Virological Events Leading to Spontaneous AKR Thymomas

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The spontaneous leukemias of AKR mice are caused by mink cell focus-forming (MCF) viruses. These viruses are generated by recombination between several endogenous murine retroviruses. The virological events leading to the generation of the leukemogenic agent were investigated by using an oligonucleotide specific for the U3 region of the leukemogenic virus and *env*-reactive oligonucleotide probes specific for the different classes of endogenous murine leukemia virus. It was shown that (i) the leukemogenic MCF virus is formed by recombination between at least three different endogenous sequences; (ii) the U3 donor for the leukemogenic virus is the inducible xenotropic virus *Bxv-1*; (iii) all spontaneous tumors contain viruses with duplicated enhancer regions in their long terminal repeats; (iv) enhancer duplication is a somatic event, since *Bxv-1* contains only one copy; (v) the first recombinant virus detectable in mass populations of thymocytes by Southern hybridization analysis contains all structural features of the ultimate leukemogenic virus; and (vi) the multiple novel viruses in a given tumor represent progeny of the same unique recombination events. On the basis of these results, an analysis of the virological events leading to AKR thymomas is presented.

Certain strains of mice, most notably AKR, suffer from a high incidence of spontaneous thymoma. The disease has a retroviral etiology and appears to involve enhancer-mediated insertional activation of one of a number of *c-onc* genes (45). Genetic studies have shown that inheritance of endogenous ecotropic proviruses is one prerequisite for leukemia (39) but that the ecotropic virus is not the proximal agent of disease induction (8). Rather, viruses formed by recombination between an ecotropic virus and one or more endogenous noncancerous proviral sequences appear to be the causative agents (8, 15, 23, 24). These recombinant viruses, which contain an altered *env* gene relative to ecotropic viruses, can replicate on both non-mouse and mouse cells and consequently have been named polytropic or dual tropic. In addition, some isolates cause cytopathic foci on mink cells, and thus the whole class of recombinant viruses has become known as mink cell focus-forming (MCF) viruses. For the purposes of this paper, we shall refer to the recombinant, exogenous proviruses found in tumors as MCF viruses and reserve the term polytropic for the class of endogenous proviruses that can potentially contribute *env* sequences to the recombinants.

A variety of different MCF viruses have been isolated from spontaneous AKR tumors, and their structural and biological properties have been analyzed to varying levels of detail. Although each isolate appears unique, a number of general conclusions may be drawn from these studies. MCF viruses can be divided into two broad classes; class I viruses replicate in the thymus and cause tumors, while class II viruses do not replicate in thymocytes and are nonpathogenic (6, 8). Class I MCF viruses consist of an ecotropic virus backbone with two areas of substitution by noncancerous viruses, one in the *env* gene, encompassing at least the N-terminal two-thirds of the SU protein (gp70)-coding sequence, and the other within the C-terminal region of TM protein (p15E) and U3 (17, 23, 40). Class II MCF viruses

appear only to contain one substituted region, usually encompassing the entire *env* gene. A few MCF isolates that replicate in the thymus but are weakly, if at all, pathogenic have also been reported (29, 46); structurally, they resemble the class I viruses.

The complex series of steps leading to the generation of the leukemogenic MCF retroviruses in AKR mice is still poorly understood, but these events presumably include inheritance of the parental proviruses, expression, recombination, and infection of the ultimate target tissues. Attempts to obtain a precise description of these events have been hampered by the large number of endogenous murine leukemia virus (MLV)-related proviruses, about 40 in AKR mice (19–21), which are inherited through the germ line in all inbred strains of mice. It seems likely that most of these proviruses play no role in the disease process, but they provide a significant background of cross-hybridizing proviruses which obscure the appearance of novel proviruses. To obtain a clearer picture of these virological events, we have used a series of oligonucleotide probes specific for the *env* gene of different classes of endogenous MLV (58), as well as one new oligonucleotide specific for the U3 sequences of class I MCF viruses, to examine the structures of recombinant viruses present in AKR thymomas and in thymuses from AKR mice of different ages. These studies are the subject of this report.

MATERIALS AND METHODS

Animals. Unless otherwise noted, all mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). Mice congenic for *Bxv-1* on a 129 background were derived as follows: (BALB/cTif × 129/Tif)_F₁ mice were backcrossed to 129/Tif mice; the progeny were splenectomized and typed for the inheritance of *Bxv-1* by assaying induction of xenotropic virus from spleen cells following treatment with lipopolysaccharide and bromodeoxyuridine as previously described (60). After nine generations of backcrossing, the *Bxv-1* locus was fixed by intercrossing followed by progeny testing for *Bxv-1* inducibility.

Tissue samples and DNA preparation. Thymuses from

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AKR/J mice of different ages and a number of spontaneous AKR/J thymomas were excised, frozen in liquid nitrogen, and stored at -70°C prior to DNA extraction. DNA was prepared from fresh spleen cell suspensions or from frozen thymuses ground under liquid nitrogen to a fine powder with a pestle and mortar as previously described (30).

Oligonucleotide synthesis and labeling. Oligonucleotides were synthesized with an Applied Biosystems model 380B DNA/Synthesizer, purified by reverse-phase high-pressure liquid chromatography, and labeled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as previously described (58, 59). Oligonucleotide JS21 consists of bases 80 to 106 of the MCF-247 clone 1b long terminal repeat (LTR) (31) and has the sequence ATTCTCAAAGT CACAAGGAAGTTTAG.

Genomic blots. DNA samples were digested, subjected to electrophoresis, transferred to nitrocellulose, and probed with labeled oligonucleotides JS4, JS5, and JS6/10 as previously described (58, 59). Hybridizations with JS21 were carried out under identical conditions except that the hybridizations were performed at 56°C and the most stringent wash was with $2\times$ SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 56°C . Hybridizations with nick-translated pE (a 400-bp ecotropic virus-specific probe) and pNE (a 622-bp nonectropic virus *env* probe) were carried out as previously described (58).

Cloning and sequencing of proviral DNA fragments. *EcoRI*-digested tumor and Charon 27 (51) DNAs were ligated, packaged in vitro, and plated on *Escherichia coli* LE392. Plate lifts were screened with ^{32}P -labeled JS21 under the conditions described above. Plate lysate DNAs were prepared from pure plaques, and the inserts were subcloned into pBS+ (Stratagene, San Diego, Calif.) for restriction enzyme analysis. DNA sequences were determined by the dideoxy method (1, 54) on restriction fragments subcloned into the M13 vectors mp18 and mp19 (New England BioLabs, Beverly, Mass.).

