes of ANF_1 origin that differentiate in the AKR thymic environment, i.e., thymocytes of $ANF_1 \rightarrow$AKR chimeras, produce markedly augmented levels of the mink-tropic and ecotropic viruses and retroviral antigens and manifest changes in differentiation patterns characteristic of late preleukemic AKR thymocytes; they also undergo leukemic transformation (7, 36). These changes are manifested from 1.5 months after construction of the chimeras and occur specifically in the thymuses of these chimeric animals and not in their spleens (7, 36). A total of 33 proven $ANF_1 \rightarrow$AKR chimeras that had been constructed by lethally irradiating 2- to 3-month-old AKR mice and then transplanting them with ANF_1 bone marrow were analyzed over a period of 1 to 5 months after reconstitution (7, 36). Here, we analyzed the T_1 oligonucleotide fingerprints of viruses grown in mink and NIH 3T3 mouse cells. These cells had been originally cocultivated with samples of thymocytes and splenocytes of five of the $ANF_1 \rightarrow$AKR chimeric animals that had manifested the preleukemic changes in their thymocytes (7, 36). Spleen cells yielded viruses with fingerprints similar to Akv virus when cocultivated with NIH 3T3 mouse cells (Fig. 2A) and similar to NZB xenotropic virus when grown in mink cells (Fig. 3A). However, thymocytes of these chimeras yielded not only those viruses but, in addition, viruses with non-Akv polytropic virus-related oligonucleotides. Representative examples are shown in Fig. 2C and D and Fig. 3B. The polytropic virus-related sequences were present in various molar yields relative to the Akv or the putative NZB xenotropic sequences and relative to one another. These findings again indicated the presence of complex

