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A Direct Demonstration of Recombination between an Injected Virus and Endogenous Viral Sequences, Resulting in the Generation of Mink Cell Focus-Inducing Viruses in AKR Mice

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We analyzed viral recombination events that occur during the preleukemic period in AKR mice. We tagged a molecular chimera between the nonleukemogenic virus Akv and the leukemogenic mink cell focus-inducing (MCF) virus MCF 247 with an amber suppressor tRNA gene, *supF*. We injected the *supF*-tagged chimeric virus that contains all of the genes of MCF 247 except the envelope gene, which in turn is derived from Akv, into newborn AKR mice to evaluate its pathogenic potential. Approximately the same percentage of animals developed leukemia with similar latent periods when injected with either the tagged or nontagged virus. DNA from tumors induced in AKR mice by the tagged chimeric virus was analyzed by Southern blotting with the *supF* gene as a probe. One set of tumors contained the injected *supF*-tagged virus. Two kinds of *supF*-tagged proviruses were found in a second set of tumors. One group of *supF*-tagged viruses had a restriction map consistent with that of the injected virus, while the other group of proviruses had restriction maps that suggested that the proviruses had acquired an MCF virus-like envelope gene by recombination with endogenous viral sequences. These results demonstrate that injected viruses recombine in vivo with endogenous viral sequences. Furthermore, the progression to leukemia was accelerated in mice that develop tumors containing proviruses with an MCF virus *env* gene, emphasizing the importance of the role of the MCF virus *env* gene product in transformation.

AKR mice develop spontaneous T-cell lymphomas between 6 and 12 months of age. Genetic studies have determined that this disease is linked to the inheritance of endogenous ecotropic proviruses (40). Although the inheritance and expression of ecotropic viruses are required, these viruses are not the proximal agents that induce disease. Rather, viruses that are formed by recombination between the inherited ecotropic virus, Akv, and two or more additional endogenous nonecotropic viral sequences appear to be the causative agents in the induction of leukemia (13, 32, 33, 40, 44). These recombinant viruses, called mink cell focus-inducing (MCF) viruses, were named for their capacity to induce cytopathic effects in mink cells (17).

MCF viruses can be isolated from spontaneous tumors (6, 17) and from spleens (11) and thymuses of mice during the preleukemogenic period (17, 40, 44). These viruses have a distinctive envelope glycoprotein (gp70) which expands their host range and classifies them as polytropic or dual-tropic viruses (13). This glycoprotein is encoded by a recombinant envelope gene (*env*) generated during the preleukemogenic period by recombination between Akv and endogenous, nonecotropic viral sequences (10, 44). RNase T_1 fingerprinting and sequence analyses of MCF 247, the prototypic MCF virus isolated from leukemia-prone AKR strain mice, reveal that the 5′ portion of the envelope gene differs significantly from the corresponding portion of the Akv genome (21, 33, 39). Similar nonecotropic substitutions have been reported in the 5′ portion of the envelope gene of spleen focus-forming virus and in other MCF viruses characterized to date (3, 4, 21, 26, 33). These nonecotropic substitutions in the 5′ portion of *env* are an important genetic hallmark of polytropic viruses.

The ability of MCF 247 to accelerate the onset of leukemia following injection into newborn AKR mice is one of the phenotypic hallmarks of the virus. In vivo studies using susceptible newborns injected with chimeric viruses between MCF 247 and the nonleukemogenic Akv have shown that four genomic regions of MCF 247 contribute to disease induction (19). One of these four regions is the envelope gene. It has been reported that the envelope gene is one of the multiple leukemogenic determinants of the Friend MCF virus as well (36).

On the basis of our previous data (19) and the proposed mechanism for the generation of spontaneous MCF viruses in AKR mice (44, 46), we predicted that an exogenous chimeric virus containing all of the genes of MCF 247 and the envelope gene derived from Akv would recombine with endogenous nonecotropic viral envelope sequences during the preleukemogenic period in AKR mice. To test this hypothesis and to identify the injected virus, we tagged the long terminal repeat (LTR) of the injected virus with a bacterial suppressor tRNA gene, *supF*. Using Southern blot analysis with the *supF* gene as a probe, we have analyzed proviruses from thymic tumors of mice injected with the chimeric virus. In this analysis, we identified proviruses derived directly from the injected virus and proviruses which obtained an MCF virus-specific envelope gene by recombination between the injected virus and endogenous viral sequences. We found that progression to leukemia was accelerated in mice that develop tumors containing proviruses with an MCF virus-specific envelope gene. By comparison, mice with tumors having proviruses with the enve-

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lope gene of the injected virus developed tumors more slowly. Our results indicate that recombination of the injected virus with endogenous, nonecotropic viral envelope sequences is an important event in acceleration of the onset of leukemia.