Nucleotide sequence accession numbers. The sequences reported in this study have been deposited with GenBank under accession numbers M59883 to M59901.

RESULTS

A probe specific for the LTR of MCF viruses. To study the virological events associated with spontaneous leukemia in AKR mice, we have developed methods for distinguishing the recombinant viruses from inherited endogenous viruses. We have recently described a series of oligonucleotide probes which react with the members of different subfamilies of endogenous nonectropic proviruses (58). These oligonucleotides, referred to as JS4, JS5, and JS6/10, detect allelic differences within the gp70-coding sequences of endogenous modified polytropic, polytropic, and xenotropic proviruses, respectively. JS5 reacts with the nonectropic *env* gene sequences present in MCF viruses and can distinguish DNA samples differing by a single provirus (59). However, a probe specific for the nonectropic LTR sequences was also desirable. With this consideration in mind, we compared the LTR sequences of a variety of MLVs. One region in which MCF-247 differed from other sequenced MLVs was identified starting 47 bp 3' of the conserved *PstI* site in U3 (see Materials and Methods). In this stretch of 27 bases, a typical MCF virus differed from other MLVs by at least 2 bases. We synthesized an oligonucleotide, JS21, corresponding to these 27 nucleotides. Conditions were determined under which this oligonucleotide reacted with cloned MCF-247 DNA but

not with representative ecotropic, polytropic, modified polytropic, or xenotropic provirus clones (data not shown).

The sequence differences which make JS21 specific for MCF-247 might represent differences characteristic of the LTR donor(s) for recombinant viruses. Alternatively, they might result from point mutations unique to MCF-247. If the former possibility was correct, then control AKR DNA would have contained one or a few proviruses reactive with this probe and tumors would have contained amplified numbers of JS21 reactive proviruses. Hence, we examined 10 spontaneous AKR thymomas and a control spleen DNA preparation for the presence of proviruses which reacted with JS21 (Fig. 1A). Control AKR DNA contained two reactive *EcoRI* fragments of 9.1 and 6.5 kb. All intact nonectropic endogenous proviruses apparently contain an *EcoRI* site within their gp70-coding sequences at map position 6.9 kb (5, 32, 57); therefore, these two fragments might correspond to the 5' and 3' halves of one endogenous provirus. The xenotropic specific *env* probe (JS6/10), which is directed against sequences 3' of the *EcoRI* site, reacted with the 6.5-kb fragment (data not shown), implying that it represents the 3' half, and that the 9.1-kb fragment represents the 5' half, of an endogenous xenotropic provirus. The 10 tumors contained between 8 and 22 reactive fragments in addition to the two endogenous bands, each corresponding to novel provirus-cell junction fragments. This result showed that viruses containing the LTR sequences detected by JS21 were amplified in all tumors and suggested that this endogenous provirus might be the sole donor for LTR sequences in recombinant MCF viruses.

To address this issue further, we probed a parallel blot with the polytropic virus-specific probe, JS5. Although this probe reacts with 17 endogenous proviruses in DNA from AKR/J mice (20), a number of novel, somatically acquired proviruses can be clearly distinguished (Fig. 1B). All additional JS5-reactive fragments appear about half as intense as the endogenous proviruses, consistent with integration into one of each chromosome pair, prior to clonal outgrowth of the tumors. Surprisingly, this does not seem to be true for the JS21 hybridization, since many of the additional JS21-reactive fragments appear as intense as the endogenous bands. This discrepancy probably can be explained by duplication of the LTR sequences which contain the region recognized by JS21 (see below). Comparison of Fig. 1A and B shows that every novel JS5-reactive fragment is similar in mobility to a JS21-reactive fragment, indicating that every somatically acquired provirus in the tumor with a polytropic *env* gene has an altered LTR sequence reactive with the JS21 probe. Similar results were obtained with *PvuII*-digested DNA samples probed with JS5 and JS21 (data not shown). These results imply that the endogenous provirus which reacts with JS21 is the only endogenous provirus that contributes LTR sequences to recombinant viruses in AKR thymomas.

It is somewhat more difficult to determine whether every new virus with altered LTR sequences has an altered *env* gene. First, the large number of JS5-reactive, endogenous sequences tends to obscure novel bands. Second, JS21 will react with more fragments than does JS5, since the former oligonucleotide will react with fragments containing either the 5' or the 3' LTR whereas JS5 will react only with the 3' halves of *EcoRI*-digested recombinant proviruses. However, the position of this conserved *EcoRI* site implies that novel fragments smaller in size than 6.9 kb should correspond to the 3' ends of proviruses. With only one or two possible exceptions, all JS21-reactive fragments within this size range

FIG. 1. Somatically acquired proviruses in AKR tumors. Parallel blots of *Eco*RI-digested DNAs from AKR spleen and AKR thymomas A to J were probed with LTR-specific JS21 (A) and *env*-specific JS5 (B).

also reacted with JS5, suggesting that the majority of new proviruses with altered LTRs indeed have altered *env* genes. Identical blots were also probed for the presence of additional *env* genes corresponding to the xenotropic, modified polytropic, and ecotropic families of endogenous proviruses. No additional JS4 (modified polytropic)- or JS6/10 (xenotropic)-reactive fragments were seen in the 10 tumors; in two cases, one additional ecotropic provirus was present (data not shown). Taken together, these results imply that the only virus needed to induce the tumors is a virus of the type typified by MCF-247: an ecotropic virus backbone with substitutions in *env* and the LTR.