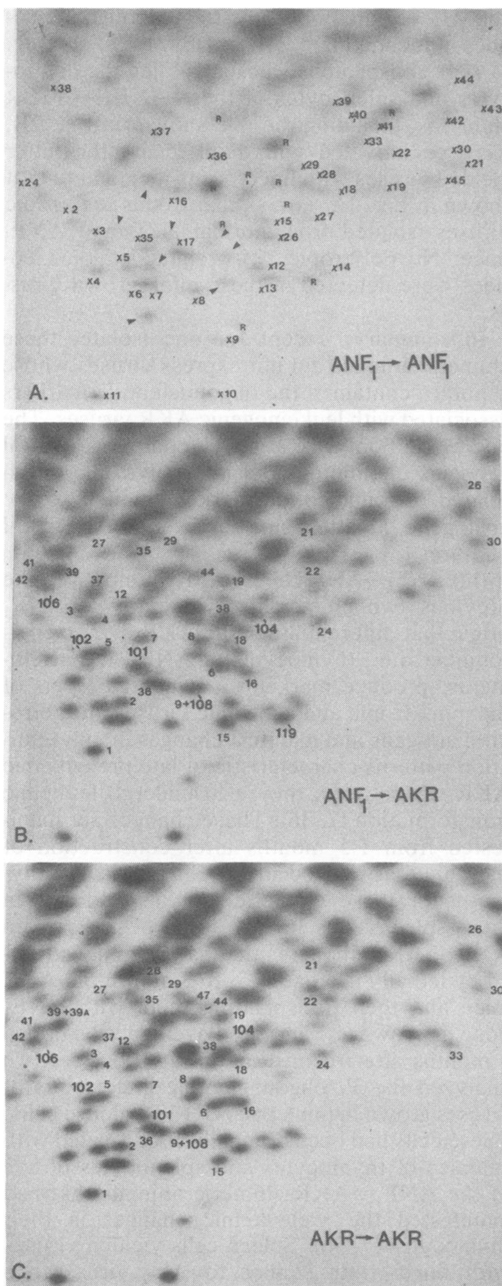


FIG. 3. Viruses from bone marrow chimera thymocytes, isolated on mink cells. (A) Fingerprint of the genomes of viruses isolated from a sample of thymocytes of the same animal as shown in Fig. 2A. No Akv ecotropic oligonucleotides are present. Identical fingerprints were obtained on mink-grown viruses isolated from spleens of intact NZB and ANF_1 mice and all types of chimeras examined (not shown). The putative xenotropic virus-derived oligonucleotides are marked with the prefix x (see text and refrences 13 and 31). Arrows represent other xenotropic virus oligonucleotides that have been found in isolates from NZB tissues cocultivated with dog or human cells (see text). None of these oligonucleotides are found in Akv ecotropic or recombinant polytropic viral genomes. "R" represents oligonucleotides of ribosomal RNA. (B) Genomes of viruses isolated from thymocytes of the same animal as shown in Fig. 2C. In this complex mixture, the xenotropic oligonucleotides seen in (A) are present (not numbered), Akv ecotropic oligonucleotides are numbered with small numbers, and the polytropic non-Akv oligonucleotides are numbered with bold-faced numbers. (C) Fingerprints of viruses isolated from the thymocytes of the same animal as shown in Fig. 2E. Again, a complex mixture of xenotropic, ecotropic, and polytropic oligonucleotides is seen. These are designated as in (B).

mixtures of viral genomes. Figure 2C shows a viral mixture isolated from NIH 3T3 mouse cells cocultivated with thymocytes from an ANF_1→AKR chimera; this consists of Akv oligonucleotides plus, in relatively lower yield, another virus with polytropic oligonucleotides 101 and 119. The latter map in the 3′ terminal portion of the p15E gene region of *env* (13, 22, 28, 31) (Fig. 1). It is noteworthy that the other polytropic oligonucleotides of the U3 region and the 5′ gp70 region are absent. This type of recombinant virus has been observed previously in the preleukemic AKR thymus (31). Surprisingly, when a portion of the same sample of thymocytes was cocultivated with mink cells (Fig. 3B), a different mixture of viruses emerged that showed: (i) the oligonucleotides of the NZB xenotropic isolate (not numbered); (ii) oligonucleotides if Akv virus (small numbers); plus (iii) polytropic oligonucleotides of all three recombinant regions that are seen in leukemogenic MCF viral genomes. These were oligonucleotides belonging to the 5′ gp70 gene (102, 104), the 3′ p15E gene (101, 119), and the U3 region (106, 108). Thus, the mink-tropic viruses contained genomes very similar to those of recombinant polytropic viruses of AKR mice. As stated above, viruses with recombinant gp70 gene sequences were not seen in the isolates cultured on NIH 3T3 mouse cells. This implies that the gp70 gene recombinants were released at low titer from ANF_1→AKR thymocytes or that during in vitro replication in NIH 3T3 mouse cells, these viruses were selected against relative to ecotropic viruses, or both. Precedents for both these hypotheses are known to exist (4, 14).

However, recombinant viruses with non-Akv oligonucleotides mapping in the U3, p15E, and gp70 gene regions of the genome could be recovered from NIH 3T3 mouse cells cocultivated with thymocytes of other ANF_1→AKR chimeras (Fig. 2D). These were also present in mink isolates from the same thymocytes (not shown). Therefore, ANF_1-derived thymocytes of these

$ANF_1 \rightarrow AKR$ chimeras released viruses that had genomic structures resembling those isolated from thymocytes of intact preleukemic and leukemic AKR mice. This implies that AKR thymocytes are not necessary for selection or for expression (or for both) of recombinant viruses containing leukemogenic virus-related sequences in their genomes.

(iv) AKR→AKR chimeras. In these bone marrow chimeras, thymocytes of AKR mouse origin are differentiating in a young (2- to 3-month-old) AKR mouse environment. They also undergo the preleukemic phenotypic changes in virus expression and differentiation patterns similar to the $ANF_1 \rightarrow AKR$ chimeras, starting 2 months after reconstitution (36). Thymocytes and splenocytes of three AKR→AKR chimeras that had undergone such phenotypic changes (36) were cocultivated with mink and NIH 3T3 mouse cells. Examples of T_1 oligonucleotide fingerprints of viruses isolated from the originally infected cells are shown in Fig. 2E and Fig. 3C. Again, thymocytes yielded mixtures of viruses that contained polytropic oligonucleotides belonging to all three recombinant regions of known leukemogenic MCF viruses (Fig. 2E and 3C), whereas their spleen cells only yielded Akv-like or NZB xenotropic virus-like oligonucleotides upon cocultivation with NIH 3T3 mouse or mink cells, respectively (like the ones shown in Fig. 2A and 3A). Thus, the genomic composition of viral mixtures from these chimeras was equivalent to those isolated from intact preleukemic and leukemic AKR mice (22, 28, 31).

