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Free and Integrated Recombinant Murine Leukemia Virus DNAs Appear in Preleukemic Thymuses of AKR/J Mice

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We studied the appearance and structure of murine leukemia viral genomes in preleukemic AKR/J mice by Southern hybridization. Up to an average of one to two copies per thymocyte of unintegrated murine leukemia virus DNA appears in the thymuses of preleukemic mice beginning at 4 to 5 months of age and disappears in leukemic thymuses. The free viral genomes are absent in the spleens, livers, and brains of preleukemic mice. Using a series of ecotropic and nonecotropic murine leukemia virus hybridization probes, we showed that the unintegrated viral genomes are structurally analogous to those of recombinant mink cell focus-forming viruses that appear as proviruses in leukemic AKR thymocytes, suggesting that these free viral DNAs are the direct precursors to the leukemia-specific proviruses. The mosaic of ecotropic and nonecotropic sequences within these unintegrated viral DNAs varies from one preleukemic thymus to another but often appears structurally homogeneous within individual thymuses, indicating that often each thymus was being infected by a unique mink cell focus-forming virus. Analysis of high-molecular-weight DNA shows that recombinant proviruses reside in the chromosomal DNA of thymocytes within the preleukemic thymus, with the number rising to an average of several copies per thymocyte, but we do not detect any preferred integration sites. These results suggest that, in general, before the development of thymic leukemias in AKR mice there is a massive infection by a unique mink cell focus-forming virus which then integrates into many different sites of individual thymocytes, one of which grows out to become a tumor.

Mice of the AKR strain exhibit a complex pattern of murine leukemia virus (MuLV) expression that is associated with the development of thymic leukemias in these mice beginning at 6 months of age (reviewed in reference 9). Two classes of MuLV proviruses reside in the AKR mouse germ line: ecotropic MuLV (called AKV), that replicate preferentially in murine cells, and a much more numerous but less well defined class of nonecotropic MuLV. Although AKV virus is produced throughout the life span of AKR mice, neither class of MuLV is able to directly induce leukemia in healthy mice. Instead, mink cell focus-forming (MCF) viruses, that appear in the preleukemic AKR thymus (10), are probably the proximal oncogenic agent.

MCF viruses arise during the development of AKR mice by recombination between the germ line ecotropic and nonecotropic MuLV (4, 5, 8, 14, 17, 19, 24). The recombination patterns within MCF genomes are variable and complex. Nevertheless, those MCF viruses that are able to induce lymphomagenesis when inoculated into young AKR mice (7, 21) carry a common mosaic pattern of ecotropic and nonecotropic sequences within the envelope (*env*) gene and the long terminal repeat (LTR) sequences. The *env* gene encodes two peptides: gp70 and p15E. In oncogenic MCF viruses certain gp70 encoding and LTR sequences are derived from the nonecotropic parent virus, whereas the amino-terminal p15E coding sequences originate from AKV, the ecotropic parent (4, 11, 17–19, 24). Somatically acquired proviruses with recombinant structures similar to the oncogenic MCF viruses appear in the DNA of leukemic thymuses (3, 13, 23).

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We recently described the isolation of a series of short ecotropic MuLV-specific hybridization probes derived from the *pol* and *env* genes of AKV (13, 14). We used these probes to study the structure of somatically acquired proviruses in AKR/J thymic leukemias (13). They showed that the genomes of leukemic thymocytes acquire numerous proviruses that are not present in the AKR germ line. These proviruses have structures similar to the lymphomagenic MCF viruses in that they contain nonecotropic gp70 coding sequences and ecotropic amino-terminal p15E coding sequences. The structure of these MCF-like proviruses vary from one leukemia to another but, in general, the structures of the multiple new proviruses within each tumor appear to be identical.

During these studies we analyzed undigested thymus DNA by Southern hybridization (25) to determine whether the viral DNAs we were studying were integrated into the genomic DNA. Our analysis of undigested DNA showed that in general leukemic thymus DNA only contained integrated MuLV proviruses, but DNA from preleukemic thymuses did contain large amounts of unintegrated MuLV DNA.

We analyzed the structure of these free viral DNA genomes with both the set of short ecotropic MuLV-specific probes and a nonecotropic gp70 probe and found that most of these MuLV genomes are structurally analogous to the tumor-specific proviruses (i.e., MCF viruses). Furthermore, in many cases each preleukemic thymus contains a homogeneous population of recombinant proviruses, whereas there is a large degree of structural heterogeneity between MuLV DNAs in different thymuses. By isolating chromosomal DNA from preleukemic thymuses we found that MCF-like proviruses are also present within the genomes of the preleukemic thymocytes. These studies identify and characterize the probable precursors to somatically acquired proviruses in AKR leukemias.

MATERIALS AND METHODS

Mice and DNA isolation. Six-week-old AKR/J mice were obtained from Jackson Laboratories and sacrificed at the ages indicated. DNAs from mice younger than 6 weeks were from our previous study of germ line proviruses (12). Mice were considered preleukemic if no enlargement of the thymus, spleen, or lymph nodes was observed. DNAs were purified as described previously (13) or as follows: to compare the copy number of unintegrated viral DNA with the chromosomal germ line proviruses we took care not to select preferentially for either the free or integrated MuLV DNAs. The tissues were frozen and ground to a fine powder with a mortar and pestle, and a small portion was solubilized at 50°C in 5 ml of 0.5 M EDTA–0.1% Sarkosyl–100 μg of proteinase K per ml (Boehringer Mannheim) for 9 h at an approximate DNA concentration of 50 μg/ml. After a single phenol-chloroform-isoamyl alcohol (25:24:1) extraction, the entire aqueous phase was dialyzed extensively against 10 mM Tris-hydrochloride (pH 8)–1 mM EDTA.

