AKR Thymic Lymphomas Involving Mink Cell Focus-Inducing Murine Leukemia Viruses Have a Common Region of Provirus Integration

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Newly acquired proviruses related to a mink cell focus-inducing murine leukemia virus were detected in low copy number in restriction endonuclease-digested DNAs from thymic lymphomas of AKR/J mice. These extra proviruses were not present in DNAs of either normal thymus or leukemic brain tissues. Extra tumor-specific DNA fragments generated by restriction endonucleases either were identical in size or fell into similar size classes, suggesting a common site(s) of provirus integration. Characterization of extra *EcoRI* DNA fragments for mink cell focus-inducing viral sequences revealed that all of them contained large terminal repeat sequences and that a significant number represented proviruses with deletions.

Murine leukemia viruses (MLVs) able to cause lymphocytic leukemias require relatively long latencies for the development of this neoplastic disease. The genomes of these slow-transforming MLVs do not contain any transduced cellular sequences besides the viral genes necessary for their replication. Therefore, transformation by these viruses does not appear to directly involve a virally encoded protein, and their mechanism of transformation remains to be elucidated. An example of a well-studied system for the involvement of MLVs in tumor development is the AKR mouse, which has been bred for a high incidence of T-cell leukemia (7).

The different types of MLV that have been implicated in AKR leukemogenesis include the AKR ecotropic, xenotropic, and dual-tropic mink cell focus-inducing (MCF) viruses (8, 18). Ecotropic viruses have a greater ability to infect murine cells in contrast to xenotropic viruses, which primarily infect heterologous cells, and the dual-tropic MLVs can grow in both cell types (8, 10, 11). It has been demonstrated that ecotropic virus expression at high titers is a requirement for the development of leukemia (12). Chattopadhyay et al. (3) have further shown that the genetic locus *Akv-1* is responsible for encoding the ecotropic virus. The MCFs are recombinant viruses that have characteristics of both ecotropic and xenotropic MLVs in their glycoprotein region, as demonstrated by RNase T₁ oligonucleotide fingerprinting (17), heteroduplexing (4), and tryptic peptide (6) analyses. The MCFs appear to be the proximal viral

agents for leukemogenesis since their expression coincides with an increase in MLV antigen expression in the preleukemic mouse (8, 10). Further support for the etiological role of the MCFs is the ability of particular isolates to accelerate the onset of leukemia when injected into newborn mice of certain strains (5). The MCFs with this ability have been shown also to be thymotropic (14).

It has been observed that there is an increase in the copy number of MLV-related sequences in the DNA of thymic lymphomas (1, 16, 19). These additional proviruses were detectable by Southern blotting because these thymic lymphomas appear to be of clonal origin, resulting in the generation of discrete bands of extra proviral DNA. By analyzing the proviruses of normal and leukemic tissues, we have demonstrated that this increase in MLV sequences was not due to amplification of either the intact genome or the envelope region of the endogenous AKR ecotropic proviruses (22). The observation has been made, however, that newly acquired viral sequences resembling the envelope region of MCF MLVs exist in DNAs from tumors but not from normal or embryonic tissues (1). It appears likely, therefore, that the MCFs are the viruses responsible for the increase in MLV sequences in tumor DNAs.

A promoter insertion mechanism has recently been advanced to explain B-lymphocyte transformation by the avian leukosis virus (ALV), another slow-transforming retrovirus. ALV has been shown to integrate adjacent to *c-myc* in

bursal lymphoma DNA, which results in a substantial increase in transcriptional activity of this cellular gene (9, 13, 15). We have observed that, similar to site-specific integration by ALV, MCF MLV proviruses also frequently integrate at a common site(s) in AKR thymic lymphoma DNAs. This integration event appears to correlate with lymphoma development.