Genomes of viruses isolated from thymic epithelium graft chimeras. Although the preleukemic virological changes in $ANF_1 \rightarrow AKR$ bone marrow chimeras occur selectively in the thymus (7, 36), signals or factors external to the thymic microenvironment of the AKR host might contribute to the process. Therefore, heavily irradiated thymuses from young (2- to 3-month-old) AKR or ANF_1 mice were grafted into thymectomized, lethally irradiated 2- to 3-month-old ANF_1 mice that had been reconstituted with syngeneic ANF_1 bone marrow. A total of 28 such thymus grafts were analyzed between 2 and 4 months after transplantation (36). Thymocytes of ANF_1 origin that differentiate in the AKR thymic epithelial grafts ($AKRTE \rightarrow ANF_1$) develop the characteristic preleukemic changes in virological and differentiation phenotypes, whereas ANF_1 thymocytes that differentiate in ANF_1 thymus grafts ($ANF_1TE \rightarrow ANF_1$) do not undergo such changes (36). Only 3 of 11 $ANF_1TE \rightarrow ANF_1$ thymuses expressed barely detectable levels of retroviruses (36). Samples of ANF_1 thymocytes that had differentiated in these thymic epithelial grafts (36) were cocultivated with mink and NIH 3T3 mouse cells which were then passaged to isolate viruses for T_1 oligonucleotide fingerprint analysis. Three samples of each type of thymus graft chimera were analyzed here. Mink and NIH 3T3 mouse cells that had been originally infected by thymocytes of $ANF_1TE \rightarrow ANF_1$ thymus graft chimeras had to be passaged more than 20 times before any viruses could be isolated for fingerprinting, whereas mink and NIH 3T3 mouse cells cocultivated with $AKRTE \rightarrow ANF_1$ thymocytes produced sufficient virus for fingerprinting after 5 to 10 passages. Examples of these viral genomes are shown in Fig. 4. ANF_1 thymocytes that had differentiated in ANF_1 thymic epithelial grafts yielded only Akv-like ecotropic virus upon cocultivation with NIH 3T3 mouse cells (Fig. 4A) and only NZB xenotropic-like virus on mink cells (not shown, but similar to Fig. 3A). By contrast, ANF_1 thymocytes that had differentiated in the AKR thymic epithelial grafts, upon cocultivation with NIH 3T3 mouse and mink cells, yielded not only the above types of viruses but also viruses with polytropic oligonucleotides that are characteristic of leukemogenic polytropic viruses (Fig. 4B and C).

Thus, thymocytes of $ANF_1TE \rightarrow ANF_1$ chimeras produced viruses that resembled those from intact ANF_1 mice and lacked the changes in viral genomes seen in preleukemic AKR thymocytes. By contrast, $AKRTE \rightarrow ANF_1$ thymocytes produced viruses with genotypes resembling isolates from preleukemic and leukemic AKR thymocytes. These data strongly indicate that the radiation-resistant thymic reticuloepithelial cells of AKR mice are capable of determining the viral genotypes selected or expressed (or both) in the thymus.

Genomic maps of viruses isolated from chimeras. Using the fingerprints described and the procedure outlined above, we constructed maps of the 3′ portion of the genomes of the viruses we observed (Fig. 5). Except for the unusual $ANF_1 \rightarrow C57BR$ isolate, the recombinant viral mixtures clearly resemble the genomic structure of isolates from preleukemic or leukemic AKR mice. The substitutions of non-Akv sequences are clustered in the 3′ portion of the p15E gene and the U3 region, and often in the 5′ part of the gp70 gene.