Chromosomal DNA from preleukemic thymuses was purified by electrophoresis of DNA samples (100 μg) through submerged horizontal 0.7% agarose gels in 50 mM Tris-acetate (pH 8.0)–20 mM sodium acetate–1 mM EDTA for 40 h at 0.5 V/cm. The chromosomal DNA was eluted by electrophoresis within dialysis tubing, followed by ethanol precipitation.

Hybridization probes. Seven individual hybridization probes were used to study the appearance and structure of MuLVs in preleukemic AKR mice. To identify unintegrated MuLV DNAs we used as probe the 8.2-kilobase (kb) AKV *Pst*I fragment that represents the entire AKV genome (27). This fragment was purified by polyacrylamide gel electrophoresis of a *Pst*I digest of the plasmid pAKV1 (11), followed by electrophoretic elution of the 8.2-kb *Pst*I fragment. The isolation of the five short ecotropic MuLV-specific hybridization probes: *pol*, gp70, Cgp70, Np15E, and Cp15E, has been described previously (13). The nonecotropic gp70 probe was a 622-base pair *Bam*HI/*Eco*RI fragment isolated from a noninfectious molecular clone of MCF 247 (generously provided by C. Holland). We first isolated the 2.9-kb *Xho*I/*Eco*RI fragment spanning the gp70 and C-terminal *pol* coding sequences by polyacrylamide gel electrophoresis. After elution, we digested the *Xho*I/*Eco*RI fragment with *Bam*HI and similarly isolated the 622-base pair *Bam*HI/*Eco*RI fragment that spans the 5′ region of the MCF 247 gp70 coding sequences deriving from the nonecotropic parent (15).

The conditions for radiolabeling of the DNA probes, Southern transfer, and hybridization were as described previously (13) except that when using the general 8.2-kb AKV probe, the final wash of the Southern blots was done at 65°C in 2× SET (300 mM NaCl, 60 mM Tris-hydrochloride [pH 8], 4 mM EDTA) to allow for cross-hybridization. Exposures of the filters were scanned by an Ortec 4310 densitometer, and the relative intensity of each band was measured by weighing the area under each peak.

RESULTS

Free MuLV DNA in the preleukemic AKR thymus. We analyzed undigested DNA isolated from AKR/J mice of different ages by Southern hybridization (25) with a nonspecific probe representative of the entire AKV genome. Under the low stringency conditions we employed (see above), this probe hybridizes to both ecotropic and nonecotropic MuLVs. Each sample contains slowly migrating high-molecular-weight chromosomal DNA that hybridizes strongly to the AKV hybridization probe because of the many endogenous germ line MuLV genomes. The unintegrated viral DNA may be one of three forms: an 8.8-kb linear molecule or 8.2- or 8.8-kb closed circles carrying either one or two LTRs, respectively (see reference 33). Under our conditions for gel electrophoresis, each of these forms migrates ahead of the chromosomal DNA.

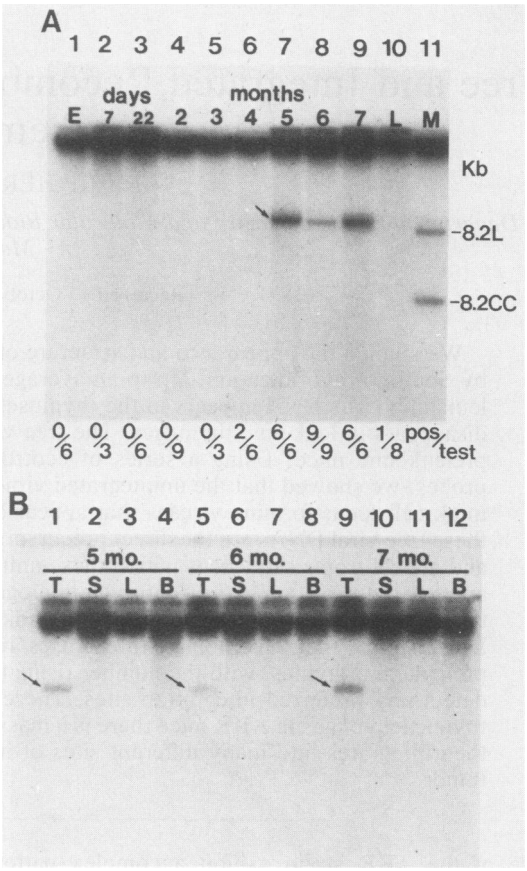

FIG. 1. Unintegrated MuLV DNAs appear in the preleukemic AKR/J thymus. Undigested DNAs (2 μg) were electrophoresed through 0.7% agarose gels, transferred to nitrocellulose filters, and hybridized to the entire genomic AKV probe as described in the text. (A) DNAs were isolated from a 17-day-old embryo (lane 1), 7- (lane 2) and 22-day-old (lane 3) livers, 2- through 7-month-old (lanes 4 through 9) preleukemic thymuses, and a leukemic thymus (lane 10). Linear and closed-circular molecules (8.2 kb) (lane 11) were used as size markers and mixed with uninfected thymus DNA to control for retardation during electrophoresis. The linear molecule is the full-length *Pst*I fragment of AKV, and the closed-circular molecule is a deleted AKV subclone in pBR322 (pAKV2-12) described elsewhere (11). The numbers below each lane show the number of samples from each age group in which we detected unintegrated MuLV DNAs (pos) over the number of samples tested (test). (B) The 5- (lane 1), 6- (lane 5), and 7- (lane 9) month-old thymus samples from (A) were probed alongside undigested DNA isolated from the respective spleen (lanes 2, 6, and 10), liver (lanes 3, 7, and 11) and brain (lanes 4, 8, and 12) tissues of these mice.