***Bxv-1* provides LTR sequences for MCF viruses.** On the basis of the similarity in restriction maps of a xenotropic provirus present in certain somatic cell hybrids (27) with a provirus identified by another MCF-specific LTR probe (49), it has been proposed that the LTR donor for MCF viruses is the inducible xenotropic virus *Bxv-1*. To test this assignment, we examined a number of different strains of mice typed for *Bxv-1* (Fig. 2). JS21 reacted with the same two fragments in DNAs from the *Bxv-1*-containing strains AKR/J, BALB/cJ, HRS/J, C57BL6/J, C57L/J, and DBA/2J but did not react with A/J, C3H/HeJ, or 129/Tif DNA, which does not contain this allele. Most importantly, it also reacted with DNA from a strain congenic for *Bxv-1* on a 129 background (Fig. 2, lane j). We also examined the segregation of the JS21-reactive fragments in 15 (129/Tif *Bxv-1*/+ × 129)_F₁ animals. The nine animals that were inducible for xenotropic virus from spleen cells by lipopolysaccharide and

bromodeoxyuridine contained the 9.1- and 6.5-kb *Eco*RI fragments, whereas the six noninducible mice did not contain these fragments (data not shown). Since *Bxv-1* is the only inducible xenotropic virus expressed in BALB/c spleen cell cultures (60), we also examined xenotropic virus-producing mink and rabbit cell lines derived by cocultivation with stimulated BALB/c lymphocytes. These also contained sequences reactive with JS21 (data not shown). Together, these data clearly show that the LTR donor for MCF viruses is the inducible xenotropic virus *Bxv-1*. This result implies that the MCF *env* and LTR sequences must be derived from different endogenous proviruses and that the generation of

FIG. 2. JS21-reactive sequences in different strains of mice. Hybridization analysis of *Eco*RI-digested DNAs from HRS/J (a), BALB/cJ (b), A/J (c), AKR/J (d), C57BL6/J (e), DBA/2J (f), C57L/J (g), C3H/HeJ (h), 129/Tif (i), and 129/Tif *Bxv-1*/+ × 129 (j). The blot was probed with ³²P-labeled JS21. The upper band corresponds to the 5' LTR of *Bxv-1*, and the lower band corresponds to the 3' LTR.

FIG. 3. Proviruses involved in AKR thymomas. The structures of proviruses participating in the leukemogenic process are shown. They include (A) the endogenous provirus, *Bxv-1*, which contributes LTR sequences to the recombinant MCF virus; (B) an ecotropic provirus (Akv) which provides the backbone to the MCF virus; (C) an endogenous polytropic provirus (*Pmv*); (D) a typical MCF virus containing *Pmv*-derived *env* and *Bxv-1*-derived LTR sequences; and (E and F) two potential intermediates in the formation of MCF viruses which represent the products of recombination between an ecotropic virus and an endogenous polytropic virus or with *Bxv-1*. Restriction sites: P, *Pst*I; E, *Eco*RI; H, *Hind*III. With the exception of *Bxv-1*, only restriction sites within the viral sequences are marked. Restriction sites in parentheses indicate sites that vary according to the precise recombination points; usually a *Hind*III site (from the ecotropic parent) is present at 3.0 kb and the *Pst*I site at 7.6 kb is absent. Also shown are sequences that react with the probes used in this study; pE is an ecotropic virus-specific *env* probe (4); pNE is a nonecotropic virus *env* probe analogous to pXenv (3); JS5, JS6/10, and JS21 are oligonucleotide probes.

MCF viruses involves at least two independent recombinational events.

Time course of MCF virus appearance. AKR mice express ecotropic virus from birth; however, MCF-induced thymomas do not start appearing until the animals reach about 7 months of age (8, 24). To assess the relative fraction of this period required for MCF virus formation and for tumor induction, we wanted to determine when during this 7-month period recombination occurred and, if possible, establish the temporal sequence of the LTR and *env* recombinations. In contrast to the problem of detecting novel proviruses in the clonal populations by tumor cells, analysis of freshly generated recombinant viruses necessitates the ability to detect novel viruses in nonclonal populations of cells with high backgrounds of endogenous proviruses. However, if one knows the structures of the progenitor viruses and uses the

LTR-specific probe, it becomes possible to design strategies for studying novel recombinants.

The structures of the proviruses involved in the leukemogenic process are shown in Fig. 3. Lines A to C show the three progenitor proviruses, line D shows the leukemogenic MCF virus, and the last lines show two possible intermediates in the formation of MCF viruses, one with only an LTR change (line E) and the other with only an *env* substitution (line F). Consideration of the restriction sites shown in Fig. 3 indicates that recombinant viruses with the structures shown in lines D and E will yield a novel internal *Pst*I fragment of 8.2 kb which reacts with JS21. The recombinant viruses shown in lines D and E can be distinguished by using the invariant *Eco*RI site present in the *env* gene of polytropic proviruses; thus, an MCF virus will give a 6.8-kb fragment which reacts with JS21 in *Eco*RI-*Pst*I-digested DNAs. A

FIG. 4. Appearance of recombinant proviruses in AKR thymus. Shown is a time course study demonstrating the presence of recombinant viruses in AKR thymuses taken from animals of different ages. In each case, the DNA samples include spleen and thymuses from two 10-week, two 16-week, two 22-week, and two 26-week-old animals. (A) *PstI*-digested DNAs probed with JS21; (B) *PstI*-*EcoRI*-digested DNAs probed with JS21; (C) *HindIII*-*EcoRI*-digested DNAs probed with pNE (the 26-week samples were badly degraded after the *EcoRI*-*HindIII* double digestion; these lanes are not shown).

virus with an *env* substitution but without a change in the LTR sequences can be identified by using a noncotropic probe for the 5' end of gp70 (pNE) in *HindIII*-*EcoRI* digests, since no endogenous virus has *HindIII* and *EcoRI* sites at 3.0 and 6.9 kb, respectively. If a novel fragment of 3.9 kb is seen with no evidence for somatically acquired JS21-reactive sequences, we would infer that only viruses of the type shown in line E of Fig. 3 were present.

We examined a panel of DNA samples extracted from the thymuses of AKR mice of different ages, digested with *PstI*, and probed with JS21. The DNA samples included a spleen cell control and thymuses from two 10-, 16-, 22-, and 26-week-old AKR mice (Fig. 4A). The fragment of 8.2 kb predicted for the somatically acquired recombinant virus was observed in samples from 16-week and older mice but was not present in the control or 10-week samples. The intensity of the 8.2-kb band varied compared with that of the 2.9-kb fragment corresponding to the 5' LTR of *Bxv-1*. In one of the two 26-week samples it was considerably darker, but a number of faint bands, presumably derived from the 3' LTR of a clonal population of cells, were also present. It seems likely that this animal had already undergone the initial stages of clonal expansion associated with thymoma development. Two of the thymus samples had an 8.2-kb fragment somewhat lighter than the 2.9-kb endogenous fragment, but they also contained a 3.0-kb somatically acquired fragment. In these samples there were probably at least two different populations of recombinant virus, one of which had retained the 3.0-kb *PstI* site of *Bxv-1* (Fig. 3, line A) and had undergone alterations in LTR size (see below). The other three samples had 8.2-kb fragments slightly darker than the endogenous 2.9-kb fragment. These hybridization intensities suggest that from 16 weeks on there are approximately one to two somatically acquired recombinant proviruses per cell in the thymus.