MATERIALS AND METHODS

Isolation of tissue DNA. Thymic lymphomas were from AKR/J mice with either spontaneous leukemias or leukemias induced by neonatal injection of 1×10^4 to 2×10^4 infectious units of MCF-13 MLV (injected mice kindly provided by G. Baldwin). DNA was isolated from mouse brain tissue as previously reported (22). Preparation of DNA from thymus or thymic lymphomas was performed by coarsely mincing the tissue and pushing it through a sterile steel mesh with the rubber end of a disposable 10-ml syringe plunger. The fibrous material retained by the screen was discarded. The remaining cells were disrupted in a Dounce homogenizer in 10 ml of ice-cold reticulocyte standard buffer (1.5 mM $MgCl_2$, 10 mM NaCl, 10 mM Tris-hydrochloride, pH 7.4) at 4°C. Nuclei were centrifuged, rinsed twice with sodium dodecyl sulfate buffer (100 mM NaCl, 10 mM EDTA, 10 mM Tris-hydrochloride, pH 7.5), and lysed in sodium dodecyl sulfate buffer containing 0.5% sodium dodecyl sulfate and 0.2 mg of proteinase K per ml at 37°C for 2 to 4 h. DNA was phenol and chloroform extracted before dialysis against TE buffer (5 mM EDTA, 10 mM Tris-hydrochloride, pH 7.4) overnight. It was then treated with 0.05 mg of RNase A per ml at 37°C for 1 h followed by proteinase K digestion after addition of sodium dodecyl sulfate to 0.5% for 8 to 14 h at 37°C. Phenol and chloroform extractions were followed by dialysis against 0.3 N NaCl in TE buffer and finally against TE buffer alone.

Restriction endonuclease digestions. A 15- μ g portion of DNA was digested with *EcoRI*, *HindIII*, or sequentially with *EcoRI* and *XbaI* (New England Biolabs or Bethesda Research Laboratories) according to the manufacturer's conditions. Completeness of digestion by *EcoRI* was aided by preincubation of the reaction mixture with 0.2 μ g of RNase A per μ g of DNA for 15 min at 37°C (S. Welch and W. Schubach, personal communication).

Analysis of DNAs by agarose gel electrophoresis and Southern blotting. Restriction endonuclease-digested DNAs were electrophoresed through 0.7 or 1% agarose gels, using low-salt E buffer (5 mM sodium acetate, 1 mM EDTA, 40 mM Tris-hydrochloride, pH 7.9). Transfer to nitrocellulose filters and hybridization conditions were described previously (22).

Preparation of virus-specific hybridization probes. Restriction endonuclease fragments of molecular clones of MCF-13 MLV (21) to be used as probes for hybridization were prepared as previously described (22). Hybridization probes included the following DNA fragments: 8.2-kilobase pair (kbp) *EcoRI* MCF_{rep}; 600-bp *PstI* MCF_{LTR}; 900-bp *EcoRI-XbaI* MCF_{3'E-X} from the *env* region; 300-bp *SmaI-EcoRI* MCF_{3'E} from the *env* region. DNA fragments were

labeled with ^{32}P by nick translation to specific activities of 0.5×10^8 to 5×10^8 cpm/ μ g of DNA.

RESULTS

Restriction endonuclease digestions of normal and leukemic tissue DNAs. We have previously shown that AKR ecotropic MLV proviruses were not amplified in DNAs from thymic lymphomas compared with normal tissues from AKR/J mice. We wished to determine whether proviruses related to another class of MLVs, the dual-tropic MCF MLVs, were newly acquired in thymic lymphoma DNAs. This class of MLVs appears to be the proximal viral agent to leukemia development in the AKR/J mouse (5, 8). DNAs were isolated from brain and thymuses of normal (4 to 6 weeks old) and leukemic AKR/J mice and were analyzed by restriction endonuclease digestions, electrophoresis through agarose gels, and Southern transfer experiments (20). For leukemic tissues we analyzed both spontaneous lymphomas from animals 8 to 12 months of age and induced lymphomas from 2- to 3-month-old animals which had been injected with MCF-13 MLV 2 to 3 days after birth. MCF-13 MLV is considered lymphomagenic by its ability to accelerate leukemogenesis in AKR mice (5).