Figure 1A shows that unintegrated MuLV DNA appears in the preleukemic AKR/J thymus. Individual 5, 6, and 7-month-old AKR/J thymuses contained unintegrated viral DNA (Fig. 1A, lanes 7 through 9, arrow), whereas DNA from an embryo (lane 1), the livers of 1- and 3-week-old mice (lanes 2 and 3), and the thymuses of 2-, 3-, and 4-month-old

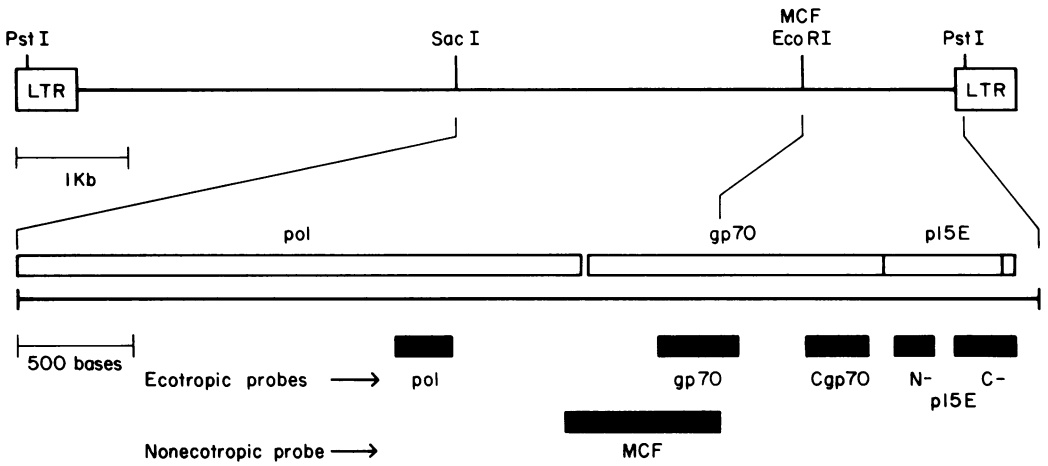

FIG. 2. Location of ecotropic and nonecotropic hybridization probes used to study the structure of free and integrated MuLV genomes in the preleukemic AKR/J thymus. The top portion shows the location of the terminal *Pst*I sites present in both AKV and MCF proviruses (4, 27) and the non-AKV *env Eco*RI site that MCF viruses acquire by recombination (4, 22). The region between the unique *Sac*I site of AKV and the 3′ LTR *Pst*I site is expanded below the provirus structure, showing the location of *pol* and *env* coding sequences within this region (11). The positions of the five ecotropic MuLV-specific probes, *pol*, gp70, Cgp70, Np15E, and Cp15E, and the one nonecotropic MuLV-specific *env* probe, called MCF, are shown at the bottom of the figure.

mice (lanes 4 through 6) did not. We also did not detect free MuLV DNA in a leukemic thymus (lane 10). Comparison with linear and closed-circular 8.2-kb size markers (Fig. 1A, lane 11) shows that the unintegrated DNA migrates as a full length 8.8-kb linear molecule. Under the conditions used for Southern transfer (i.e., with an acid depurination step [32]), both closed-circular and linear DNA molecules are detected equally well (data not shown). The majority of the unintegrated MuLV genomes are, therefore, of the linear form in the preleukemic thymuses of AKR mice. Long exposures, however, of some of the samples analyzed revealed small amounts of the circular form of the provirus.

We analyzed DNA from many individual mice of each age group; the results are shown below each lane of Fig. 1A. We did not detect free proviral DNA in tissues from mice younger than 4 months, but the preleukemic thymuses from 5- to 7-month-old mice always contained some unintegrated DNAs. Two of six thymuses from 4-month-old mice and one of eight leukemic thymuses had unintegrated viral DNA, indicating that the free MuLV DNA appears in the thymuses of AKR mice when they are 4 to 5 months old and generally disappears in the grossly leukemic thymus.

To study the tissue specificity of the unintegrated MuLV DNA, we isolated DNA from the spleens, livers, and brains of preleukemic mice. Figure 1B shows that the unintegrated DNAs observed in the thymuses of the three positive samples in Fig. 1A (shown in lanes 1, 5, and 9 of Fig. 1B; see arrows), are not detectable in the spleens (lanes 2, 6, and 10), livers (lanes 3, 7, and 11), or brains (lanes 4, 8, and 11) of these mice. Therefore, among these four tissues the free viral DNAs are preferentially located in the preleukemic thymus.

Recombinant structure of free MuLV DNAs. Figure 2 shows the location within the MuLV genome of the six hybridization probes that we used to study the structure of MuLV DNAs found in the preleukemic thymus. Each probe hybridizes uniquely to short regions of either ecotropic or nonecotropic MuLVs. The five ecotropic MuLV-specific probes have been described previously (13) and derive from within *pol* (*pol*), the central (gp70) and C-terminal (Cgp70) gp70 coding sequences, and the N-terminal (Np15E) and C-terminal (Cp15E) p15E coding sequences of the AKV genome. The nonecotropic probe originates from the 5′ gp70 coding sequences of MCF 247 (see above).