To determine whether the somatically acquired proviruses

had undergone both LTR and *env* substitutions, we next examined *EcoRI*-*PstI*-digested DNA samples (Fig. 4B). In the 16-week and older samples, a 6.8-kb *PstI*-*EcoRI* fragment which reacted with JS21 was seen, implying the presence of viruses with the structure shown in line D of Fig. 3. However, trace amounts of an 8.2-kb band are seen in three samples, suggesting that viruses with only the LTR substitution were also present. Analysis with other enzymes confirmed this observation (data not shown).

In light of reports that class II MCF viruses can be isolated from 3-week-old AKR mice (16, 17), we next investigated whether we could detect a virus with the *env* but not the LTR substitution in the 10-week thymus samples (Fig. 4C). *EcoRI*-*HindIII*-digested DNAs were hybridized with a general noncotropic *env* probe. Although a large number of endogenous proviruses reacted with this probe in the spleen sample, none showed the mobility predicted for a recombinant virus. As expected from the other analyses, the 3.9-kb fragment was observed in samples taken from 16-week or older mice; there was no evidence for the presence of a recombinant virus in the 10-week samples.

PstI digests of the same DNA samples were also probed with our *env*-specific oligonucleotide probes (data not shown). Although this analysis was complicated by the presence of large numbers of endogenous proviruses, we could conclude that neither modified polytropic nor xenotropic *env* sequences were amplified. We had hoped to detect an 8.2-kb *PstI* fragment reactive with JS5, the polytropic specific *env* probe; however, this fragment was not well resolved from a very intense 7.6-kb band, the product of most, if not all, of the endogenous polytropic proviruses. However, in the two samples that seemed to have acquired the 3.0-kb *PstI* site from *Bxv-1*, we did observe a novel 5.2-kb JS5-reactive *PstI* fragment, the size predicted for a fragment running between the *PstI* sites in *pol* and the 3' LTR (data not shown). This fragment was not seen in the

FIG. 5. Demonstration that tumors contain duplicated LTR sequences. Tumor DNA (A to J) and DNA from MCF clones containing one or two copies of the LTR direct repeat (dr) were digested with *Pst*I plus *Kpn*I, separated by electrophoresis on a 1.2% agarose gel, transferred to nitrocellulose, and probed with labeled JS21.

other samples, ruling out the possibility that our failure to detect a novel 3.9-kb *Eco*RI-*Hind*III fragment in the 10-week samples was due to the absence of the ecotropic *Hind*III site.

Taken together, these results imply that high levels of recombinant viruses are not present in AKR animals less than 10 weeks old, even though recombinant viruses with *env* and/or LTR substitutions have been isolated from such animals (7, 16, 64). On the basis of hybridization intensities, we estimate that such viruses cannot be present at levels above 0.1 copy per cell. At some stage during the next 6 weeks, a recombinant virus replicates within the thymus to reach an average level corresponding to at least one provirus per cell.

Duplication of LTR sequences in MCF viruses. Not all thymotropic MCF viruses are leukemogenic (46). Recent studies of the acceleration of AKR leukemia by injection of cloned viruses have suggested that the leukemogenic potential of MCF viruses is dependent on the number of copies of the U3 enhancer element; viruses with two copies of the enhancer accelerate disease, whereas those with one copy do not (29). To test whether the MCF viruses involved in spontaneous tumor formation also contained duplicated LTR sequences, we examined our panel of tumor DNAs. The thymoma DNAs were digested with *Pst*I and *Kpn*I, enzymes which show highly conserved sites within the MLV LTR at the 5' end of U3 and within R, respectively, and then probed with JS21. As controls, we used two molecular clones of MCF-247 with one or two copies of an LTR direct repeat which yield *Pst*I-*Kpn*I fragments of 370 and 489 bp, respectively (29). All tumor DNAs contained at least two different-size *Pst*I-*Kpn*I fragments (Fig. 5). One band was similar in mobility to that of the cloned MCF provirus containing one copy of the direct repeat. The other fragment was larger and generally gave a much more intense hybridization signal. The size of the upper band varied somewhat from tumor to tumor but was similar to that of the clone with two direct repeats. A few tumors (e.g., Fig. 5, lanes B and I) also yielded a band which might represent three or more repeats within their viral LTRs. Thus, all tumors appear to contain proviruses with duplicated enhancer sequences within their LTRs, but the variability in size implies that they must have been somatically acquired, presumably from a donor with one copy of the enhancer sequence. This vari-

FIG. 6. Appearance of duplicated LTR sequences in AKR thymuses. DNA from spleen and the eight thymus samples shown in Fig. 4 were digested with *Pst*I plus *Kpn*I, separated by electrophoresis on a 1.2% agarose gel, transferred to nitrocellulose, and probed with labeled JS21. dr, Direct repeat.

ability also suggests that the precise nature of the duplication event varies from animal to animal.

To test directly whether the LTR donor, *Bxv-1*, contains one or more copies of the enhancer sequence and whether duplication occurs during the life of an AKR mouse, we used JS21 to probe *Pst*I-*Kpn*I-digested spleen and thymus DNAs (Fig. 6). Control spleen DNA contained one *Pst*I-*Kpn*I fragment which reacted with JS21. This fragment was identical in size to the corresponding fragment from the one-direct-repeat clone of MCF-247 (data not shown). Thymus samples from 10-week-old mice yielded only one reactive fragment, identical in size to the spleen cell sample. By contrast, the 16-week and older samples all showed at least one larger band which reacted with JS21, implying that duplication of LTR sequences accompanies recombinant virus formation. Taken together, these results suggest that in addition to the LTR and *env* recombination events, duplication of LTR sequences is necessary for tumor induction.

Structural analyses of proviruses cloned from tumors. A number of considerations suggested that sequence information from recombinant proviruses present within spontaneous tumors might provide insights into the virological events leading to tumor formation. By comparing the recombination junctions from different proviruses cloned from the same tumor, it might prove possible to order the recombination events. For example, the presence of different viruses with the same p15E/U3 substitution but with different *env* substitutions would provide compelling evidence that the LTR change preceded the *env* change. Similarly, by examining the nature of the LTR duplication in different proviruses, the timing of this event could also be ordered. The LTR sequences would also reveal the regions of U3 which play a role in tumor formation and provide a direct test of the hypothesis that a specific duplication event, consistently generating a novel sequence at the boundary of the duplicated sequences, must take place.