MATERIALS AND METHODS

Construction of infectious chimeric viruses tagged with the *supF* gene. Chimeric viral genomes constructed by using infectious molecular clones of MCF 247 and Akv 623 (19, 20) were tagged in the U3 region of the LTR with an amber suppressor tRNA gene, *supF*, using the following scheme. A modified pUC19 vector, which lacks the *Pst*I site, was constructed by first cleaving with *Pst*I and then removing the protruding ends by using the exonuclease activity of T4 DNA polymerase. The vector was then religated and used to transform *Escherichia coli* DH5. The resulting vector, pUC19Pst$^-$, was used to subclone permuted, chimeric viral genomes (19) at the *Xba*I site. Transformation of *E. coli* DH5 and plasmid isolations were performed by using standard protocols (34). After subcloning of the chimeric viral genomes into pUC19Pst$^-$, the plasmids were cut at the *Pst*I site in the U3 region of the viral LTR and blunt ended with T4 DNA polymerase. An *Fnu*DII fragment containing the *supF* gene isolated from plasmid piAN7 (gift of B. Seed) was ligated into the LTR to generate *supF*-tagged chimeric viral genomes. Ligated DNAs were transformed into *E. coli* CC114 and plated onto MacConkey agar (Becton Dickinson Microbiology Systems) by using standard protocols. Colonies containing recombinant plasmids were chosen by their red color, and plasmids were characterized by restriction digests.

Viruses. To produce infectious virus, DNA inserts were isolated from recombinant plasmids and ligated to form concatemers. *Mus dunni* cells were transfected by calcium phosphate precipitation (15) with 1 μg of plasmid DNA mixed with 19 μg of calf thymus DNA. Two days after transfection, and every 2 days thereafter, the cells were trypsinized and reseeded at a density of 5×10^6 cells per 100-mm-diameter dish. Fourteen days following transfection, supernatants were assayed for virus production by a reverse transcriptase assay (2). Virus stocks were stored at −80°C.

Mice and tumor induction. Newborn (<2-day-old) AKR/J mice were injected by both intrathymic and intraperitoneal routes with 0.05 ml of undiluted tissue culture-grown virus. Mice were monitored daily for evidence of frank leukemia (scruffy fur, labored breathing, lymph node enlargement) and were sacrificed by cervical dislocation when the disease was far advanced. Mice were diagnosed with leukemia if necropsy results revealed grossly enlarged lymphoid organs. Lymphoma development before 180 days of age was considered an accelerated response to the injected virus.

Southern hybridization analysis. Twenty micrograms of genomic DNA was digested to completion with the restriction endonucleases *Pst*I, *Eco*RI, and *Kpn*I or *Eco*RI and *Kpn*I under the conditions recommended by the suppliers (New England Biolabs). The samples were electrophoresed in a 1% agarose gel in running buffer (0.8 M Tris borate, 1 M sodium acetate, 0.02 M EDTA). The gels were treated for 10 min with 0.25 N HCl prior to transfer of the DNA to nylon membranes (Zetabind; CUNO), using 0.4 N NaOH. Following prehybridization for 2 h at 65°C, membranes were hybridized to ^{32}P-labeled (random prime method; Boehringer Mannheim) *supF* sequences (10^6 dpm/ml) that were concatenated by ligation prior to labeling. The prehybridization solution contained 4× SSCP (1× SSCP is 120 mM NaCl, 15 mM sodium citrate, 15.4 mM dibasic sodium phosphate, and 5.3 mM monobasic sodium phosphate), 1× Denhardt's solution (34), 1% sodium dodecyl sulfate (SDS), and 125 μg of denatured salmon sperm DNA per ml. The hybridization solution contained 0.25× SSCP, 1% SDS, 1× Denhardt's solution, and 1% sodium dextran sulfate. The final high-stringency wash was with 0.1× SSCP–0.1% SDS at 65°C for 15 min. Membranes were exposed to Kodak XAR-5 film with intensifying screens at −80°C.

Northern (RNA) hybridization analysis. Total cellular RNA from the cell lines transfected with the *supF*-tagged recombinant viral DNAs was extracted by the guanidinium hydrochloride procedure (24). Approximately 20 μg of RNA was electrophoresed in formaldehyde-agarose gels and transferred to nylon membranes according to the protocol provided by the manufacturer. DNA-RNA hybridizations were carried out with ^{32}P-labeled *supF* sequences prepared as described above.

Statistical analysis. Chi-square analysis (52) was used to compare the disease incidence of the *supF*-tagged and non-tagged chimeric viruses. The Mann-Whitney test (52) was used to compare the latent periods, measured in days to thymoma development, for animals having tumors that contained proviruses with the genotype of the injected virus and for animals with tumors that contained *supF*-tagged proviruses with an MCF virus-like *env*.