Figure 3 shows the hybridization patterns of unintegrated viral DNAs from 20 preleukemic thymuses with the AKV genomic probe (Fig. 3A), five ecotropic MuLV-specific probes (Fig. 3B through F), and the nonecotropic MCF 247 gp70 probe (Fig. 3G). Only that portion of each Southern blot that contained the free viral DNA is shown. DNA from a molecular clone of AKV (lane C) and *Bam*HI-digested AKR chromosomal DNA (not shown) served as controls to determine whether during the hybridization each probe had been either ecotropic or nonecotropic MuLV specific. As expected, the AKV-derived probes each hybridized to the AKV DNA (Fig. 3A through F, lane C), but the MCF 247 gp70 probe did not (Fig. 3G, lane C).

The hybridization probe representing the entire AKV genome showed that unintegrated virus was present in each of these preleukemic thymuses (Fig. 3A, lanes 1 through 20). The level of MuLV DNA in three of the samples (lanes 5, 13, and 18) was too low to detect with the short ecotropic virus-specific probes. The five ecotropic MuLV-specific hybridization probes (Fig. 3B through F) identify complex and variable recombination patterns within the free MuLV genomes. Consistent with the structure of MCF-like proviruses in the leukemic thymus (13), these unintegrated DNAs always hybridize to the Np15E probe (Fig. 3E) and seldom to the gp70 probe (3C), whereas the *pol* (3B), Cgp70 (3D), and Cp15E (3F) probes identify structural heterogeneity. The nonecotropic MCF-derived gp70 probe hybridizes to the unintegrated viral DNA in each sample (Fig. 3G). These results are consistent with the suggestion that the majority of the free viruses detected in the preleukemic thymus are MCF MuLVs. We cannot prove, however, that some of the unintegrated DNAs are not entirely derived from nonecotropic MuLVs because we do not have available a nonecotropic probe that would distinguish these from MCF viruses.

In many cases, the hybridization patterns generated by the short ecotropic MuLV-derived probes indicate a unique population of recombinant viral DNAs within individual thymuses. For example, the unintegrated DNA in lane 2

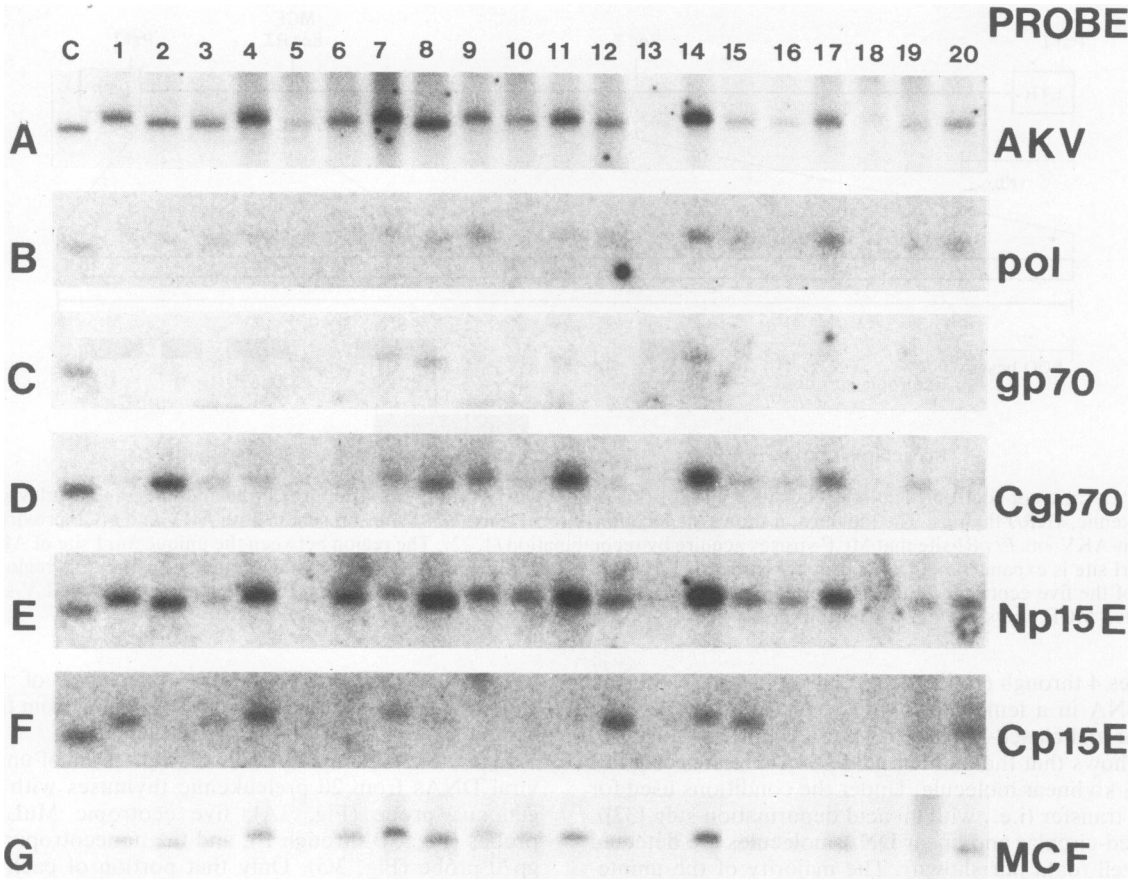

FIG. 3. Most unintegrated MuLVs in preleukemic thymuses are MCF-like recombinants. Southern blots of undigested DNAs (5 μg) that were separated by electrophoresis through 0.7% agarose gels were hybridized to the genomic AKV (A), *pol* (B), gp70 (C), Cgp70 (D), Np15E (E), Cp15E (F), and MCF (G) probes as described in the text. The hybridization to the chromosomal DNA has been omitted from each panel. The ecotropic hybridization control (lane C) contains the 8.2-kb genomic AKV *Pst*I fragment. The DNAs were isolated from 4- (lanes 1 and 2), 5- (lanes 3 through 8), 6- (lanes 9 through 14), and 7- (lanes 15 through 20) month-old preleukemic thymuses.