We therefore set out to clone a number of different JS21-reactive proviral fragments from two different tumors from our panel. In addition to cloning somatically acquired proviruses, we hoped to clone the viral sequences corresponding to *Bxv-1* for direct tests of its structural and functional properties. *Eco*RI-digested DNA from tumors B and E was cloned into the lambda vector Charon 27, and 200,000 recombinant plaques from each library were

A

B

	Pst 1
TE2	CTGCAGTAACGCCATTTTGAAGGCATGAAAAAGTACCAGAGCTGAGTTC
MCF247-1b	A..... js21
TE2	TCAAAAGTCACAAGGAAGTTTAGTTAAAGAATAAGGCTGAACAAAAGCTGG
MCF247-1b	
TE2	GACAGGGGCCAAACAGGATATCTGTGGTCGAGCAGCTGGGCCCGGCTCA
MCF247-1b	
TE2	GGCCCAAGAACAGATGGTACTCAGATAAAGCGAAACTAGCAACAGTTTCT
MCF247-1b	
TE2	GGAAAGTCCCACCTCAGTTTCAAGTTCCCAAAAGACGGGAAAAACCCC
MCF247-1b	
TE2	AAGCCTTATTAACTAACCAATCAGCTCGCTTCTCGCTTCTGTAACCGC
MCF247-1b	 R→
TE2	GCTTTTGTCTCCCGAGCCCTATAAAAGGGTAAAACCCCACTCGGTG
MCF247-1b	A..... C.
TE2	CGCCAGTCATCCGATAGACTGAGTCGCCCGGGTACC
MCF247-1b	C..... Kpn 1

FIG. 7. Structure of *Bxv-1*. The clone TE2, which corresponds to the 5' half of *Bxv-1* and associated flanking mouse DNA, was characterized by restriction mapping and limited sequence analysis. (A) Restriction map of this clone. The stippled box indicates mouse sequences, the filled box represents the LTR, and the line represents the viral body sequences. Restriction sites: E, *EcoRI*; B, *BamHI*; S, *SacI*; P, *PstI*; K, *KpnI*; X, *XbaI*. There were no *HindIII* sites. (B) Comparison of the LTR sequences (from the *PstI* to the *KpnI* site) from this clone with the published sequence of MCF-247 clone 1b (31). Only bases differing from TE2 are shown in the MCF-247 sequence. The bases corresponding to the oligonucleotide JS21 are underlined. The U3/R junction (29) is indicated with an arrow.

screened with JS21. Four independent clones were obtained from the tumor B library (TB1 to -4), and three were obtained from tumor E (TE2 to -4). Clones TE3 and -4 and all clones obtained from tumor B represent the 3' halves of recombinant viruses, running from the conserved *EcoRI* site in the middle of gp70 through p15E and the LTR into flanking cellular sequences. Each clone had unique flanking sequences and corresponded in size to one of the somatically acquired proviral fragments visible in Fig. 2. We therefore assume that they represent integrations into the cell prior to the origin of the tumor and not the result of subsequent reinfections. All six clones reacted with the polytropic virus-specific probe JS5 as well as the LTR probe JS21 and thus correspond to recombinant MCF viruses of the type typified by MCF-247. By contrast, TE2 (a 9.1-kb *EcoRI* fragment) did not react with one of the class-specific *env* probes. Detailed restriction mapping indicated that it corresponded to the 5' half of a xenotropic provirus. Moreover, the restriction sites in the mouse flanking sequence (Fig. 7A) were identical to those previously determined for the potential LTR donor (49). Taken together with the reactivity with JS21, these findings show that TE2 corresponds to the 5' *EcoRI* fragment of *Bxv-1*. It is worth noting that TE2 grew very poorly in Charon 27 and that several attempts to plaque purify the clone were necessary before a pure clone could be obtained. Even after transfer to plasmid, poor yields of DNA were observed. This finding presumably explains the previ-

ous lack of success that we, and we believe others, have experienced in trying to clone *Bxv-1*.

The LTR sequence (between the conserved *PstI* and *KpnI* sites) of *Bxv-1* was determined. A comparison of this sequence with that of MCF-247 clone 1b further supports the idea that *Bxv-1* contributed LTR sequences to MCF-247. There are four differences between the two sequences (Fig. 7B). Two, shown in lowercase, presumably reflect *Akv*-derived sequences in MCF-247 (see below). The other two changes are probably point mutations which occurred during MCF-247 replication, since neither is seen in other MCF viruses. One of these differences (the G-to-A transition at base 47 of the sequence shown) creates the unique *EcoRI* site in MCF-247. This change is also responsible for a one-base-pair mismatch between JS21 and *Bxv-1*. However, it does not appear to impair the ability of JS21 to discriminate between *Bxv-1* and other endogenous viruses, presumably because it represents the 5' nucleotide of JS21. *Bxv-1* shows 20 single-nucleotide substitutions and a 6-bp deletion (just upstream of the TATA box) compared with a xenotropic virus cloned from NZB mice (47). The sequence shown in Fig. 7 is identical to that of the corresponding region of a xenotropic virus recently isolated from CWD mice and believed to be derived from *Bxv-1* (41).

Knowing the sequences of the viruses involved in generating the recombinant viruses, we decided to determine the precise points of recombination of the tumor-derived proviruses as well as the structures of the LTR duplication in the hope that this information might shed light on the order and mechanism of these processes. After a preliminary restriction mapping using *PstI* and *KpnI* (Fig. 8A) to determine the approximate 3' recombination point for the *env* substitution, appropriate *KpnI-EcoRI*, *PstI*, and *PstI-KpnI* fragments were subcloned into M13 vectors and sequenced. Short comparisons illustrating the deduced recombination junctions are shown in Fig. 8B to D. A number of conclusions may be drawn from these results. First, since all three recombination junctions in the four viral fragments cloned from tumor B are identical, it is likely that they represent the progeny of the same two recombination events. This is probably also true for the two viruses from tumor B despite the one-base-pair mismatches adjacent to the recombination points in TE4 (Fig. 8B and C), which can more easily be accounted for by mutations following recombination rather than recombination at virtually the same base between the ecotropic virus with differing noncancerous viruses. Taken together with the observations of Herr and Gilbert (26), who showed by restriction mapping that different proviruses from the same tumor had very similar structures, these results suggest that prior to tumor formation a unique recombinant virus has already been selected within the thymus of each AKR mouse. Second, the 3' end of the *env* recombination can vary by at least 500 bp (Fig. 8B). This implies that the C-terminal quarter of gp70 and the first 25 amino acids of p15E from ecotropic and endogenous polytropic viruses are functionally equivalent and unlikely to play a major role in tumorigenesis. Third, the 3' ends of p15E/U3 recombination in the proviruses from the two tumors appear to be identical one to the other and also to those of the recombinant viruses MCF-247 and MCF 30-2. This recombination point appears to lie at or very near the U3/R junction, strongly suggesting that the recombination event which lead to the acquisition of *Bxv-1* LTR sequences by these viruses was initiated by the first jump (48) occurring during the synthesis of viral DNA in a virion containing heterodimeric RNA.