RESULTS

Structures of the recombinants. To study the function of the MCF virus *env* gene in the acceleration of leukemia in AKR/J strain mice, we constructed chimeric viruses between molecular clones of the leukemogenic virus MCF 247 and the nonleukemogenic virus Akv. We tagged the chimeric viruses with an amber suppressor tRNA gene, *supF*, in order to identify the injected virus in vivo. The structure of a prototypic retroviral provirus and the predicted proviral structures of MCF 247, Akv 623, and the chimeras tagged with *supF* are shown in Fig. 1. We used two restriction sites shared by MCF 247 and Akv 623 clones, *Xho*I and *Xba*I, to exchange the gp70-containing fragments between MCF 247 and Akv. The molecular clone MCF/Akv(gp70)*supF* contains most of the genes of MCF 247 except for a fragment from the *Xho*I site in the *pol* gene to the *Xba*I site in the *env* gene. In this clone, the portion of the envelope gene which encodes gp70 is ecotropic and derived from Akv (Fig. 1C); Akv/MCF(gp70)*supF* is the reciprocal chimera and contains a polytropic *env* gene derived from MCF 247 (Fig. 1D).

Maintenance and expression of the *supF* tag in chimeric viruses. Molecular clones of MCF/Akv(gp70)*supF* and Akv/MCF(gp70)*supF* were transfected into *M. dunni* cells to generate cells producing infectious virus. After the cells were cultured for approximately 6 months, we analyzed the structures of the *supF*-tagged proviruses by Southern blotting. Figure 2 shows a Southern blot analysis of DNA extracted from *M. dunni* cells transfected with *supF*-tagged MCF/Akv(gp70)*supF* (Fig. 2A) or Akv/MCF(gp70)*supF* (Fig. 2B) and then digested with either *Pst*I, *Kpn*I, or *Eco*RI and hybridized with a *supF*-specific probe. Since insertion of the *supF* gene into the U3 region of the LTR destroyed the two *Pst*I sites in the chimeras, the genome of each tagged provirus as well as flanking DNA sequences were contained on a single *Pst*I fragment. As expected, multiple bands were

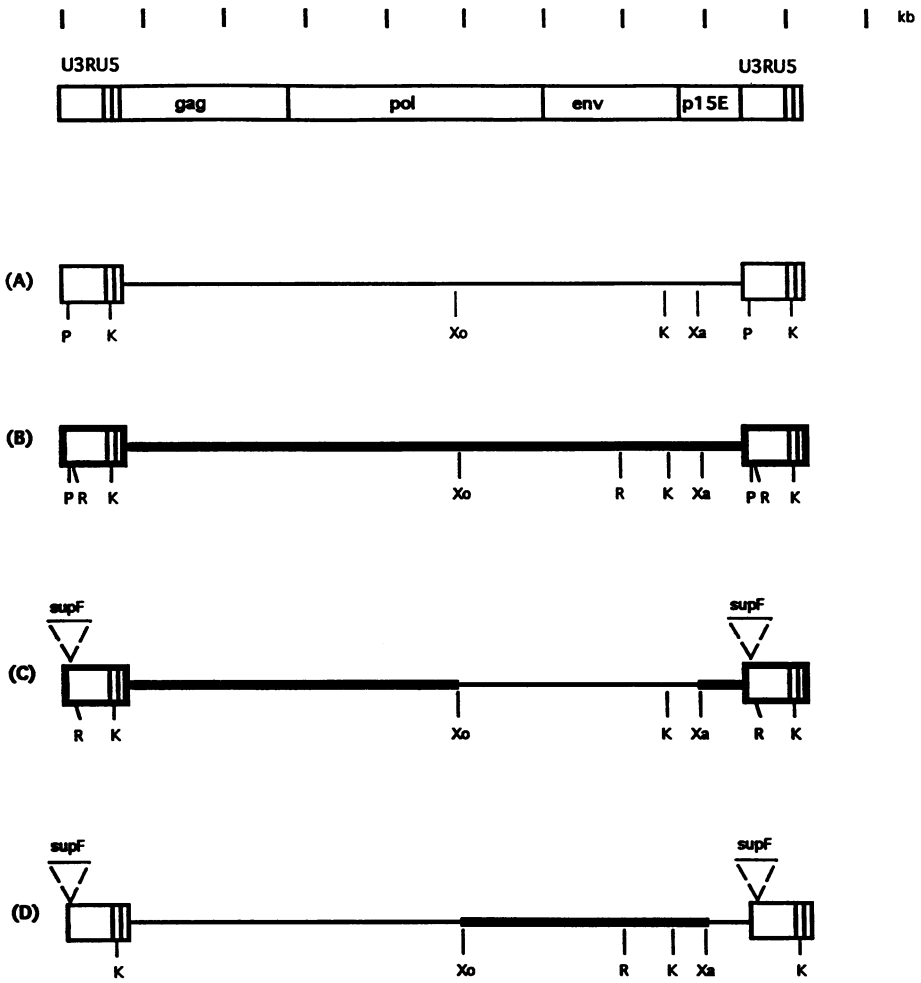

FIG. 1. Predicted proviral structures of *supF*-tagged chimeric viruses. A diagrammatic representation of a prototypical retroviral genome (top) and the predicted proviral structures of the *supF*-tagged recombinant viruses used in this study are shown. Restriction sites (Xo, *Xho*I; R, *Eco*RI; K, *Kpn*I; Xa, *Xba*I; P, *Pst*I) in the DNA clones of Akv 623 (A) and MCF 247 (B) used to generate the recombinants are shown relative to the proviruses. Chimeras MCF/Akv(gp70)*supF* (C) and Akv/MCF(gp70)*supF* (D) were constructed from the sequences derived from Akv (▬) and MCF 247 (▮).

present in *Pst*I-digested DNAs, indicating that both cell lines contain numerous proviruses.