(Fig. 3) hybridizes strongly to the Cgp70 and Np15E probes but does not hybridize to the *pol*, gp70, and Cp15E probes. Because the recombinant structures vary greatly, the homogeneous structure of the viral genomes in sample 2 suggests that these recombinant viruses derived from a single parent. By the same criterion, the free MuLVs in samples 1, 3, 6, 9, 10, 12, 17, 19, and 20 (Fig. 3) also appear to be homogeneous populations. In some cases the extent of hybridization by the *pol*, Cgp70, and Cp15E probes to the recombinant viruses is less than that of the Np15E probe; for example, compare the Cgp70 and Np15E hybridizations in lanes 15 and 16. This can either reflect a heterogeneous population of free viral DNAs or result from a crossover point between nonecotropic and ecotropic parent viruses within these probe sequences (e.g., the Cgp70 sequences in the proviruses in lanes 15 and 16), thus causing the ecotropic MuLV-specific probe to hybridize less efficiently.

In this experiment we detected free viral DNA in four preleukemic thymuses with the ecotropic gp70 probe (Fig. 3C; lanes 7, 8, 11, and 14). The gp70 hybridization is always weaker than the Np15E hybridization and probably results from a subpopulation of viruses that contain ecotropic gp70 sequences. In other experiments, where we had long exposures of Southern hybridizations with purified unintegrated MuLV DNAs, we invariably detected weak hybridization by the ecotropic gp70 probe to ecotropic MuLV-specific restriction fragments (data not shown). These results are consistent with the appearance in some AKR leukemias of a small number of somatically acquired proviruses carrying the ecotropic gp70 probe sequences (13).

Copy number of unintegrated viral DNAs. To determine the average number of copies of free viral DNA per thymocyte, we compared the extent of hybridization by the unintegrated DNAs to that of the germ line ecotropic proviruses that are present twice per cell. To be able to compare the unintegrated and integrated viral genomes, we carefully extracted total DNA from preleukemic thymuses so that neither of these two forms was preferentially selected (see above). The DNAs were then digested with *Eco*RI and hybridized to the Np15E probe.

*Eco*RI does not cleave the germ line ecotropic proviruses (26). Therefore, each provirus lies on an integration-specific restriction fragment. AKR mice from Jackson Laboratories all carry three ecotropic germ line proviruses that lie on 50-, 12-, and 11-kb *Eco*RI fragments, whereas two other germ line ecotropic proviruses that lie on 17- and 14-kb *Eco*RI fragments are scattered throughout the Jackson Laboratory AKR mouse colony (1, 12, 20). MCF viruses, on the other hand, acquire from the nonecotropic parent virus an *Eco*RI site within the gp70 coding sequences that lies 1.9-kb from the 3′ terminus of the free linear genome (see Fig. 2) (4, 22).

Figure 4 shows the Southern blot of *Eco*RI-digested

preleukemic thymus DNA from 4-, 5-, 6-, and 7-month-old mice (lanes 1 through 4, respectively). The Np15E probe hybridizes to the 1.9-kb 3′ terminal fragment from the unintegrated viruses in each sample. We measured the relative intensities of the germ line ecotropic proviruses and unintegrated 1.9-kb band by scanning the film with a densitometer. Comparisons of the 11- and 12-kb bands (the lower doublet at the top of Fig. 4) with the 1.9-kb unintegrated virus band indicated that there were, on the average, 1.1, 1.6, 0.8, and 1.1 free MuLV genomes per thymocyte in lanes 1 through 4, respectively. If these free viruses were present in only a subpopulation of thymocytes, then the copy number per cell would be correspondingly higher in the infected thymocytes. A qualitative examination of the intensity of unintegrated provirus bands in Fig. 3E (the Np15E hybridization) suggests that the number of free viral genomes reaches a maximum at 5 to 6 months and then begins to decrease at 7 months.

Integrated somatically acquired MCF-like proviruses in the preleukemic thymus. To examine integrated proviruses in the genomes of preleukemic thymocytes, we separated chromosomal DNA from unintegrated MuLV DNAs by preparative agarose gel electrophoresis (see above) and digested the purified chromosomal DNA with *Eco*RI, either alone or in combination with *Pst*I. *Eco*RI generates integration-specific provirus fragments, but *Pst*I cuts within the LTR and therefore separates the proviruses from their flanking cellular sequences. *Eco*RI and *Pst*I double digestion generates an 8.2-kb germ line ecotropic MuLV *Pst*I restriction fragment, whereas somatically acquired MCF proviruses are cut into two pieces; the 5′ region lies on a 6.8-kb fragment, and the 3′ terminal sequences lie on a 1.4-kb fragment (see Fig. 2).