Relationship of phenotypes and genotypes of thymocytes and viruses in intact and chimeric mice. Table 1 represents a summary of data compiled from this and previous reports (6, 7, 18, 31, 35–37). The most interesting observation is the consistent linkage between the AKR thymic epithelial genotype and the manifestation of thymocyte differentiation phenotype, virus phenotype, and virus genotype that are characteristic of preleukemic (or leukemic) AKR mice.

DISCUSSION

These results establish clearly that the genetic factor(s) derived from NZB mice that suppresses the development of preleukemic changes and thymic leukemia in ANF_1 mice is also associated with factors that prevent the formation of recombinant retroviruses with sequences related to known leukemogenic AKR viruses. Also, the suppressive effects of the NZB gene or genes are not expressed in the target thymocytes (thymic lymphocytes), but are expressed by the thymic reticuloepithelial cells of ANF_1 mice. Finally, the structural analysis of the genomes of viruses isolated from the chimeras studied here confirms and extends previous observations that during in vivo generation of recombinant polytropic viruses the acquisition of polytropic virus-related sequences in the p15E-U3 genetic region and in the gp70 gene region may occur independently.

Animals that demonstrated the preleukemic phenotypic changes in the thymus (as manifested by cell surface markers and levels of virus and viral antigen production by thymocytes) also produced viruses with recombinant genomes (Table 1). Work by other investigators has shown that injected leukemogenic recombinant AKR viruses are thymotropic and induce the typical preleukemic phenotype in thymocytes (13, 24, 29). These two observations taken together strongly imply that during the lifetime of AKR mice, recombinant viruses undergo selective replication in thymocytes and are responsible for the observed phenotypic changes.

From our studies on the reciprocal bone marrow and thymus graft chimeras, it appears that the thymic reticuloepithelial component in the AKR thymus, and not the thymic lymphocytes, determines the production of viruses with recombinant genomes and the subsequent manifestations of preleukemic phenotypic changes in thymocytes. The ANF_1 mice used in these studies do not develop leukemia or preleukemic changes in their thymuses (6, 7, 35, 37), nor do they produce viruses with genetic structures associated with leukemogenic recombinant AKR viruses despite the inheritence of relevant viral genes from their parents. Instead, they release viruses with the phenotype and genotype of the non-leukemogenic AKR ecotropic and NZB xenotropic viruses (Fig. 2A, 3A, and 5). These could be isolated only with great difficulty from the ANF_1 thymocytes. However, when ANF_1 bone marrow precursor cells are introduced into irradiated AKR mice, typical AKR-like preleukemic changes are seen in the ANF_1 thymocytes that are now differentiating in the AKR thymic environment (7, 36) (Table 1).

TABLE 1. Comparison of phenotypes and genotypes of cells and viruses from mice examined

Mouse strain	Cellular genotype		Preleukemic thymocyte phenotype[a]	Preleukemic virus genotype[b]
	Thymocytes	Thymic epithelium		
Intact				
AKR (young)[c]	AKR	AKR	0	0/+
AKR (old)[c]	AKR	AKR	+	+
NZB	NZB	NZB	0	0
ANF_1	ANF_1	ANF_1	0	0
Bone marrow chimeras				
$ANF_1 \rightarrow ANF_1$	ANF_1	ANF_1	0	0
AKR→AKR[c]	AKR	AKR	+	+
$ANF_1 \rightarrow$AKR[c]	ANF_1	AKR	+	+
AKR→ANF_1	AKR	ANF_1	0	0[d]
$ANF_1 \rightarrow$C57BR	ANF_1	C57BR	0	0[e]
Chimeras with thymic grafts				
ANF_1TE→ANF_1	ANF_1	ANF_1	0	0
AKRTE→ANF_1[c]	ANF_1	AKR	+	+

[a] Preleukemic thymocyte phenotype (+) as defined by the amplified expression of retroviruses and retroviral antigens and changes in the expression of cell surface differentiation antigens by thymic lymphocytes (6, 7, 18, 31, 35–37). The latter are manifested by augmented expression of H-2 and Ia antigens, by a decrease in Thy-1 antigens, and by variations in the expression of Lyt antigens in the thymic lymphocytes.