DNA from cells transfected with MCF/Akv(gp70)*supF* and cleaved with *Kpn*I produced a 1.7-kb band derived from the 3′ end of the provirus that hybridized to *supF*. The DNA digested with *Eco*RI produced a 8.6-kb band (Fig. 2A). The 5′ end of each provirus and its' flanking sequence were represented by a population of heterogeneously sized bands derived from proviruses integrated into multiple sites in the cellular DNA. DNA from cells transfected with Akv/MCF(gp70)*supF* DNA also was cleaved with *Kpn*I or *Eco*RI (Fig. 2B). *Kpn*I-cleaved DNA produced a 1.7-kb band that hybridized with the *supF* probe. This size band is predicted to be a proviral fragment containing the carboxy terminus of *env*, p15E, and the 3′ LTR of the provirus shown in Fig. 1D. The provirus derived from this chimera contains only one *Eco*RI restriction site, which is located in the polytropic *env* gene. Heterogeneously sized bands were found when cellular DNA was cleaved with *Eco*RI, suggesting that each of the fragments represents a unique integration site. The analyses of genomic DNA from cells transfected with either of the *supF*-tagged chimeras demonstrate that the transfected chimeric viruses are the major type of virus replicating in culture. These data suggest that viral recombination events are undetectable following transfection of viral DNA into *M. dunni* cells and following long-term propagation of the chimeric virus in cell culture.

We next analyzed RNA from cells transfected with the chimeric viruses and from viral particles for the presence of the *supF* tag (Fig. 3). Total cellular RNAs from *M. dunni* cells transfected with cloned viral DNAs and cultured for 2 to 6 months were analyzed by Northern blotting. *supF* hybridized to two mRNAs (Fig. 3, lanes A to C) presumably representing full-length unspliced (8.6-kb) and spliced (3.6-kb) viral RNAs. In addition, full-length viral RNA (8.6 kb) extracted from particles in the culture supernatants also hybridized with the *supF* probe (Fig. 3, lane E). RNA from *M. dunni* cells transfected with calf thymus DNA as a control did not hybridize with the *supF* probe (data not shown). These data demonstrate that the *supF* tag is transcribed and retained in the viral genome upon passage in vitro.

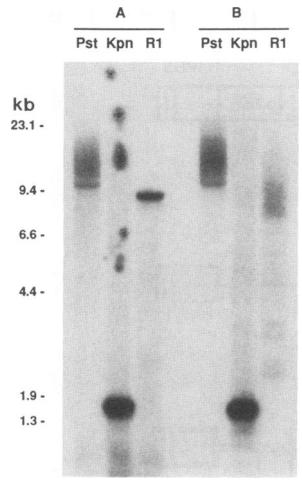

FIG. 2. Southern blots of DNA from cells expressing *supF*-tagged chimeric viruses. DNA extracted from cells transfected with molecular clones of MCF/AKV(gp70)*supF* (A) and AKV/MCF (gp70)*supF* (B) were cut with *Pst*I (Pst), *Kpn*I (Kpn), and *Eco*RI (R1) and analyzed as described in Materials and Methods.

To determine the stability of the *supF* tag, we isolated four clonal cell lines. To isolate these clonal lines, *M. dunni* cells were transfected with a molecular clone of MCF247 which was tagged in the LTR with the *supF* gene (MCF 247*supF*) and cultured for 2 months. The infected cells were cloned by endpoint dilution. After allowing the cloned cells to divide for an additional 2 months in culture, we prepared cellular RNA from one clonal cell line (Fig. 3, lane D) and viral genomic RNA from the particles in the supernatant (Fig. 3, lane F) and analyzed these RNAs by Northern blotting. The *supF*-specific probe hybridized to both the cellular RNA and viral genomic RNA, demonstrating that the *supF* gene was