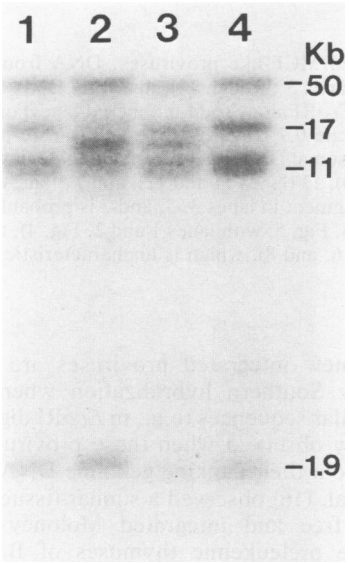

FIG. 4. Quantitative comparison of unintegrated MuLV DNAs and integrated germ line ecotropic proviruses. DNAs isolated as described in the text from 4-, 5-, 6-, and 7-month-old thymuses (lanes 1 through 4, respectively) were digested with *Eco*RI and hybridized, after electrophoresis through an 0.7% agarose gel and transfer to a nitrocellulose filter, to the Np15E probe as described in the text. The samples are the same as the 4-, 5-, 6-, and 7-month-old thymus DNAs shown in lanes 1, 4, 10, and 17, respectively, of Fig. 3. The sizes of the integrated and unintegrated (1.9-kb) *Eco*RI fragments are given in kb.

Figure 5 shows the purified chromosomal DNAs from six preleukemic thymuses (lanes 3 through 14) hybridized to the Np15E probe. The *Eco*RI digests are the odd-numbered lanes, and the *Eco*RI/*Pst*I double digests are the even-numbered lanes. For comparison, we show the results for DNA isolated from the thymus of a 3-month-old AKR mouse (lanes 1 and 2), the unfractionated DNA (lanes 17 and 18) from the 7-month-old preleukemic thymus shown in lanes 13 and 14, and a leukemic thymus (lanes 15 and 16).

The *Eco*RI-cut DNA from the 3-month-old thymus (lane 1) shows a germ line pattern of ecotropic proviruses: the ubiquitous 11-, 12-, and 50-kb fragments and the 14-kb fragment which is present in some AKR/J mice. This sample lacks the variable 17-kb *Eco*RI provirus fragment, but this provirus does appear in all of the preleukemic thymus DNA samples (lanes 3, 5, 7, 9, 11, and 13). Digestion with *Pst*I produced the common 8.2-kb ecotropic MuLV *Pst*I fragment (lane 2). In this experiment the Np15E probe hybridized weakly to nonecotropic proviruses, which explains the very faint series of bands between about 2- and 8-kb in the control *Eco*RI digest (lane 1) and a 700-base pair fragment that appears in all of the *Eco*RI/*Pst*I double digests (even-numbered lanes).

The unfractionated DNA from a preleukemic thymus, when digested with *Eco*RI, displayed two restriction fragments at 1.9- and 2.0-kb (lane 17); these represent the 3′ terminus of the unintegrated viral DNA. (The larger fragment of the doublet probably contains a duplication within the U3 region, as has been observed frequently in MuLV proviruses [see reference 29].) As expected, these fragments disappear when the chromosomal DNA has been purified from the unintegrated DNAs (compare lane 17 with lane 13).

The leukemic thymus DNA, when digested with *Eco*RI (lane 15), displays a series of individual somatically acquired provirus fragments, the majority of which (six) are between 2 and 8 kb long. Because the bands representing the somatically acquired proviruses are about half the intensity of the germ line provirus fragments, each provirus is probably present once in each leukemic thymocyte representing a monoclonal tumor (13). When digested further with *Pst*I, the leukemia-specific MuLV *Eco*RI fragments and the unintegrated MuLV fragments from the preleukemic thymus (lane 17) were cleaved to generate the common 1.4-kb *Eco*RI/*Pst*I fragment (lanes 16 and 18).

In contrast to the leukemic thymus DNA, we only observed somatically acquired proviruses in one (lane 7) of the *Eco*RI-digested preleukemic thymus DNAs. (The 4.3-kb *Eco*RI fragment in lanes 3, 5, and 7 is probably an artifact [see the legend to Fig. 5].) Because the bands in the one 6-month-old thymus sample (lane 7) are quite faint, they probably represent new provirus integrations into one or more subpopulations of thymocytes in a developing tumor. Although not visible in the *Eco*RI digests, each preleukemic thymus carries integrated recombinant proviruses because in each case further digestion of these DNAs with *Pst*I produced the MCF-derived 1.4-kb *Eco*RI/*Pst*I fragment that is missing in the 3-month-old thymus DNA sample (compare lanes 4, 6, 8, 10, 12, and 14 with lane 2). These results suggest that the free proviruses in the preleukemic thymus integrate at different sites in different thymocytes.

