

Influence of Enhancer Sequences on Thymotropism and Leukemogenicity of Mink Cell Focus-Forming Viruses

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Oncogenic mink cell focus-forming (MCF) viruses, such as MCF 247, show a positive correlation between the ability to replicate efficiently in the thymus and a leukemogenic phenotype. Other MCF viruses, such as MCF 30-2, replicate to high titers in thymocytes and do not accelerate the onset of leukemia. We used these two MCF viruses with different biological phenotypes to distinguish the effect of specific viral genes and genetic determinants on thymotropism and leukemogenicity. Our goal was to identify the viral sequences that distinguish thymotropic, nonleukemogenic viruses such as MCF 30-2 from thymotropic, leukemogenic viruses such as MCF 247. We cloned MCF 30-2, compared the genetic hallmarks of MCF 30-2 with those of MCF 247, constructed a series of recombinants, and tested the ability of the recombinant viruses to replicate in the thymus and to induce leukemia. The results established that (i) MCF 30-2 and MCF 247 differ in the numbers of copies of the enhancer sequences in the long terminal repeats. (ii) The thymotropic phenotype of both viruses is independent of the number of copies of the enhancer sequences. (iii) The oncogenic phenotype of MCF 247 is correlated with the presence in the virus of duplicated enhancer sequences or with the presence of an enhancer with a specific sequence. These results show that the pathogenic phenotypes of MCF viruses are dissociable from the thymotropic phenotype and depend, at least in part, upon the enhancer sequences. On the basis of these results, we suggest that the molecular mechanisms by which the enhancer sequences determine thymotropism are different from those that determine oncogenicity.

Mink cell focus-forming (MCF) retroviruses have been implicated as the proximal leukemogenic agents in the induction of spontaneous leukemia in certain inbred strains of mice (19, 21, 23, 34). MCF viruses have been isolated from the thymus and other tissues of AKR mice during the preleukemic period (9, 10, 15, 16). Some MCF isolates when injected into newborn animals accelerate the onset of leukemia (10, 34). The ability to replicate to high titers in the thymus and express high levels of murine leukemia virus (MuLV)-related cell surface antigens on infected thymocytes is tightly correlated with the oncogenic phenotype of this class of MCF viruses (20, 28, 36, 37). MCF 247 is the prototype of thymotropic, oncogenic MCF viral isolates (23-25, 34).

MCF viruses with biological phenotypes distinct from those of MCF 247 have been isolated from thymocytes of preleukemic AKR mice (36, 37). These viruses are unique because they replicate to high titer and express MuLV-related cell surface antigens on infected thymocytes and do not accelerate the onset of leukemia in AKR mice. MCF 30-2 is the prototype of this class of MCF viruses (37). Since previous studies showed that the ability of MCF as well as radiation-induced MuLV to replicate in thymocytes is positively correlated with the leukemogenic phenotype (10, 11), the isolation of this class of viruses presents a paradox. We were interested in this paradox and have used MCF 30-2 as the prototype of this group of exceptional viruses to define the genetic determinants which distinguish it from MCF 247.

Studies using MCF 247, Friend, and Moloney MuLVs, as well as a number of other viruses have demonstrated that

MuLV enhancer elements, present as duplicated sequences within the long terminal repeats (LTRs) of these viruses, are important to the tissue tropism and oncogenic properties of these viruses (3-6, 12-14, 17, 24, 32, 33, 43, 46, 52). To determine whether these or other sequences determine the difference in the oncogenic phenotypes of MCF 247 and MCF 30-2, we have cloned MCF 30-2, compared the viral genomes of MCF 30-2 and MCF 247, and constructed and characterized a set of recombinants between MCF 247 and MCF 30-2. We show here that the major differences between MCF 30-2 and MCF 247 are in the p15E region of the *env* gene and in the LTR. In addition, we demonstrate that the differential virulence of MCF 30-2 and MCF 247 is due to sequences in the LTR.