Restriction endonuclease-digested DNAs from brain and normal thymus or lymphoma from the same animal were compared in adjacent lanes of an agarose gel. Hybridization probes used for the analysis of viral sequences were DNA fragments isolated from a molecular clone of MCF-13 MLV (21). Although MCF-13 MLV is one of several MCF viruses that have been isolated from AKR mice, its genome is very similar to other MCFs by RNase T₁ oligonucleotide and restriction endonuclease mappings (2). Because of these similarities we have used molecular clones of this particular MCF virus to generate probes for our analysis of MCF-related sequences in thymic lymphomas. We expected that different but sufficiently related MCF sequences would be detectable with these probes.

DNAs digested with *EcoRI* were hybridized with a probe representing the entire MCF-13 MLV genome (MCF_{rep}). *EcoRI* cleaves a number of different MCF and xenotropic MLVs once in the *env* region, which should generate cellular and viral junction fragments (2, 21). The MCF_{rep} probe hybridized predominantly to endogenous viral sequences, giving rise to a background of bands in both normal and leukemic tissue DNAs (Fig. 1). These endogenous viral bands were contributed by ecotropic and xenotropic retrovirus sequences which share homology with each other and with MCF MLVs and which are present in the genomes of many different mouse

FIG. 1. Hybridization of *Eco*RI-digested tissue DNAs with MCF_{rep} or MCF_{LTR} probes. Tissue DNAs (15 µg) digested with *Eco*RI at 37°C for 14 to 16 h were electrophoresed through 0.7% horizontal agarose slab gels in low-salt E buffer (see text) at 2.1 V/cm for 19 h. After transfer to nitrocellulose filters, DNAs were hybridized with a nick-translated ³²P-labeled MCF_{rep} probe (A) and an MCF_{LTR} probe (B). The same nitrocellulose filter was used for both probes after melting off the MCF_{rep} probe and rehybridizing with the MCF_{LTR}. Marker DNA fragment sizes (*Hind*III-digested lambda DNA) are indicated by the dashed lines to the left of the lanes of different agarose gels. Br, Brain from a leukemic animal; 2S, spontaneous lymphoma; 1I, induced lymphoma; 8S, spontaneous lymphoma; 6S, spontaneous lymphoma; 16S, spontaneous lymphoma; T, normal thymus. Arrowheads denote extra proviral bands.

strains. In spite of the endogenous background, however, we were able to detect extra proviruses in DNAs from lymphomas but not from the thymus of a normal animal or brain of a leukemic animal. These extra proviruses are denoted by arrowheads in Fig. 1. A few of the extra proviruses appeared very faintly with the MCF_{rep} probe (Fig. 1A) but were more easily detectable when we hybridized the same nitrocellulose filters with an MCF-13 long terminal repeat (LTR) probe (MCF_{LTR}, Fig. 1B). Extra *Eco*RI proviruses for which this was observed were the 2.2- and 3.0-kbp fragments in lymphoma 2S, the 2.4-kbp fragment in lymphoma 8S, and the 4.0-kbp fragment in lymphoma 6S (Fig. 1A and B). The explanation for this difference in

detectability by the two probes arose from further analysis of the viral sequences present in the *Eco*RI proviruses, where we determined that these proviruses contained little more than LTR sequences as discussed below. Since the LTR is only a small percentage of the entire genome, these proviruses would barely be detectable with the MCF_{rep} probe.

The difference in intensities of the extra proviruses in each lane of Fig. 1 made it difficult to present all of them with ideal exposures. A few of them (e.g., 2.2-kbp fragment in 2S and 4.6-kbp fragment in 1I) either do not appear well resolved or are overexposed in Fig. 1B but were discernible at lighter exposures of the same autoradiograms (Fig. 2A). DNA samples in lanes

FIG. 2. *Eco*RI-digested brain and thymic lymphoma DNAs hybridized with various MCF virus-specific probes. DNAs were digested with *Eco*RI and treated as described in the legend to Fig. 1. The same nitrocellulose filters were reused for the different hybridization probes, which are: (A) MCF_{LTR}; (B) MCF_{3'E-X}; (C) MCF_{3'S-E}; and (D) MCF_{gag-pol}. DNAs are as follows: Br, brain of a leukemic animal; 2S, spontaneous lymphoma; 1I, induced lymphoma; and 16S, spontaneous lymphoma.