[b] Preleukemic virus genotype (+) as defined by the recovery of polytropic virus-related oligonucleotides on fingerprints of viral RNA.

[c] Preleukemic phenotypes and genotypes are found in these animals.

[d] From reference 7.

[e] One isolate contained polytropic virus-related oligonucleotides that mapped within the gp70 gene (see text).

These transplanted ANF_1 thymocytes also release viruses with genetic structures known to be associated with the development of leukemia in AKR thymus. These include viruses that contain recombinant sequences relative to Akv in the p15E-U3 region with or without gp70 gene substitutions (Fig. 2C, 2D, 3B, and 5). Similar findings were observed in the thymocytes of AKR→AKR chimeras (Fig. 2E, 3C, and 5; Table 1). The presence of recombinant oligonucleotides in the viruses isolated from both of these chimeras was thymus specific since their spleen cells yielded viruses with Akv-like and NZB xenotropic virus-like oligonucleotides (Fig. 2A, 3A, and 5). These preleukemic changes in the ANF_1→AKR chimera thymocytes are not due to nonspecific effects of radiation because thymocytes of ANF_1→ANF_1 and ANF_1→C57BR chimeras do not manifest such changes (Fig. 2A, 2B, 3A, 5; Table 1; references 7, 36). Furthermore, when bone marrow precursor cells of AKR origin are allowed to repopulate irradiated ANF_1 hosts (AKR→ANF_1 chimeras), no such preleukemic phenotypic changes in the thymocytes of AKR origin are observed (7). Therefore, ANF_1 thymocytes are susceptible to developing the preleukemic phenotype and will release viruses with recombinant genomes when they have undergone differentiation in the thymic microenvironment of the AKR host. This occurs despite the inheritence of NZB haplotype by these cells. Conversely, transplantation of AKR prothymocytes into the irradiated ANF_1 mice does not bring about the preleukemic phenotypic changes in the thymus. Thus, we conclude that AKR genes that are responsible for the formation of recombinant viruses and leukemogenesis, and NZB genes that suppress such events, are not primarily expressed in the thymocytes of AKR and ANF_1 mice, respectively. Since the preleukemic changes in the ANF_1→AKR bone marrow chimeras occurred selectively in their thymuses, it implicated the radioresistant cells of the AKR thymic environment, but this did not exclude the possibility that other signals or factors in the irradiated AKR hosts were contributing to the process. Therefore, we grafted irradiated thymuses from young AKR mice into ANF_1 mice that had been thymectomized, lethally irradiated, and reconstituted with syngeneic ANF_1 bone marrow (and hence prothymocytes). These mice did develop preleukemic changes in their thymus grafts (36) and produced viruses with recombinant genomes (Fig. 4B, 4C, and 5; Table 1). These changes did not occur when irradiated ANF_1 thymuses were grafted into the irradiated, thymectomized, and reconstituted ANF_1 recipients (Fig. 4A and 5; reference 36). The only difference between these two groups of thymus-grafted chimeras is the origin of the irradiated thymic epithelial tissue graft (AKR versus ANF_1). In the heavily irradiated thymus grafts used in our experiments, only the epithelial reticular cells remain viable (8, 11, 12, 38). The proliferating lymphocytes that repopulate the graft are entirely derived from the bone marrow precursor cells that were injected to reconstitute the recipients (8, 11, 12, 38). Moreover, we used young (2- to 3-month-old) AKR mice as the source of thymic epithelium for our prospective studies; at this age no preleukemic cellular changes or leukemic cells are detectable in the AKR thymus (18, 35, 37). These results, therefore, indicate that the suppressive effect of NZB-derived genes in ANF_1 mice is expressed in the ANF_1 thymic epithelium.