We next determined the nucleotide sequences of the

FIG. 8. Structures of tumor-derived recombinant viruses. Cloned MCF viruses from tumors B and E were characterized by restriction mapping and by sequencing across the recombination junctions. (A) Overall structures of the clones and their origins. Stippled areas indicate sequences derived from the endogenous polytropic parent, filled areas are of ecotropic origin, unfilled areas come from *Bxv-1*, and the dotted line indicates mouse flanking DNA. Restriction sites shown (E, *EcoRI*; K, *KpnI*; P, *PstI*) are those used for subcloning into M13 for sequencing studies; the *env* *KpnI* and *PstI* sites are characteristic of the ecotropic and polytropic parents, respectively. (B) Sequence at the recombination junctions at the 3' end of the *env* substitution for the different clones. (C and D) The 5' and 3' ends of the p15E/U3 substitution, respectively. Parental virus sequences are shown at top and bottom. Only bases differing from at least one parent are shown. The deduced recombination junctions are underlined. In most cases, we have extensive additional sequence data from either side of the recombination points (data not shown). On the 5' side of the recombination junctions, the sequenced clone was identical to the parent shown at the top of each comparison and 3' sequence was the same as the lower parent (data not shown). Sources of sequence data: MX27, Stoye and Coffin (57); Akv, Herr (25); MCF 30-2, Holland et al. (29); and *Bxv-1*, Fig. 7. No sequence data are available for the p15E of *Bxv-1*; however, MCF 30-2 seems a reasonable substitute in light of the fact that its LTR is *Bxv-1* derived and it contains nonecotropic sequences, presumably of *Bxv-1* origin in p15E. The arrow in panel D shows the U3/R junction.

enhancer-containing region within the LTRs of the proviral fragments that we had cloned from tumors B and E. A schematic comparison of these sequences with those of *Bxv-1* and three previously sequenced MCF viruses is shown in Fig. 9. Each recombinant was clearly derived from *Bxv-1*; however, each clone contained a duplication, or in one case a triplication, of *Bxv-1* sequences in addition to one or two random point mutations. The duplication events each generated two perfect copies of *Bxv-1* sequences which were separated by between one and seven nucleotides of unknown origin. The mechanism of duplication is unclear but might occur in the following way. Reverse transcriptase, upon encountering a break in the RNA strand that it is transcribing, marks time by adding a few random nucleotides before locating and jumping to the other strand at a point upstream of the original pause. Different sequences are duplicated in each case; the 5' and 3' ends of the duplicated region can vary by at least 60 nucleotides in each direction. The junction between the 3' end of the first copy of the direct repeat and the 5' end of the second copy was always different, and the interval between repeated sequence motifs varied between 62 (MCFr35) and 115 (TE4) bp. The duplicated sequences always included the LVB binding site and the core motif. The GRE binding site was not duplicated in three cases, the proviruses from tumor B as well as MCFr35 and MCF-13, suggesting that these sequences need not be duplicated for tumor formation. The NF1 site was not duplicated in MCFr35 and was only partially duplicated in the TB proviruses. Interestingly, there was variation in the LTR structures in different proviruses from the same tumor, even though they were derived from the same recombination events. This finding provides suggestive evidence that the LTR duplication event occurs after both recombinations have taken place.

DISCUSSION

Virological steps leading to AKR thymomas. A complex series of virological events culminating in thymic lymphoma characterizes certain strains of mice, such as AKR, HRS, and C58. These steps include (i) inheritance of three parental proviruses, (ii) expression and replication of ecotropic virus, (iii) infection of a cell expressing *Bxv-1*, (iv) recombination to acquire altered LTR sequences, (v) infection of a cell expressing polytropic virus, (vi) recombination to acquire altered *env* sequences, (vii) duplication of enhancer sequences, (viii) replication of recombinant virus in the thymus, and (ix) transformation of T cells and growth of a tumor. They must involve at least three parental proviruses and two specific recombinational events as well as the duplication of enhancer-containing LTR sequences. Despite the complexity of this process, at least 90% of all AKR mice die of an MCF-induced thymoma, implying that recombinant viruses of a precisely defined structure are generated in a very consistent and reproducible fashion. Since there is no reason to believe that recombination between retroviruses occurs in a directed fashion, this implies that certain products of recombination are strongly selected on the basis of the biological properties conferred by their component parts.

Endogenous parents for MCF viruses. MCF viruses are formed by recombination between at least three different endogenous proviral sequences. One parent is clearly a replication-competent ecotropic virus that is expressed early in the life of the animal and replicates to high titers. In AKR mice this might be the product of either *Env-11* or *-12* (11,

FIG. 9. LTR duplications in MCF viruses. The structures of a number of recombinant viruses are compared schematically with that of the LTR donor, *Bxv-1*. Sequence features shown include the position of JS21 as well as the Lvb, core, NF1, and GRE binding sites (22, 55). Numbers shown correspond to the sequence data of TE2 shown in Fig. 7. Open boxes indicate the duplicated regions, with the number of zig-zags indicating the number of duplications in individual clones. The first and last bases duplicated in each clone are numbered. Inserted bases in the center of each duplication are shown. Sequences for MCFr35, MCF-13 and MCF-247W come from references 50, 68, and 29, respectively. The sequence of *Bxv-1* (TE2) is shown in Fig. 7. The sequences of TB1, -2, -3, and -4 and of TE3 and -4 were determined in this study. Each LTR shows an average of one random point mutation compared with TE2 (not shown in this figure for the sake of simplicity).