presented adjacent to each other in Fig. 1 were electrophoresed through the same agarose gel. Usually DNAs from the brain and lymphoma of the same leukemic animal were analyzed in adjacent lanes, resulting in easier detection of the extra proviruses in the tumor DNAs. Only one of these brain DNA lanes, however, is presented in Fig. 1, and as a result the endogenous viral bands of this control DNA may not always have the exact same alignment with the endogenous bands in the tumor DNA lanes.

The high-molecular-weight *Eco*RI fragments in Fig. 1 were overexposed to allow the detection of the lower-molecular-weight bands, where the majority of extra proviruses were observed. We did not detect a large number of extra bands in the higher-molecular-weight region (Table 1) either in lighter exposures of the same autoradiograms or when gels were electrophoresed for longer periods of time, resulting in a greater resolution of bands in this size range (22). One example of an agarose gel in which *Eco*RI-digested DNAs were electrophoresed further is shown in Fig. 3, where no differences could be detected between brain and lymphoma DNAs

with an MCF_{LTR} probe. A band of about 6.5 kbp, which appears to be specific for brain DNA in this figure, was also present in lymphoma DNA in the original autoradiogram. We cannot

TABLE 1. Extra *Eco*RI DNA fragments detected with an MCF probe in spontaneous and induced lymphomas

Lym- phoma ^a	Provirus size (kbp)				
1S	5.4				
2S		5.1	3.5	3.0	2.2
6S			4.0		
8S			3.5		2.8 2.4
16S					2.8
17S			3.5		2.8
19S			3.5		
1I		4.6	3.5		
5I	7.5		3.5		2.8
6I			3.5 3.3		
8I		4.6			
9I		5.1	3.5		

^a S, Spontaneous lymphoma; I, MCF-13 MLV-induced lymphoma.

completely rule out the possibility, however, that the large number of endogenous bands may still be masking extra proviruses that might be migrating in this high-molecular-weight region of the gels.

To examine the extent of this phenomenon, we analyzed *Eco*RI-digested DNAs from 13 AKR/J thymic lymphomas and found extra proviruses compared with normal tissues in 12 of them (Table 1). The copy number of extra proviruses in these 12 lymphomas ranged from one to four at the most. One of the striking results from this analysis was the similarity of sizes of the extra *Eco*RI DNA fragments that we observed in the 12 lymphomas. We discovered that these extra MCF-related proviruses fell roughly into three major size classes of 4.0 to 5.4, 3.5, and 2.8 to 3.3 kbp. A fourth and minor class had sizes from 2.2 to 2.4 kbp.

Extra proviruses of identical size, 3.5 kbp, were found in 8 of 12 lymphomas (Table 1). Other classes did not contain a provirus of a single size in each case but had proviruses with very similar sizes. The greatest range in size within any of the classes was 1.4 kbp. Six lymphomas had proviruses in the 4.0- to 5.4-kbp range; six, in the 2.8- to 3.3-kbp range; and two, in the 2.2- to 2.4-kbp range. All 12 lymphomas had proviruses which fell into at least one of these four size classes, suggesting a common site(s) of integration in lymphoma DNAs. We chose to group the extra MCF-related proviruses into these size classes because extra proviruses, in spite of having similar sizes in the same lymphoma, could not be integrated at exactly the same site. For example, proviruses of 3.5 and 3.0 kbp observed in lymphoma 2S (Table 1) were placed into two different size classes.