From the data presented, we believe that the NZB-derived genetic influence blocks the formation of recombinant viruses and therefore prevents ANF_1 thymocytes from expressing the AKR preleukemic phenotype. The ANF_1 thymocytes, when compared with those of the AKR parents, produce none or barely detectable levels of ecotropic viruses, and this restriction to retrovirus expression is specifically localized in the thymus but not in the other lymphoid organs of the ANF_1 mice (6). However, the significantly high levels of ecotropic virus production by thymocytes of young preleukemic AKR mice (2, 6, 15, 23) has been a paradox because the ecotropic Akv virus does not infect or replicate well in thymocytes (16, 24). Moreover, the DNA of AKR thymomas does not show any evidence of reinfection by Akv virus, but it does contain newly integrated copies of recombinant viral genomes (3, 26, 34). The young AKR thymocytes, therefore, could be releasing viruses that have already undergone recombination in the p15E-U3 region but still have an ecotropic host range (25, 31). Such recombinant viruses have been observed in very young AKR thymuses (31); they could contribute to the high levels of ecotropic virus production by the young AKR thymocytes as detected by biological assays. Our studies with the chimeras here emphasize the role of AKR thymic epithelium in the expression of such recombinants. Therefore, the NZB-derived gene(s) that causes the thymus-specific restriction of ecotropic virus expression by ANF_1 mice probably also blocks the formation or expression of such recombinant viruses with ecotropic host range. As discussed above, this block is not apparent in the ANF_1 thymocytes themselves but is probably expressed by the ANF_1 thymic epithelium. It is known that NZB mice are $Fv\text{-}1^{nn}$, but they bear a variant allele of $Fv\text{-}1^{n}$ ($Fv\text{-}1^{n/r}$) (30). This gene could determine the thymus-specific restriction in ANF_1 mice (6) since a similar restriction has been observed in

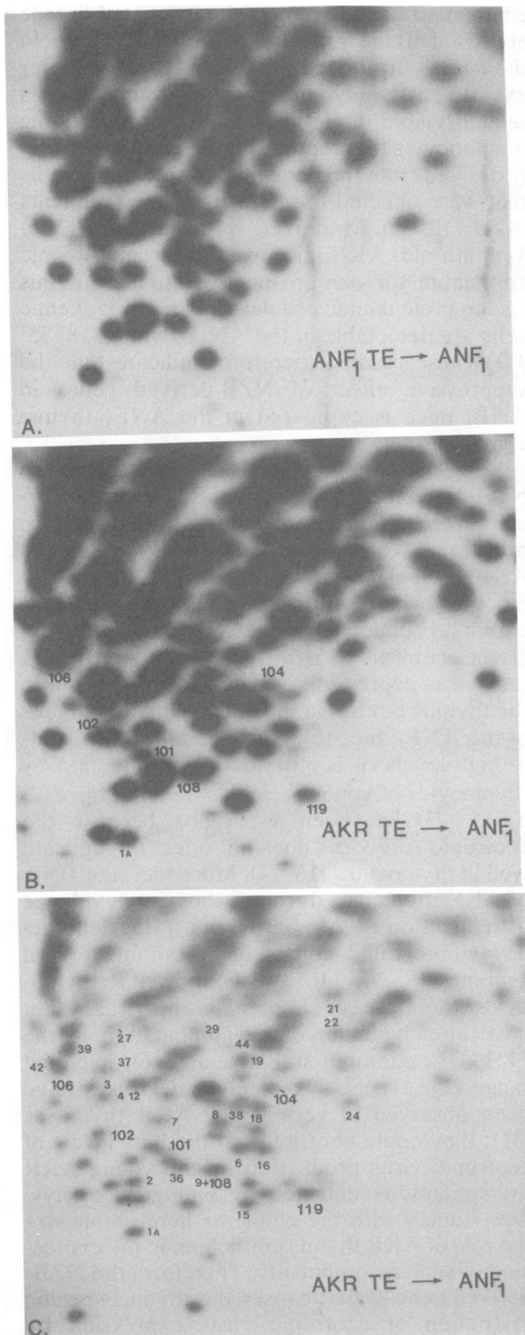
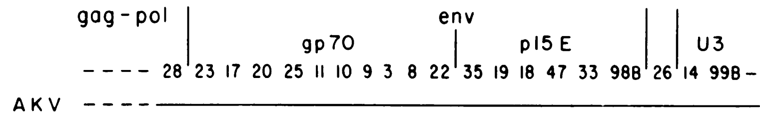