MATERIALS AND METHODS

Molecular cloning of MCF 30-2. Hirt extracts of two separate cultures of *Mus dunni* cells infected with MCF 30-2 virus were prepared, and molecular clones were isolated as described previously (25). Briefly, viral DNA was digested with *EcoRI* and cloned into pBR322. Clones were identified by hybridization to ³²P-labeled DNA from a molecular clone of MCF 247. Inserts from 10 clones were isolated, concatenated, and transfected into SC-1 or *M. dunni* cells (22, 31). Medium from separate cultures transfected with 3 of the 10 molecular clones (p30-2-C1128-1, p30-2-1464-1, and p30-2-1464-8A) contains retroviral reverse transcriptase activity (1). Two of the clones (p30-2-1464-1 and p30-2-1464-8A) were derived from a single Hirt extract, and the third (p30-2-C1128-1) was cloned from a separate infection. One clone, p30-2-1464-8A, was used to generate the site-specific recombinant viruses described below. The three clones of

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MCF 30-2 were mapped by digestion with 12 restriction enzymes (*Pst*I, *Kpn*I, *Sma*I, *Pvu*I, *Pvu*II, *Hind*III, *Sau*3A, *Xho*I, *Cla*I, *Hpa*I, *Eco*RI, and *Xba*I).

Construction of recombinants. Recombinants were constructed as described previously (25). Restriction sites used to generate recombinants were conserved in both MCF 30-2 and MCF 247 (7, 8). The structures of the recombinants were verified by using unique restriction sites diagnostic for the fragments present in recombinants. MCF 247 has a unique *Eco*RI site in the LTR (7, 8). This site is not present in the LTR of MCF 30-2 (data not shown). The structures of the recombinant viruses produced after transfection of the clones were confirmed by digesting viral intermediates with *Eco*RI and analyzing the digest with a Southern blot. All recombinants with an LTR of MCF 247 consistently contained an *Eco*RI site, whereas the site was absent from recombinants with an MCF 30-2 LTR. In some cases, the structures of the recombinants were confirmed by analyzing RNase T₁ fingerprints of viruses produced after transfection of the recombinants. The fingerprints were analyzed for the presence or absence of the unique MCF 247 or MCF 30-2 RNase T₁-resistant oligonucleotides in the precursor to the p15E gene (Prp15E gene) (29, 34).

Two independent clones of MCF 247 were used to generate the recombinants. One, p247-W, produced infectious virus when transfected into *M. dunnii* cells as described previously (25). Another molecular clone of MCF 247 (p247-1b) did not produce infectious virus when transfected. The LTR of p247-1b was biologically functional when used to generate an infectious recombinant and has been sequenced (29).

Viruses and viral assays. Infectious viruses were generated from the DNA constructs of parental viruses or recombinants after DNA transfection into *M. dunnii* cells. These cells are permissive for the replication of MCF viruses (31). The presence of infectious virus in culture supernatants was assayed by the S⁺L⁻ assay on mink cells or by the presence of reverse transcriptase activity (1).

RNase T₁ fingerprints. Preparation of ³²P-labeled viral RNA, its digestion with RNase T₁, and separation of the resulting oligonucleotides by two-dimensional gel electrophoresis were performed as described previously (18).

DNA sequencing. All DNA sequencing was performed as previously described, using M13mp18 or M13mp19 vectors (35).

Pathogenicity tests. Female AKR/J mice (40 to 45 days of age) were anesthetized by intraperitoneal injection of avertine (aqueous solution of 0.29 mg of 2,2,2-tribromoethanol-160 mg of isoamyl alcohol per g of body weight). The thymus was exposed surgically as described previously (27), and the left lobe was injected with virus or tissue culture medium as a control (0.05 ml per lobe normalized to 500 to 1,000 pmol per ml of reverse transcriptase activity, representing approximately 5×10^4 infectious units). Injected mice were observed for signs of frank leukemia (scruffled fur, hunched appearance with chest enlargement, labored breathing, lymph node enlargement). Induction of thymic lymphoma was confirmed by gross pathologic examination of affected tissues at necropsy. In some experiments, virus-injected mice were not observed after 180 days of age, at which time the incidence of spontaneous leukemia in AKR/J control mice becomes significant (24, 37, 38).