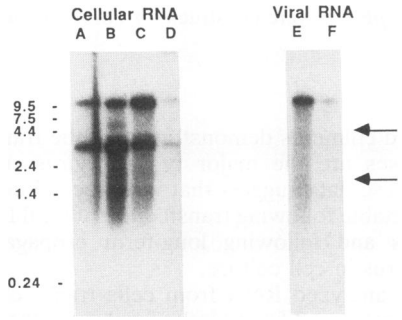

FIG. 3. Maintenance and expression of the *supF* tag in the recombinant viruses. Total cellular RNA was extracted from cells cultured between 2 and 6 months following transfection with cloned viral DNA from Akv/MCF(gp70)*supF* (lane A), MCF/Akv(gp70)*supF* (lane B), and MCF 247*supF* (lane C). Cells infected with MCF 247*supF* were cloned after 2 months in culture (lane D), and cellular RNA was prepared. Viral RNA was prepared from supernatants of cells cultured 2 months (lane E) and 4 months (lane F) after transfection with MCF 247*supF* DNA. Approximately one-fifth of each RNA preparation was electrophoresed, transferred to the nylon membrane, and analyzed by Northern blotting. The Northern blot was hybridized with a *supF*-specific probe. Molecular weight markers (in kilobases) are indicated on the left; the arrows at the right indicate the positions of 28S and 18S RNAs in the cellular RNA.

TABLE 1. Leukemogenicity of chimeric viruses in AKR/J mice

Virus[a]	No. injected	% Disease incidence[b]	Mean latent period[c] (days)
Control[d]	30	3	
MCF 247	33	91	103 ± 23
MCF/Akv(gp70)	51	82	134 ± 27
MCF/Akv(gp70)*supF*	34	71	149 ± 31

[a] Virus stocks were prepared from *M. dunni* cells transfected with the cloned viral DNAs described in Fig. 1.
[b] Percentage of animals injected as newborns with virus that were diagnosed at autopsy as having thymic lymphoma at or before 180 days.
[c] Calculated as the mean of all animals observed with disease at or before 180 days ± standard error.
[d] Newborn mice injected with medium. For disease incidence of medium-injected control animals, see Fig. 4a in reference 19a.

maintained as a stable tag in the viral LTR through many infectious cycles of the virus.

Leukemogenicity of the *supF*-tagged recombinant viruses in AKR/J mice. Viruses prepared from cells transfected with MCF/Akv(gp70)*supF* DNA (Fig. 1C) were injected into newborn (<48-h-old) AKR/J mice to determine their leukemogenic potential (Table 1). As previously reported (19), MCF/Akv(gp70) virus induced a lower incidence of leukemia (82%) with an increased latent period of disease induction (134 ± 27 days) relative to MCF 247 (91% disease; mean latent period of 103 ± 23 days). Specifically, we compared the pathogenic potential of the *supF*-tagged chimeric virus (71%), MCF/Akv(gp70)*supF*, with the nontagged chimeric virus (82%). The pathogenic potential (percent disease incidence) of these chimeric viruses did not differ statistically as determined by chi-square analysis. Therefore, the *supF*-tagged virus induced leukemia with an incidence and latency comparable to those of the nontagged virus.

Recombination of MCF/Akv(gp70)*supF* in vivo. We used the *supF* gene as a probe to identify *supF*-tagged proviruses in the DNA of thymic tumors or tissues from mice injected with MCF/Akv(gp70)*supF*. The *supF* probe does not hybridize with genomic DNA from uninfected cells or untagged viral sequences (data not shown). *supF*-tagged proviruses having the envelope gene derived from the injected virus (Fig. 4A) can be discriminated from *supF*-tagged proviruses that have acquired an MCF virus-specific *env* gene (Fig. 4B) by *Eco*RI cleavage of tumor DNAs. The proviral envelope gene derived from the injected virus lacks the *Eco*RI restriction site, while an MCF virus-specific *env* gene is predicted to contain this site (Fig. 4). This difference allows us to discriminate between the injected virus and a virus that acquires the MCF virus-specific *env* gene by recombination. When proviruses containing an MCF virus-specific *env* gene are cleaved with *Eco*RI, the digestion produces a 1.7-kb fragment derived from the 3′ end of provirus that hybridizes to *supF*.

A typical Southern blot analysis of DNA from the thymus, spleen, and lymph nodes of a mouse injected with MCF/Akv(gp70)*supF* virus is shown in Fig. 5. At least seven *supF*-tagged proviruses (detected as bands greater than 9.4 kb) were detected in the *Pst*I-digested DNAs from all three tissues of this animal. The 1.7-kb band is the major band when each DNA is digested with *Eco*RI. This size is consistent with the size predicted for the fragment derived from the 3′ end of a *supF*-tagged provirus that had acquired an MCF virus envelope gene. Fragments containing the 5′ end of each provirus and flanking cellular sequences are represented as a population of heterogeneously sized bands