FIG. 4. Viruses isolated from thymocytes of thymic epithelium-grafted chimeras. (A) Fingerprints of genomes of viruses isolated on NIH 3T3 mouse cells from the thymocytes of ANF_1 origin that had differentiated in ANF_1 thymic grafts. The fingerprint is identical to that of Akv ecotropic virus. (B and C) Fingerprints of genomes of viruses grown on NIH 3T3 mouse and mink cells, respectively, that were cocultivated with thymocytes of ANF_1 origin that had differentiated in AKR thymic grafts. Polytropic oligonucleotides are numbered with bold-faced numbers in both panels, Akv oligonucleotides are not numbered in (B), but are numbered with small numbers in (C), and x oligonucleotides present in (C) are not numbered.

(AKR × RF)F_1 mice (23). However, other genes might also be involved (5). It is noteworthy that both ecotropic and xenotropic viruses are expressed unrestricted in high levels in nonthymic lymphoid organs of the ANF_1 mice; however, no recombinant polytropic viruses can be isolated from those tissues (6, 7) (Fig. 2A and 3A). This is probably because the xenotropic virus-like sequences (Fig. 3A) expressed in those tissues are not found in any polytropic virus-related sequences, and therefore, they do not participate in these recombinational events, as discussed above and in the legend to Fig. 5.

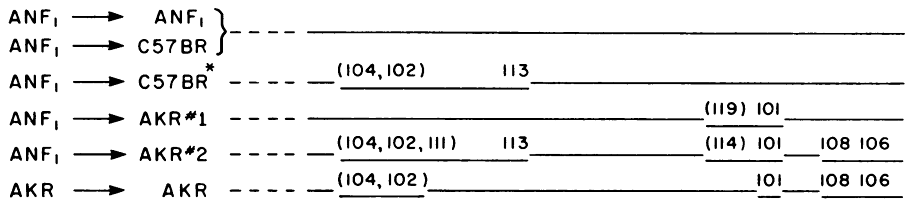
Our results also confirm an earlier report that preleukemic thymocytes can produce viruses that resemble the nonleukemogenic Akv virus in structure and host range but contain polytropic virus-related oligonucleotide markers limited to the p15E and U3 regions. We observed viruses with such structures in isolates from intact AKR mice (31) and ANF_1→AKR chimeras (Fig. 2C and 5). The biological role of these p15E-U3 recombinants during the development of spontaneous thymic leukemia in AKR mice remains speculative. However, several observations suggest they may be important in leukemogenesis. First, several leukemogenic AKR viruses with ecotropic host range and Akv-like gp70 gene sequences have been found to differ from Akv virus only in the 3′ end of p15E and in the U3 region (25). These viruses may undergo further recombination in vivo to generate viruses with non-Akv gp70 gene sequences (10). Second, the expression of ecotropic viruses with recombinant p15E-U3 sequences precedes the expression of viruses with non-Akv gp70 gene sequences or polytropic viruses during the development of spontaneous thymic leukemia in AKR/J mice (31). Whether ecotropic viruses with the non-Akv p15E-U3 region sequences can directly induce preleukemic changes in thymocytes or are involved in the generation of leukemogenic polytropic viruses through a stepwise recombination process as proposed by Thomas and Coffin (31), or both, remains to be shown.