RESULTS

Characterization of molecular clones of MCF 30-2. To define the viral genes that distinguish MCF 247 from MCF

30-2, the genomes of the viruses were compared by RNase T₁ fingerprinting and restriction mapping. We used these techniques to reveal significant differences between the two viruses. Shown in Fig. 1 are RNase T₁ fingerprints of the viruses produced after transfection of the molecular clones of MCF 247 (p247-W) and MCF 30-2 (p30-2-1464-8A). The two viruses share all commonly analyzed oligonucleotides localized to the sequences encoding gp70 and the LTR. Isolated, unique oligonucleotides are present in both MCF 247 and MCF 30-2 (i.e., 30-2B, 107, and 29; Fig. 1A and B). Since these unique oligonucleotides are in separate genes, it is most likely that they represent single-base changes in the viral genomes. Conversely, a major oligonucleotide substitution was located in the p15E region of the envelope gene (Fig. 1E). Two oligonucleotides that are present in the p15E gene of MCF 30-2 (i.e., 30-2A and 119) are missing in MCF 247 and are replaced by three other oligonucleotides (i.e., 18, 33, and 47; Fig. 1E).

To determine whether the oligonucleotide substitutions in the p15E gene represent major amino acid differences in the gene products of MCF 30-2 and MCF 247, the DNA sequence of the p15E gene of MCF 30-2 was determined and compared with that of MCF 247 (29; Fig. 2A). The nucleotide sequences of the p15E genes of MCF 247 and MCF 30-2 differ by 9.3% (56 differences in a total of 603 nucleotides; Fig. 2A). Third-base changes account for 6% of the differences. The predicted amino acid sequences of the proteins differ by ~5% (10 amino acid differences in 201 amino acids). Therefore, the oligonucleotide differences in the p15E genes of MCF 30-2 and MCF 247 reflect a series of single-amino-acid differences in the two proteins.

To extend the results of the RNase T₁ oligonucleotide mapping, the molecular clones of MCF 247 and MCF 30-2 were compared by restriction mapping. Differences in three restriction sites were found between clones of MCF 30-2 and MCF 247. A *Hpa*I site located in the *gag* region and an *Eco*RI site in the LTR of MCF 247 were missing in MCF 30-2 (data not shown). In addition, three infectious molecular clones of MCF 30-2 were digested with *Pst*I and *Kpn*I. The double digest of the three clones of MCF 30-2 produced a fragment derived from each LTR that was approximately 100 nucleotides smaller than the same fragment derived from a clone of MCF 247 (p247-W).

To define the structure of the MCF 30-2 LTR, the LTR of MCF 30-2 was sequenced and compared with the sequences of two clones of MCF 247. Shown in Fig. 2B are the LTR sequences of two molecular clones of MCF 247 (p247-W and p247-1b) and a clone of MCF 30-2 (p30-2-1464-8A). The sequences of the two clones of MCF 247 are identical except that one contains two copies of a sequence present as a direct repeat in p247-W, whereas the second (p247-1b) contains a single copy of this sequence. The LTR of MCF 30-2 has one copy of the enhancer sequence that is duplicated in MCF 247 (p247-W) as well as four other nucleotide sequence differences. The enhancer sequence of MCF 30-2 is identical to the second copy of the enhancer of one clone of MCF 247 (p247-W) and has a single-nucleotide sequence difference with the other clone of MCF 247 (p247-1b). Together, the RNase T₁ fingerprints and DNA sequencing results show that the major differences that distinguish MCF 247 and MCF 30-2 lie in the p15E region of the *env* gene and in the LTR.