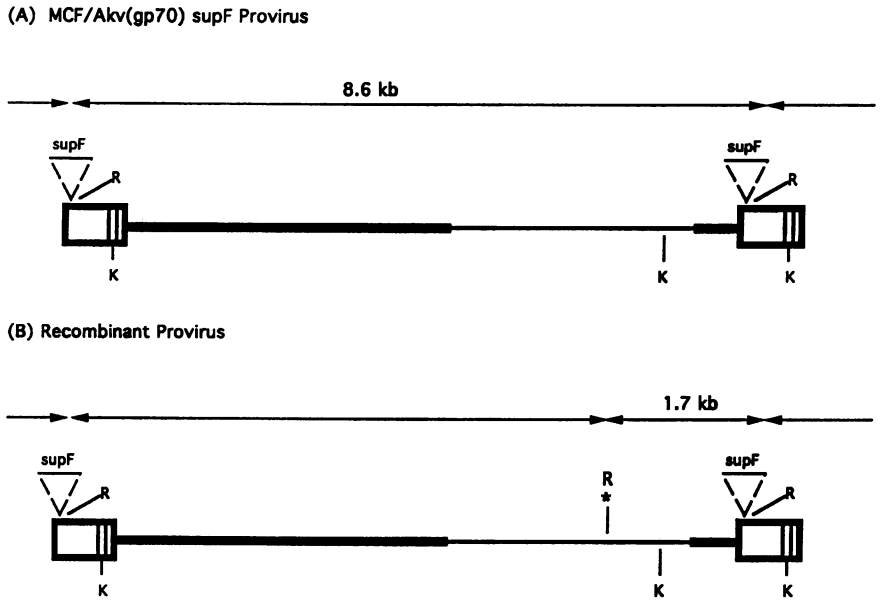

FIG. 4. Restriction map of a provirus having the genotype of the injected virus, MCF/Akv(gp70)*supF* (A), and of a provirus having the genotype of virus containing an MCF-specific envelope gene (B). *Eco*RI (R) and *Kpn*I (K) restriction sites are indicated relative to the proviral DNA. The predicted size of the *Eco*RI restriction fragment that hybridizes with the *supF* probe is indicated above each provirus. The asterisk indicates the position of the *Eco*RI restriction site in the putative recombinant viruses.

between 2 and 8.6 kb in size. *supF*-hybridizing bands of identical size are present in *Eco*RI-digested DNA from the spleen, thymus, and lymph nodes, indicating that the three tissues contain similar proviruses and that tumor cells containing the *supF*-tagged proviruses undergo a clonal expansion. In addition to the 5′-end-derived fragments and the 1.7-kb band, an 8.6-kb band in the *Eco*RI-digested DNAs also hybridized to the *supF* probe. This band represents a provirus derived from the *supF*-tagged injected virus containing an ecotropic *env* gene (Fig. 4A). The intensity of the 8.6-kb band relative to that of the 1.7-kb fragment suggests that the majority of the *supF*-tagged proviruses in these tumors contained an MCF virus-specific *env* gene. These data indicate that these recombinant proviruses may have a significant role in the induction of leukemia.

A Southern blot of two tumor DNAs, each taken from a thymus of a mouse injected with MCF/Akv(gp70)*supF*, is shown in Fig. 6. As indicated by the *Pst*I-digested tumor DNAs, multiple *supF*-tagged proviruses are present in each tumor. The 1.7-kb band that hybridizes to the *supF* probe in DNAs digested with *Kpn*I is of the size predicted for a

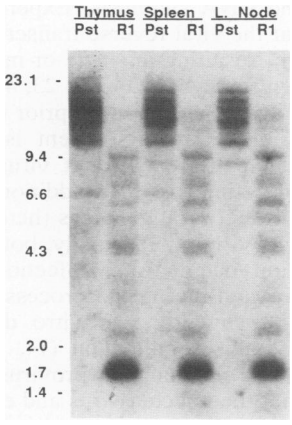

FIG. 5. Southern blot analysis of high-molecular-weight DNAs from the thymus, spleen, and lymph nodes from one animal injected with MCF/Akv(gp70)*supF*. Each DNA was cut with *Pst*I (Pst) or *Eco*RI (R1) and probed with the *supF*-specific probe. The presence of a 1.7-kb *Eco*RI fragment which hybridized with the *supF* probe indicates that proviruses present in these tissues have acquired an MCF virus-specific envelope gene. Molecular weight markers (in kilobases) are indicated at the left.

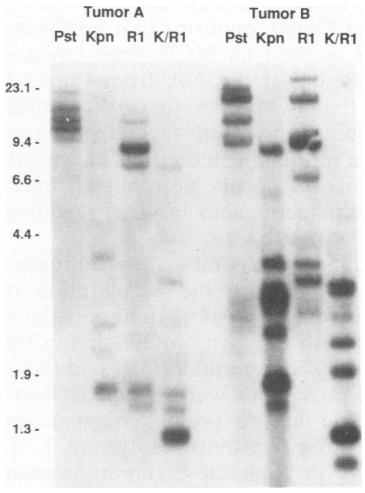

FIG. 6. Southern blot analysis of high-molecular-weight DNA from thymic lymphomas induced with MCF/Akv(gp70)*supF*. Each tumor DNA was cut with *Pst*I (Pst), *Kpn*I (Kpn), *Eco*RI (R1), or *Kpn*I and *Eco*RI (K/R1) and analyzed as described in Materials and Methods. Molecular weight markers (in kilobases) are indicated at the left.