Another observation made during this study also suggests that the p15E-U3 region of recombinant viruses is important in leukemogenesis. One of the ANF_1→C57BR chimeric animals produced viruses that were recovered on mouse NIH 3T3 cells and contained unique genomic structures. The viral mixture contained at least two genomes, one representing the endogenous

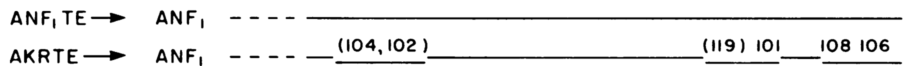


I. NIH3T3 ISOLATES

A. Bone Marrow Chimeras

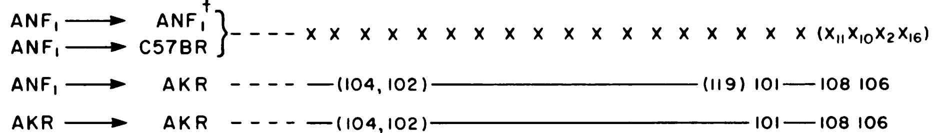


B. Thymus Graft Chimeras



II. MINK ISOLATES

A. Bone Marrow Chimeras



B. Thymus Graft Chimeras

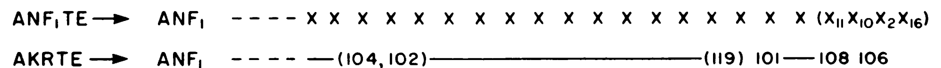


FIG. 5. T_1 oligonucleotide maps of viral isolates from thymocytes of chimeras. Maps were constructed as outlined in the text. 3′ portions of the linear RNA genomes are shown, using conventions as in Fig. 1. Akv oligonucleotides are numbered at the top. The presence of oligonucleotides identical to those of Akv virus in the viral genomes is shown by a solid line. Allelic substitutions by non-Akv polytropic virus-related oligonucleotides are numbered above 100 and placed in appropriate map positions. In cases in which viral mixtures were present, non-Akv and Akv oligonucleotides mapping in or near the same region of the genome are shown just above and below the center line of the map. *, Only the thymocytes from one $ANF_1 \rightarrow C57BR$ chimera produced this type of virus mixture (see text). †, Putative NZB xenotropic virus oligonucleotides are designated by X, with numbers for those that have been mapped at the 3′ end. These X oligonucleotides are not present in Akv virus or in the recombinants. These X oligonucleotides were also present in the mink isolates from the other chimeras shown in this figure. Splenocytes of all chimeras and thymocytes and splenocytes of intact ANF_1 mice yielded viruses with oligonucleotides identical to those of Akv virus and the $ANF_1 \rightarrow ANF_1$ mink-tropic isolate.

Akv-type virus and another with substitutions of polytropic virus-related oligonucleotides limited to the gp70 gene (Fig. 2B and 5). Despite the presence of these recombinant virus sequences, the thymocytes did not show any evidence of preleukemic changes (Table 1) (7, 36). In our experience with a number of fingerprints of viruses isolated from AKR mice, we have never seen this particular pattern (C. Thomas and J. Coffin, unpublished observations). To date, we have been unable to isolate mink-tropic virus from this mixture, and it is possible that the oligonucleotides present are from a defective virus present in the isolate, or represent a family of viruses with similar non-Akv gp70 gene sequences but with differing types of non-Akv sequences in the p15E-U3 and other regions. However, since this virus did not induce the typical preleukemic changes in the thymus, it would suggest that viruses containing polytropic virus-related sequences only in the gp70 gene but not in the p15E-U3 region are not leukemogenic. This hypothesis is being tested in our and other laboratories (C. Holland and N. Hopkins, personal communication).

Finally, our observations have certain implications concerning the cellular mechanisms of spontaneous leukemogenesis in AKR mice. It

would appear that the formation of leukemogenic recombinant viruses can be mediated solely by AKR thymic epithelium. Such viruses then infect target thymocytes and induce preleukemic changes in the cellular phenotype and, ultimately, leukemic transformation. Since thymic epithelium plays a role in regulating T-cell differentiation and maturation (11, 12, 32, 38), it is possible that the AKR thymic epithelium induces abnormal differentiation patterns with expression of retroviruses and formation of recombinants in the ANF_1 as well as AKR thymocytes. Alternatively, this tissue may be the source of recombinant viruses (33) that are capable of infecting the target thymocytes. However, we cannot exclude the possibility that thymic epithelium itself is susceptible to selective infection by and replication of preformed recombinant viruses that were generated elsewhere in the young AKR mice used as donors in our transplantation experiments.

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