We estimated the number of copies of integrated MCF-like proviruses in the six preleukemic thymuses shown in Fig. 5 by comparing the intensity of the MCF-derived 1.4-kb band with the germ line 8.2-kb *Pst*I fragment with a densitometer. The 6- and 7-month-old samples in lanes 8, 10, 12, and 14 had, on the average, 5.3, 3.2, 0.3, and 2.2 somatically

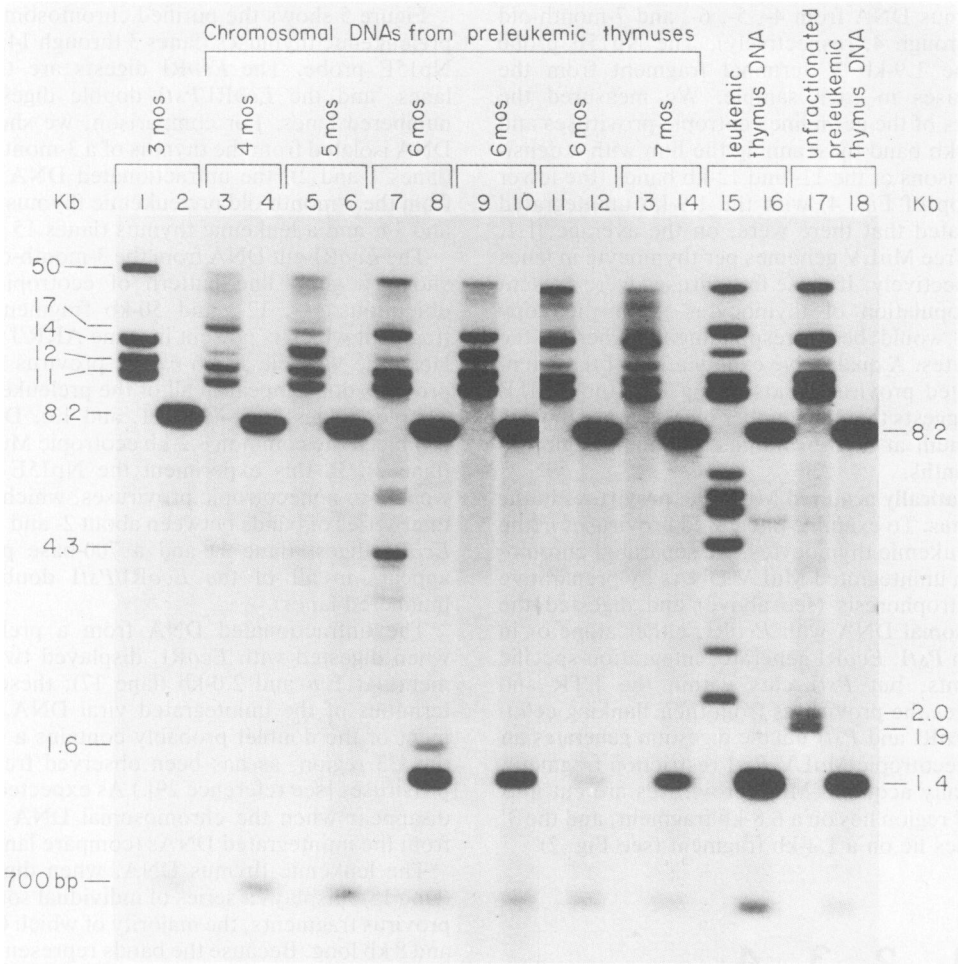

FIG. 5. Chromosomal DNAs from preleukemic AKR/J thymuses contain integrated MCF-like proviruses. DNA from a 3-month-old thymus (lanes 1 and 2), purified chromosomal DNAs from six preleukemic thymuses (lanes 3 through 14), and total DNA from a leukemic (lanes 15 and 16) and a preleukemic (lanes 17 and 18) thymus were digested with either *Eco*RI alone (odd-numbered lanes) or in combination with *Pst*I (even-numbered lanes) and analyzed, after electrophoresis through two separate 0.7% agarose gels and transfer to nitrocellulose filters, by hybridization to the Np15E probe as described in the text. The purified chromosomal DNAs were from the following samples shown in Fig. 3: samples 1 (lanes 3 and 4), 4 (lanes 5 and 6), 9 (lanes 7 and 8), 10 (lanes 9 and 10), 12 (lanes 11 and 12), and 17 (lanes 13 and 14). The sizes of restriction fragments discussed in the text are given in kb. The 4.3-kb *Eco*RI fragment in lanes 3, 5, and 7 is probably an artifact because it is not present in other *Eco*RI digests of the same samples (compare lanes 3 and 5, Fig. 5, with lanes 1 and 2, Fig. 4), and it appears to correlate with the 1.6-kb fragment in the *Eco*RI/*Pst*I digests of these samples (lanes 4, 6, and 8), which is uncharacteristic of these MuLV proviruses. bp, Base pairs.

acquired proviruses per thymocyte, whereas the new recombinant proviruses in the 4- and 5-month-old thymuses were so few (the bands were barely visible in the autoradiogram shown in Fig. 5, lanes 4 and 6) that they could not be quantitated. Comparison with the appearance of unintegrated viral DNAs suggests that at 4 to 5 months the majority of the MCF-like viral genomes in the thymus are unintegrated, but with time the free DNAs disappear as integrated genomes increase in number.

DISCUSSION

We have described the appearance and structure of free and integrated MuLVs in the preleukemic thymuses of AKR/J mice. Unintegrated linear MuLV genomes first appear in the thymus at 4 to 5 months, rise to a level of, on the average, about one per thymocyte, and then disappear in the leukemic thymus, which is dominated by a monoclonal tumor. The new integrated proviruses are generally not detectable by Southern hybridization when they remain linked to cellular sequences (e.g., in *Eco*RI digests), but they can be readily observed when these proviruses have been dissociated from their flanking genomic DNA.

Jaehner et al. (16) observed a similar tissue-specific accumulation of free and integrated Moloney-MuLV-related DNAs in the preleukemic thymuses of BALB/Mo mice carrying the *Mov-1* allele. These mice have a single germ line Moloney provirus and develop thymus-derived leukemias analogous to those observed in AKR mice (30, 31). Although not addressed by Jaehner et al. (16), the unintegrated viruses in the BALB/Mo preleukemic thymus probably represent the MCF-like MuLVs that are found integrated in the leukemic thymuses of these mice (30).