TABLE 2. Molecular analysis of the envelope genes of proviruses in thymic tumors induced in AKR mice by MCF/Akv(gp70)*supF* virus

Tumor group	Envelope gene of *supF*-tagged proviruses[a]		*n*	No. of days to thymic lymphoma development[b]
	Injected	Recombinant		
I	−	−	2	180, 180
II	+	−	9	70, 138, 145, 175, 178, 178, 180, 180, 180[c]
III	+	+	5	101, 105, 125, 145, 146[c]

[a] Southern blots of tumor DNA were hybridized with a *supF*-specific probe. +, present; −, absent.

[b] Newborn mice were injected intrathymically and intraperitoneally with virus and were monitored weekly for signs of frank leukemia (scruffy fur, labored breathing, lymph node enlargement).

[c] Mann-Whitney nonparametric test of latent period for tumors containing *supF*-tagged proviruses indicated that the tumors in group III occurred earlier than the tumors derived from animals in group II ($P < 0.05$).

fragment derived either from the 3′ end of a provirus having the *env* gene of the injected virus (Fig. 4A) or from the 3′ end of a recombinant provirus having an MCF virus *env* gene (Fig. 4B). Additional fragments in the *Kpn*I-digested DNAs represent viral integration events and were derived from the 5′ end of the *supF*-tagged proviruses and the flanking cellular sequences.

We used an analysis of tumor DNA cleaved with *Eco*RI and hybridized to the *supF* probe to discriminate between *supF*-tagged proviruses having an ecotropic *env* gene (Fig. 4A) and *supF*-tagged proviruses that have acquired an MCF virus-specific *env* gene (Fig. 4B). *Eco*RI-digested DNAs from tumor A and tumor B each contain an 8.6-kb band that hybridizes to a *supF* probe, demonstrating the presence of proviruses with the envelope gene of the injected virus in the tumors. Tumor A DNA, when cleaved with *Eco*RI and hybridized to the *supF* probe, also contains a 1.7-kb fragment. This fragment is the size predicted for the 3′ end of a provirus having an MCF virus *env* gene. Furthermore, the 1.7-kb fragment is reduced in size to 1.3 kb when the DNA is cleaved with both *Kpn*I and *Eco*RI. The presence of this band, and the absence of a 1.7-kb fragment in DNA digested with *Kpn*I and *Eco*RI, confirms that *supF*-tagged proviruses in this tumor contain an MCF virus envelope gene. Conversely, *Eco*RI-digested DNA from tumor B contains an 8.6-kb band that hybridizes to *supF*, consistent with this tumor containing only *supF*-tagged proviruses having the envelope gene of the injected virus. These data demonstrate that recombination occurs between an injected virus and endogenous viral sequences in vivo, resulting in the generation of MCF viruses.

The significance of recombinant proviruses containing the MCF virus *env* gene in the acceleration of leukemia is underscored by our analysis of 16 tumors derived from AKR mice injected with the virus MCF/Akv(gp70)*supF*. Tumors were analyzed by Southern blotting, and the envelope genes of the tagged proviruses were mapped. Two tumors which arose quite late in the preleukemogenic period did not contain *supF*-tagged proviruses (group I, Table 2). It is possible, however, that these tumors contained spontaneously arising MCF viruses. Fourteen tumors contained proviruses having the *supF* tag (groups II and III, Table 2). At least five *supF*-tagged proviruses were present in each tumor. The tumors containing *supF*-tagged proviruses were divided into two groups. Group II ($n = 9$) consisted of tumors that contained proviruses having the *env* gene of the injected virus. It is unclear from this analysis whether these tumors contain spontaneously arising MCF viruses. In addition to containing proviruses with the *env* gene of the injected virus, tumors in group III ($n = 5$) contained recombinant proviruses having an MCF virus *env* gene. A nonparametric comparison (52) of the latent period (measured in days to thymic lymphoma development) for the tumors in the two groups indicated that the tumors in group III occurred earlier than the tumors derived from animals in group II ($P < 0.05$). The statistically shorter latent period to tumor induction for the animals in group III indicates that the presence of a recombinant virus with the MCF virus *env* gene significantly accelerates the onset of virally induced leukemia. The presence of *supF*-tagged proviruses containing an MCF virus-specific *env* gene extends the findings of Holland et al. (19) and supports the hypothesis that the MCF virus-specific *env* gene plays a pivotal role in the leukemogenic process.

DISCUSSION

It is well known that retroviruses have a propensity to recombine. This phenomenon was described in vitro following the coinfection of cells with two distinct viruses having stable phenotypic markers (48). Recombination has also been demonstrated between retroviruses used to infect cells and endogenous retroviral sequences expressed in those cells (37, 43, 49). Moreover, recombination between genetically unique endogenous retroviruses has been proposed as a mechanism to explain the appearance of novel retroviruses in vivo (10, 44). Our findings demonstrate that recombination of an injected virus with endogenous retroviral sequences occurs in vivo, resulting in the generation of MCF viruses.