30, 53). By contrast, in HRS/J mice it is formed by recombination between the partially defective proviruses *Emv-1* and -3 (65). The studies reported here provide direct evidence confirming previous reports (27, 49) that the second parent, the LTR donor, is the xenotropic virus *Bxv-1*. However, *Bxv-1* may not be involved in the generation of all recombinant viruses, since two leukemogenic MCF viruses isolated from HRS/J mice, PTV-1 and PTV-2 (23), do not react with JS21 (data not shown). Indeed, recent studies suggest that mutation of the ecotropic LTR can sometimes substitute for this recombination event (63a). *Bxv-1* is not normally expressed at high levels, though B-cell stimulation can lead to its activation (60, 62). Though replication competent, the virus product of *Bxv-1* cannot grow to high titers in murine cells because these cells lack a receptor for xenotropic virus. The third parent, the *env* donor, remains something of a mystery since all strains of mice contain numerous endogenous proviruses that could potentially con-

tribute gp70 to a MCF virus. Slightly under half of these fall into the modified polytropic class; these do not seem to play a role in AKR leukemias since we see no evidence of amplification of sequences reactive with the JS4 phenotype probe in tumors. Furthermore, the number of potential *env* gene donors is also clearly lower than the number of endogenous polytropic proviruses, since some proviruses contain point mutations which would prevent translational initiation of the *env* gene product (44, 57), while others contain large deletions in their *env* (32, 36, 57) genes. Expression of endogenous polytropic virus has been demonstrated in a number of tissues, including thymus (35, 36), and is enhanced by immune stimulation (33, 34). However, none of the endogenous proviruses has been shown to encode an infectious virus. No single AKR-specific provirus is uniquely associated with MCF virus formation in the AKXD series of recombinant inbred mice (20). It therefore remains an open question whether there is a single, unique *env* donor or

whether multiple endogenous polytropic viruses can fulfill this role.

Role of the nonectropic substitutions. MCF viruses contain nonectropic sequences in *env* and the p15E/LTR region. Comparison of the growth properties imparted by these sequences in MCF and ectropic viruses might therefore be expected to illuminate some of the factors influencing the appearance of recombinant viruses. In a number of retroviral systems the LTR sequences are important in determining the tissue tropism and pathogenicity of the virus (2, 13, 14, 38, 66). These effects seem to be mediated by the enhancer sequences within the LTR. Comparisons of the host range properties of class I (LTRs of *Bxv-1* origin) and class II (LTRs of Akv origin) MCF viruses are consistent with this view (6). Similarly, a direct test of enhancer function in a stable transformation assay of murine T lymphoid cells using Neo^r as an indicator gene showed that the LTR of MCF-13 contained a significantly more powerful enhancer than did an Akv-derived LTR (68). However, a potential role for the C-terminal region of p15E in allowing thymus growth cannot be ignored. It is invariably coinherited with the U3 region of *Bxv-1* in all class I MCFs (40). (It has recently been suggested that at least some MCF viruses from CWD mice may contain p15E regions of polytropic origin [41]. However, a comparison of the sequences of the genomes of these viruses with those of ectropic MLV and the sequence determined here argues strongly against this possibility [data not shown].) In addition, studies of the tissue tropism and pathogenicity of in vitro-generated recombinants between MCF-247 and Akv revealed that the MCF-247 LTR alone conferred minimal pathogenicity to Akv but the addition of p15E sequences markedly increased the thymotropism and pathogenic potential of the recombinant virus (28).

Thymoma induction in AKR mice is associated with (Fig. 5) and appears to be dependent on (29) the generation of MCF viruses containing duplicated enhancer sequences within the acquired LTR. Viruses with one copy of the viral direct repeat seem to replicate reasonably well in the thymus, although perhaps not quite as well as viruses containing two or more copies (29). Changes in LTR size must occur consistently, since all tumors examined in this study contained viruses with LTRs larger than that of *Bxv-1*. On the other hand, the frequency of this event, or the growth advantage that it provides, must be relatively low, since the MCF 30-2, which contains one copy of the direct repeat, appears genetically and phenotypically stable (29). It remains to be determined which feature(s) of the duplicated sequence is important for pathogenicity. The most likely possibility is that two enhancers function better than one; an alternative suggestion that the duplication event generates a novel binding site for a regulatory protein (29) is inconsistent with the polymorphic nature of the duplication event (Fig. 9). From the data presented in Fig. 9, we can identify a 36-bp stretch of nucleotides which are common to all duplications; this includes the LVb binding site and the core region but not the NF1 binding site. Within this sequence there are only four single-base changes from Akv, one at the 3' end of the LVb binding site, one which lies within NF1, and two potentially within the region identified as LVt/LVc in Moloney MLV (Mo-MLV) (22, 55, 56). In the absence of functional data for the enhancers of the sequenced clones, we cannot determine which, if any, of these differences is important. In any event, the p15E/LTR substitution seems to play a crucial role in spontaneous AKR leukemogenesis, a

function which the corresponding Akv sequences apparently cannot provide even after enhancer duplication.

Thymotropic viruses such as Mo-MLV and SL3-3 seem invariably to generate MCF viruses after injection of newborn animals (63, 67). This finding suggests that replication of a virus with an ectropic gp70 within the thymus inevitably leads to recombination with an endogenous polytropic sequence to yield an MCF virus. Not only is such a virus generated, but it also seems to have the capacity to outgrow the ectropic progenitor virus, as evidenced by the absence of novel ectropic proviruses in the AKR tumors that we studied. The *env* gene alteration might provide a method of overcoming interference by ectropic gp70 synthesized from the endogenous proviral loci or of infecting cells that do not express the ectropic receptor. Alternatively, the expression of recombinant gp70 might actually stimulate the outgrowth of infected cells (42, 43), thus causing the apparent outgrowth of the recombinant virus itself. In turn, the stimulated cells might then represent targets for insertional activation. Consistent with this view, the ability of in vitro recombinants between MCF-247 and Akv to generate novel MCF viruses correlates with the ability to induce thymomas (28). Recently it has been shown that the deleted *env* glycoprotein, gp52, of Friend spleen focus-forming virus can interact with the erythropoietin receptor, stimulating erythroid growth (37), and it does not seem unreasonable to speculate that the MCF gp70 might act similarly via a T-cell-specific growth factor receptor.