To confirm that these DNA fragments of similar sizes were actually corresponding to a common integration site(s) rather than to fortuitously comigrating bands, we digested the lymphoma DNAs with other restriction enzymes, such as *Hind*III alone or double digestion with *Eco*RI and *Xba*I, to determine whether the extra proviruses generated by other enzymes would again fall into similar size classes. *Hind*III does not cleave MCF-13 but does cleave other MCF viruses, usually once at 3 kbp from the 5' end of the provirus DNA (2). *Hind*III digestion of five lymphoma DNAs revealed, by hybridization with MCF_{LTR}, a common size class of extra proviruses of 5.2 to 5.35 kbp in four of the samples (Fig. 4). These extra bands were not observed in brain DNA (Fig. 4). Besides a 5.2- to 5.35-kbp fragment, other extra bands of 2.85 or 3.1 kbp were also observed for lymphomas 2S and 16S. A comparison of the extra proviruses generated by *Hind*III with those generated by *Eco*RI revealed that the four lymphomas (2S,

FIG. 3. *Eco*RI-digested tissue DNAs hybridized with the MCF_{LTR} probe. Tissue DNAs (15 µg) digested with *Eco*RI as described in the legend to Fig. 1 were electrophoresed through a 0.7% horizontal agarose slab gel in low-salt E buffer at 2.1 V/cm for 44 h. DNAs were transferred to nitrocellulose and hybridized to a ³²P-labeled MCF_{LTR} probe. Marker DNAs were as given in the legend to Fig. 1. Brain (Br) and spontaneous lymphomas (6S) were from the same leukemic AKR mouse.

19S, 11, and 5I) sharing a *Hind*III fragment of about 5 kbp also shared the 3.5-kbp *Eco*RI fragment (Table 1). Lymphoma 16S, which lacked the 3.5-kbp *Eco*RI fragment, also lacked

an extra *Hind*III band of about 5 kbp but contained an extra band of 3.1 kbp instead (Fig. 4).

Double digestion with *Eco*RI and *Xba*I resulted in DNA fragments again seen only in lymphoma DNAs (Fig. 4). *Xba*I cleaves lymphomagenic MCF MLVs once in the *env* region at 7.8 kbp from the 5' end of the provirus (2). These fragments were once more of nearly identical sizes for four different lymphomas (Fig. 4). The *Eco*RI-*Xba*I extra DNA fragments of 1.15 to 1.4 kbp in size were present in DNAs which shared extra *Eco*RI fragments in the 4.0- to 5.4-kbp size class. The results with *Hind*III or *Eco*RI-*Xba*I digestions lend further support to the notion of a common site(s) of provirus integration in thymic lymphomas since it would be highly improbable

that digestions with different enzymes would continue to yield fortuitously comigrating bands.

Characterization of *Eco*RI DNA fragments with MCF-13 MLV probes. To identify the regions related to the MCF MLV genome that were present in the extra proviruses generated by *Eco*RI digestion of lymphoma DNAs, we used viral probes specific for different regions of the MCF-13 MLV genome for which we had previously generated molecular clones (21). The regions of the MCF-13 MLV genome that these specific probes recognize are shown in Figure 5. All new proviruses detected with the MCF_{rep} probe also hybridized with an LTR probe (MCF_{LTR}) (Fig. 1; Table 2). To analyze envelope-related sequences, we used two different

FIG. 4. *Hind*III single-digested or *Eco*RI-*Xba*I double-digested brain and thymic lymphoma DNAs hybridized with an MCF_{LTR} probe. Tissue DNAs (15 µg) were digested with either *Hind*III alone or sequentially with *Eco*RI and *Xba*I at 37°C for 14 to 16 h. DNAs were electrophoresed through 0.7% agarose gels for *Hind*III digestion or 1% agarose gels for *Eco*RI-*Xba*I digestions at 2.1 V/cm for 18 h. DNAs were analyzed by Southern blotting and hybridized with ³²P-labeled MCF_{LTR}. Marker DNA fragments are as described for Fig. 1. Lanes for *Hind*III digestions are: Br, brain from a leukemic animal; 1I, induced lymphoma; 2S, spontaneous lymphoma; 19S, spontaneous lymphoma; 16S, spontaneous lymphoma; and 5I, induced lymphoma. Lanes for *Eco*RI-*Xba*I digestions are: Br, brain; 1S, spontaneous lymphoma; 6S, spontaneous lymphoma; 2S, spontaneous lymphoma; 8I, induced lymphoma.