Two models explain the molecular mechanism of retroviral recombination: forced-copy choice, or minus-strand recombination (8), and strand displacement/assimilation, also known as plus-strand recombination (25). The forced-copy choice model (8) predicts that recombination occurs during minus-strand DNA synthesis. This mode of recombination occurs most frequently when one of the RNA heterodimers is nicked or damaged. Conversely, the strand displacement/assimilation model (25) predicts that recombination occurs during plus-strand DNA synthesis. Experimental evidence demonstrates that the viral reverse transcriptase can utilize either mechanism, resulting in single or multiple crossover events with a high frequency (14, 22, 23, 45).

Several initial events are required prior to recombination by either mechanism. The first event is the coinfection and/or coexpression of two distinct viruses or retroviral sequences within a single cell. In addition, virus particles containing two distinct viral genomes (heterodimeric) must be packaged in one virion (22). Finally, both models predict that recombination occurs after infection with the heterodimeric virions and during the process of reverse transcription (14, 45). Given the in vitro data defining the mechanisms of viral recombination (14, 22, 45, 48), our demonstration that a recombinant provirus exists in tumor DNA suggests that the injected virus and endogenous MCF envelope sequences were coexpressed in one cell and copackaged in one virion, recombined, and generated infectious recombinant progeny.

One important consequence of retroviral recombination is the emergence of viruses having unique biological properties. The envelope gene is one region of the viral genome that is frequently involved in recombination. Genetic changes in the envelope gene often generate a virus having increased

pathogenicity and expanded infectivity to different cell types (5, 12, 16, 37, 42, 50). Thus, the process of retroviral recombination is likely to be important for the evolution of viral species in vivo. In this study, 31% (5 of 16) of the tumors contained detectable recombinant proviruses (Table 2). This finding suggests that either (i) there is a biological selection for proviruses having an MCF virus-specific *env* gene that is important in the transforming event or (ii) the prevalence of these viruses makes them consistent passengers during tumor development.

One role of the MCF virus envelope gene product, gp70, is to interact with cellular components on the surface of the host cell, enabling the virus to overcome receptor interference by ecotropic viruses (7, 9, 38). In addition, the MCF virus gp70 may be cytotoxic and suppress the growth of a specific cell type (31). Another role of the MCF virus envelope glycoprotein is suggested by recent studies that show that the MCF virus envelope protein may provide a growth advantage by stimulating cell proliferation (27, 28, 30, 35). One group (28, 30) used interleukin-2 (IL-2) and erythropoietin-dependent or IL-3-dependent established cell lines to demonstrate that MCF virus infection promotes growth factor-independent cell proliferation in the absence of growth factor; ecotropic and xenotropic viruses did not stimulate factor-independent growth (28, 30, 41, 47, 51). The altered proliferative capacity of erythropoietin-dependent cells infected with MCF viruses appears to be due to the association of the glycoprotein with the erythropoietin receptor (30, 51). This association between viral glycoprotein and growth factor receptor presumably induces cell proliferation. MCF virus infection also promotes IL-2- and erythropoietin-independent growth of one erythropoietin-dependent lymphoid cell line transfected with genes for both the IL-2 and erythropoietin receptors (28). The interaction of the MCF virus gp70 with the IL-2 receptor is proposed to initiate a series of events that result in cell mitosis. However, immunoprecipitation studies have not demonstrated a physical association between the IL-2 receptor and the MCF virus gp70 (28). More recently, however, Al-Salameh and Cloyd (1) demonstrated that MCF viruses activate signaling pathways different from those activated by the binding of gp70 to the IL-2 receptor. Therefore, autocrine stimulation of the IL-2 receptor by MCF virus gp70 alone does not explain uncontrolled cell proliferation. Additional events, such as insertional mutagenesis and/or activation of protooncogenes (18), may be required for transformation T lymphocytes in vivo by MCF viruses.

Thus, the contribution of the MCF virus gp70 to the induction of leukemia is not completely understood. However, it is clear that the envelope gene substitutions are required for disease induction. Our data demonstrate that the onset of viral leukemia is accelerated in animals containing proviruses derived from an injected chimeric virus that has recombined in vivo to acquire an MCF virus-like envelope gene and clearly demonstrate that an injected retrovirus recombines with endogenous retroviral sequences in vivo. This finding is consistent with the hypothesis that retroviral recombination facilitates the evolution of viral species in vivo and results in a biological selection for viruses that induce cell proliferation. Our results suggest that T-cell transformation occurs earlier in vivo when the target cell is infected with a virus that contains as MCF virus-specific *env* gene than when the cell is infected with a chimeric virus containing an ecotropic *env* gene. These results underscore the importance of the MCF virus envelope glycoprotein in the progression to leukemia.

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