Generation of MCF viruses. The structures of the class I MCF viruses that have been isolated from leukemic tissues are remarkably similar to one another, with relatively short patches of nonectropic sequences on an ectropic backbone and a duplication in the LTR. This similarity raises a number of interrelated questions. First, how are recombinant viruses generated and how many recombinational events are involved? Second, since it is reasonable to assume that the initial recombinants with this structure are relatively rare, how are they selected out of the total population of replicating viruses? Third, since coreplicating retroviruses recombine at very high frequencies, how are viruses with these structures maintained?

Retroviral recombination (reviewed in references 9 and 10) occurs during the synthesis of viral DNA by reverse transcriptase following infection of cells by virus particles containing two different RNA strands. Such heterozygous viruses result either from infection of a given cell by two different viruses, following infection by one virus of a cell type expressing a different, endogenous sequence, or possibly by coexpression of two endogenous viruses. It seems most likely that this second mechanism is operative in AKR mice, at least for the second recombination step, since the nonectropic proviruses involved do not seem to replicate in mice. It therefore follows that the patterns of expression of the nonectropic proviruses are critically important and may represent an important factor in determining which proviral loci participate in the formation of MCF viruses. The precise mechanism of recombination remains poorly understood, but estimates of retrovirus recombination rates are very high. For example, two oligonucleotides present in one strain of avian leukosis virus and physically separated by only 800 bp appear genetically unlinked after a few rounds of replication in the presence of a second virus (9). This finding implies that individual crossover events are frequent and provides an explanation for the relatively short patches of nonectropic sequences found on the Akv backbone. On the other hand, the substituted regions must be long enough to

provide the selective advantages necessary for outgrowth of the virus containing them.

As MCF viruses contain genetic information contributed by three different parents, generation of these viruses must involve at least two different recombination events (64). The practical difficulty in analyzing the recombinants present in an individual mouse at different ages complicates the task of trying to obtain a precise description of the events that lead to the leukemogenic virus, since it necessitates deducing a temporal sequence from a set of single time points. Some workers have hypothesized that three or more recombinations take place (17, 35), mainly to provide a role for all of the transcripts or recombinant viruses observed in AKR tissues. We favor a more parsimonious explanation, with only two recombinations, which places greater emphasis on the selection of particular recombinants. In the next paragraphs we outline some of these ideas.

The first recombination step leading to MCF virus generation in AKR mice will occur as the result of an ecotropic virus infecting a cell expressing either *Bxv-1* or an endogenous polytropic virus. At present we cannot be certain which noncancerous virus parent participates in the first recombinational step or even whether the two recombinations occur in a fixed order. In addition, it is not possible to tell in which cell or tissue type the recombinational events occur. However, there are a number of lines of evidence which suggest that (a) *Bxv-1* is involved in the initial recombination and (b) generation or selection of the recombinant viruses might occur in cells other than thymocytes. First, viruses with the structures predicted from recombination between ecotropic virus and *Bxv-1* have been isolated by cocultivation from the cells of thymuses from young AKR mice, whereas Akv with polytropic gp70 substitutions characteristic in size of those found in type I MCF viruses have not been observed (12, 16, 17, 64). Second, genome-length RNAs with xenotropic LTR sequences but lacking altered *env* genes have been detected in RNA extracted from the thymuses of 2-month-old AKR mice (35). Full-length Akv was also observed, but it remains unclear whether this corresponds to expression of the endogenous Akv loci or results from infection of thymocytes. The third and perhaps most intriguing piece of evidence stems from the fact that the endogenous ecotropic provirus is not particularly thymotropic (28, 52). Thus, the simplest way to generate a thymotropic ecotropic virus would be to recombine with *Bxv-1* in nonthymic cells to acquire novel p15E/LTR sequences. In addition to replication within different cell types following injection, different MLVs apparently recombine with different endogenous proviruses (18). Mo-MLV-derived MCF viruses show *env* genes identical to those of MCFs isolated from AKR mice (57), and Mo-MLV is thymotropic. If selection of the endogenous polytropic *env* gene donor is determined by the tissue tropism of the ecotropic provirus, then this would suggest that Akv is replicating in the same cell type as Mo-MLV, presumably following recombination with *Bxv-1*.

At present, we can only speculate where the two recombinational events are occurring. In vitro studies of activated murine lymphocytes have shown that stimulated B cells (but not T cells) produce *Bxv-1* (43, 60, 62). Furthermore, lipopolysaccharide-stimulated AKR spleen cells apparently produce phenotypically mixed ecotropic and xenotropic viruses (61), which would be one step toward generating the LTR recombinant. These findings suggest the B cells present in spleen or bone marrow might represent the initial site of formation of mixed viruses. However, other cell types cannot be ruled out by these experiments.

The site of the second recombination also remains open. However, studies of chimeric animals have shown that the thymic epithelial cells play a crucial role in the leukemogenic process (12). Thus, it seems possible that recombination with the polytropic *env* gene occurs within this cell type. Alternatively, thymic epithelium may serve as a source of MCF-producing cells, capable of infecting immature thymocytes as they move to the thymus.

Each AKR tumor contains multiple copies of a unique class I MCF virus. These proviruses do not seem to be acquired subsequent to cell transformation, since they are present at equivalent levels. How does this virus come to predominate in the tumor, outgrowing Akv and all the other recombinant viruses generated during the lifetime of that animal? It seems reasonable to assume that the specific noncancerous sequences of the MCF viruses confer a considerable growth advantage in thymocytes compared with viruses that do not contain them. These would include AKV and other products of the recombination reactions which do not contain sequences that promote growth in the thymus. However, this notion does not explain the absence of other class I recombinants from the tumor cells. One can imagine two general mechanisms that might lead to an absence of polymorphism among the somatically acquired proviruses in a given tumor. It is possible, albeit somewhat unlikely, that the first MCF virus to start replicating in the thymus prevents infection by subsequent viruses. If this were the case, then one would predict that multiple viruses isolated from thymus of individual preneoplastic mice would all have the same structure. Alternatively, selection might be associated with tumor formation itself. Suppose that a population of immature thymocytes is infected with a number of different viruses and that individual cells are more susceptible to self-infection than infection from other cells. This would lead to the integration of a large number of identical proviruses in the same cell, and if one of these activated a *c-onc* gene, a tumor could result. A variation of this theme, which does not run into the difficulties associated with viral interference, is that target cells are simultaneously infected by the progeny of a cell producing just one virus, perhaps by virtue of close physical proximity. This hypothesis would predict that no appreciable selection of proviruses would occur within the animal until a tumor clone develops. Resolution of this issue will require detailed structural analyses of multiple recombinant proviruses cloned directly from preneoplastic mice.

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