FIG. 5. MCF-13 MLV DNA fragments used for probes specific for different regions of the genome. Viral probes specific for different regions of the MCF-13 MLV genome are indicated by labeled boxes. Restriction endonucleases used to cleave the MCF-13 cloned DNA to generate the specific fragments are: Sm, *SmaI*; RI, *EcoRI*; and Xb, *XbaI*.

env probes recognizing different parts of the glycoprotein region of MCF-13 MLV. Probes MCF_{3'S-E} and MCF_{3'E-X} are *SmaI-EcoRI* and *EcoRI-XbaI* DNA fragments, respectively, of MCF-13 (Fig. 5). Both of these 3'-end probes recognize xenotropic MLV-related sequences as well (21; unpublished data).

All of the extra proviruses in the major size class (3.5 kbp) that were analyzed with these *env* probes hybridized with the MCF_{3'E-X} probe (Fig. 2; Table 2). Half of the 4.0- to 5.4-kbp and both of the 2.2- to 2.4-kbp proviruses lacked MCF_{3'E-X} sequences. However, the majority of 2.8- to 3.3-kbp proviruses did contain these 3'-end sequences. These results suggested that the majority of extra *EcoRI* fragments contained sequences with homology to the *env* region of MCF MLV. Only two extra proviruses hybridized with the MCF_{3'S-E} probe (Table 2). This probe recognizes the *EcoRI* 6.9-kbp fragment which represents the 5' portion of the genome of a number of different MCF and xenotropic MLVs (21). One of these extra proviruses was smaller than 6.9 kbp, suggesting a deletion in this region of its genome, and the other was larger.

To determine whether any of the extra proviruses not hybridizing with 3'-end probes contained 5'-end sequences instead, we used a 4.65-kbp *SmaI* fragment representing the *gag-pol* region of MCF-13 MLV (MCF_{gag-pol}, Fig. 5). We could not detect any hybridization of extra proviral bands with this probe (Fig. 2; Table 2). It appeared, therefore, that a few of the extra proviruses generated by *EcoRI* digestion consisted mainly of LTRs since they did not hybridize to viral probes representing either *env* or *gag-pol* sequences. These extra proviruses, however, may also contain sequences immediately 5' to the right LTR since these sequences were not recognized by either MCF_{3'} probe. These results led us to conclude that a significant number of the newly acquired proviruses observed in lymphoma DNAs represented highly deleted genomes since they lacked most viral sequences. A small minority of extra proviruses, however, may represent intact genomes. Their 5'-half *EcoRI* fragments would be migrating in

the region of the gel where the contribution of endogenous viral sequences to the background is the greatest, which may be obscuring any additional bands.

DISCUSSION

We have analyzed the proviruses of MLVs to study their involvement in the development of thymic lymphomas in AKR/J mice. By using a cloned DNA fragment as a probe specific for the AKR ecotropic MLV, we have previously shown that the intact genome of this virus or at least its envelope region is not amplified in DNAs from spontaneous thymic lymphomas (22). We have extended these studies of MLV proviruses in lymphoma DNAs by using DNA fragments specific for different regions of a lymphomagenic MCF virus, MCF-13, which we have molecularly cloned (21). Using an MCF_{rep} probe, we have detected newly acquired MCF-related proviruses in lymphoma DNAs digested with various restriction endonucleases. These additional proviruses were not observed in normal tissues. We detected extra proviruses in 12 of 13 lymphoma DNAs analyzed by *EcoRI* digestion, suggesting that they may play an important role in the development of thymic lympho-