We extended our previous structural analysis of somatically acquired recombinant proviruses in AKR thymic leukemias (13) to these preleukemic thymus-specific MuLVs.

Hybridization to the series of short ecotropic MuLV-specific *pol* and *env* probes shows that the large majority of these free proviruses have recombinant structures analogous to leukemogenic MCF viruses. Although the recombination patterns of the free MuLV genomes vary from one preleukemic thymus to the next, frequently individual thymuses appear to contain MuLVs that are structurally homogeneous. The unintegrated viruses are analogous to somatically acquired proviruses present in leukemic thymuses; both acquire certain nonecotropic gp70 coding sequences but retain ecotropic N-terminal p15E coding sequences. These results suggest that the free MuLV DNAs we begin to detect in 4- to 5-month-old AKR/J thymuses are the direct precursors to the somatically acquired proviruses that appear in leukemic AKR/J thymuses.

When we purified and examined the chromosomal DNA of the preleukemic thymus, we detected an increase in new integrated proviruses, rising to a level of several copies per thymocyte at 6 months. We did not detect preferred sites for the integration of these somatically acquired proviruses. The increasing amount of viral DNA integrated over the 4 to 6 months either reflects further integrations at new sites or the polyclonal growth of leukemias. The grossly leukemic thymus, however, is taken over by a monoclonal cell population which has unit copies of several proviruses (13). Earlier work with AKR leukemias also suggests that there are not preferred sites for MuLV integration because each leukemia has a different pattern of provirus integrations (2, 3, 13, 23, 26). It is still possible, however, that tumorigenesis does not occur until a provirus has integrated into one or more specific sites or regions within the genome.

Our previous study showed that AKR/J thymic leukemias often contain numerous structurally identical somatically acquired proviruses that appeared to be the descendents of a unique MCF virus (13). There were several possible explanations for this: (i) the tropism of an individual MCF virus may be specific for a small subset of thymocytes such that any one cell may be infected by only one type of MCF virus; (ii) individual preleukemic thymocytes may be infected by a single MCF virus and the numerous proviruses arise by intracellular reintegration of viral genomes expressed from the initial provirus; or (iii) MCF viruses may arise infrequently but then infect many different thymocytes. The appearance of a large number of unintegrated MCF-like viruses in the preleukemic thymus that frequently appear to be structurally identical suggests that structural homogeneity among new proviruses in leukemic thymuses is due to the rare appearance of thymotropic MCF viruses in AKR mice. Consistent with this, purified MCF virus isolates are able to infect the majority of cells within the thymus after injection into young AKR mice (6, 21).

Of four tissues tested in preleukemic AKR mice (thymus, spleen, liver, and brain), only the thymus contained detectable quantities of unintegrated viral DNA. Cloyd (6) analyzed the appearance of MCF-infected cells in a variety of tissues after inoculation of young AKR mice with oncogenic MCF viruses (i.e., 247 and C58LI) and found that virtually only thymus cells were infected. These results further identify both the relatedness between lymphomagenic MCF viruses and the unintegrated viral genomes we describe here and the strict tissue tropism of leukemogenic MCF viruses.

There are two major classes of lymphocytes within the thymus: a dominant population (80 to 90%) of immature T cells that are incompetent immunologically and steroid sensitive and a minor population (10%) of apparently mature T cells that are insensitive to steroids. We have not identified which thymocytes contain the unintegrated MuLV DNAs, but Cloyd (6) has shown that thymocytes infected by inoculated MCF viruses are sensitive to the steroid dexamethasone, suggesting that the unintegrated DNAs are present in the major thymocyte population of immature T cells.

Thomas and Coffin (28) have analyzed by RNase T_1 oligonucleotide fingerprinting the genomes of murine leukemia viruses isolated from the thymuses of preleukemic AKR/J mice after cocultivation in vitro with NIH 3T3 cells. As early as 1.5 months, they detected MuLVs that appeared to have recombined within the carboxy-terminal p15E coding sequences and U3 region but not the sequences encoding gp70. Because the appearance of recombinant 3′ terminal U3 sequences seemed to be independent of the appearance of nonecotropic gp70 oligonucleotides, Thomas and Coffin (28) hypothesized that the generation of MCF viruses required two separate recombination events.

We did not detect any unintegrated viral DNAs in the thymuses of AKR/J mice younger than 4 months. The reason we did not detect MuLVs similar to those identified by Thomas and Coffin (28) is probably because we were studying products which arise after broad infection in the thymus, whereas they analyzed viruses that had been amplified in vitro by cocultivation with NIH 3T3 cells. This difference suggests that the viruses which they identified in young AKR thymuses do not infect thymocytes efficiently and that it is not until an AKV virus has recombined in at least two separate locations (gp70 and p15E/U3) to generate a leukemogenic MCF virus that the massive infection we observe in the preleukemic thymus occurs.

Taken together, the results described above and our results suggest that after a complicated period of recombination between the endogenous ecotropic and nonecotropic MuLVs a leukemogenic MCF virus is produced that, beginning at 4 to 5 months, produces a massive infection of the major thymocyte population of immature T cells. From this infection and its multiple integrations, a monoclonal tumor develops containing many somatically acquired MCF proviruses. Although the appearance of MCF viruses in AKR/J mice and their involvement in AKR leukemogenesis have been well documented, it is still not known how they induce the transformation of thymocytes.

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