TABLE 2. MCF sequences present in extra *EcoRI* DNA fragments in tumors

<i>EcoRI</i> proviruses		MCF probes			
Lym-phoma ^a	Size (kbp)	LTR	3'(<i>EcoRI</i> - <i>XbaI</i>)	3'(<i>SmaI</i> - <i>EcoRI</i>)	<i>gag-pol</i> (<i>SmaI</i>)
1S	5.4	+	—	—	—
2S	5.1	+	+	—	—
	3.5	+	+	—	—
	3.0	+	—	—	—
	2.2	+	—	—	—
6S	4.0	+	—	—	—
8S	3.5	+	+	—	—
	2.8	+	+	—	—
	2.4	+	—	—	—
16S	2.8	+	+	—	—
17S	3.5	+	+	—	—
	2.8	+	+	—	—
19S	3.5	+	+	—	—
1I	4.6	+	+	—	—
	3.5	+	+	—	—
5I	7.5	+	ND ^b	+	ND
	3.5	+	ND	—	ND
	2.8	+	ND	—	ND
6I	3.5	+	+	—	—
	3.3	+	+	—	—
8I	4.6	+	ND	—	ND
9I	5.1	+	ND	—	ND
	3.5	+	ND	+	ND

^a S, Spontaneous lymphoma; I, MCF-13 MLV-induced lymphoma.

^b ND, Not determined.

mas. Other investigators have also observed new proviruses in AKR/J tumors (1, 16, 19).

The *EcoRI* extra tumor-specific DNA fragments fell into four size classes (Table 1), suggesting a common site(s) of provirus integration in thymic lymphoma DNA. Especially striking was the presence of an identical 3.5-kbp fragment in 8 of 12 tumor DNAs, which may reflect a highly preferred site for provirus integration. The other size classes included fragments of similar, although not identical, sizes which differed by 1.4 kbp at the most within each class. To demonstrate that these results were not due to fortuitously comigrating *EcoRI* fragments, we digested the DNAs with other restriction endonucleases. Double digestion with *EcoRI* and *XbaI* revealed extra bands in tumor DNAs, again with practically identical sizes (Fig. 4). *HindIII* digestion also generated a common size class of fragments about 5 kbp. Our ability to detect these commonly shared MCF-related proviruses in different lymphoma DNAs digested with three different enzymes supports the notion that a common site(s) may be involved in provirus integration. It would be highly unlikely that three different enzymes would generate proviral fragments which would fortuitously comigrate with each other in each instance. Additional restriction endonuclease analysis of these proviruses would always be complicated by the fact that the MCF MLVs that appear to be responsible for these proviruses are not a single virus but a class of viruses which have similar but not identical restriction endonuclease cleavage sites. To more convincingly demonstrate that a common site(s) of integration is involved in thymic lymphoma development, we are presently analyzing the flanking cellular sequences of the extra proviruses by molecular cloning.

Because different enzymes have generated different numbers of extra proviruses from the same DNA sample, it is difficult to determine whether one or more sites of integration are involved. Since we have been able to detect single extra proviruses, however, in a few DNAs, this would suggest that a single integration event is sufficient for transformation to occur. The multiple extra proviruses we have observed in one DNA sample could be accounted for by a single large domain of cellular DNA with multiple preferred integration sites. Integration at any one or more of these sites in this chromosomal domain might play an important role in thymic lymphoma development. This explanation appears more likely than the alternative one that there is more than one completely different chromosomal site that may be involved in tumor formation, although this is a possibility that cannot be ruled out at this time.

Further analysis of the extra proviruses gener-

ated by *EcoRI* with probes recognizing specific regions of MCF-13 MLV revealed that a significant number of these proviruses represented deleted genomes since they did not hybridize to envelope sequences and corresponded primarily to LTR sequences. These deleted proviruses resemble the defective ALV genomes observed in bursal lymphomas, which mainly contained 3'-end sequences (13, 15). The majority of extra proviruses that we have observed did contain *env* sequences (Table 2). That all of the extra proviruses contained LTR sequences suggests that these sequences may play an important role in transformation as in the activation of the *c-myc* gene in bursal lymphomas by the ALV LTR (9). Although our studies suggest that MCF MLVs may integrate into DNA of T lymphocytes at a common site(s), our preliminary analysis of virus-related RNAs in thymic lymphomas suggests that a promoter insertion mechanism is not functioning in the development of these tumors.

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