Two separate experiments suggest that the original MCF 30-2 virus contained a single copy of the duplicated enhancer sequence of MCF 247. First, the restriction mapping data showed that each of the three clones of MCF 30-2 had a

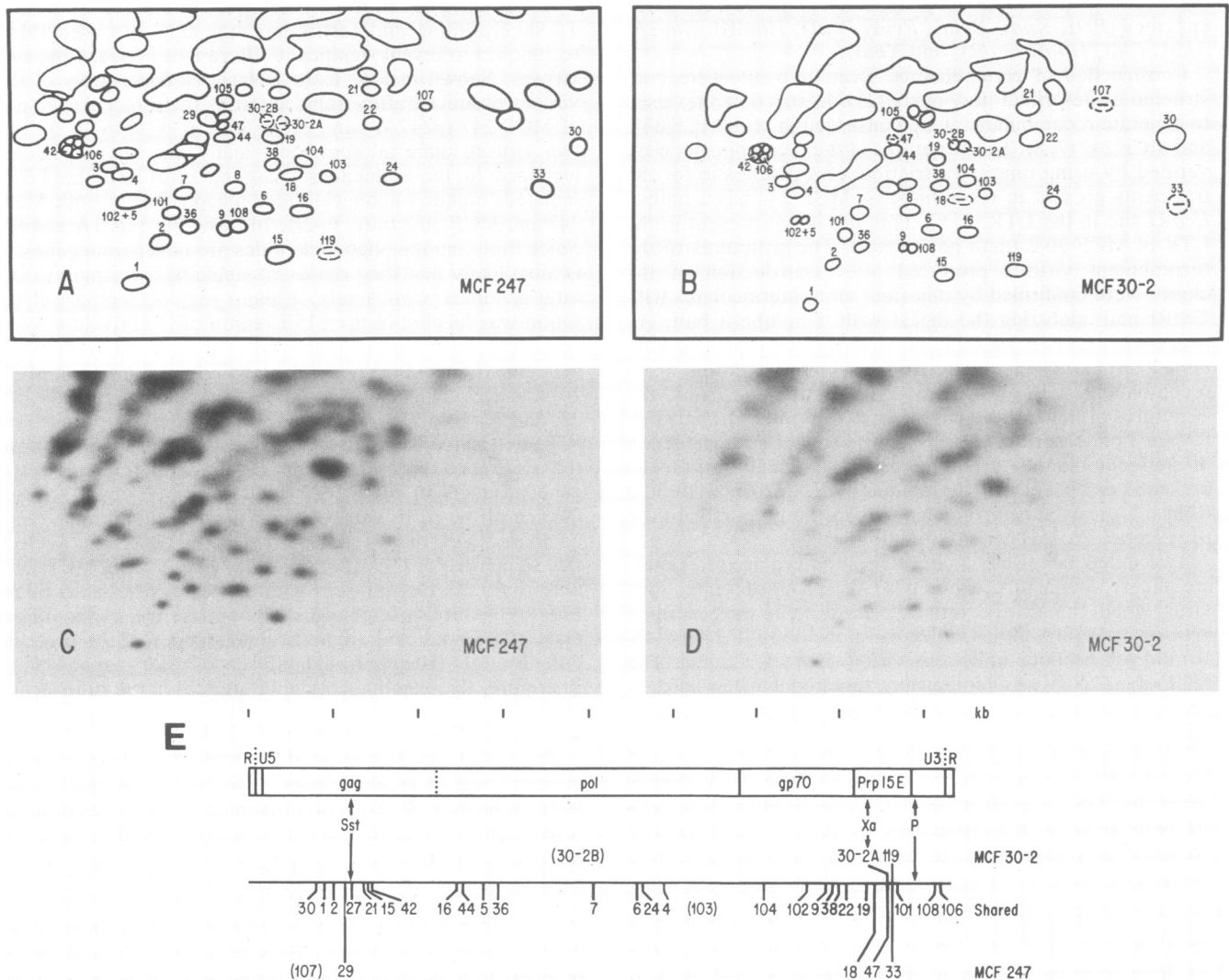


FIG. 1. (A through D) RNase T₁ fingerprints of MCF 247 and MCF 30-2. Viral RNAs obtained after transfection of the molecular clones of MCF 247 or MCF 30-2 were analyzed by RNase T₁ digestion and two-dimensional gel electrophoresis. Panels A and B are schematic diagrams of the T₁ fingerprints shown in panels C and D and were traced from those fingerprints. The numbers assigned to specific oligonucleotides are according to Rommelaere et al. (42). RNase T₁ oligonucleotides specific for MCF 30-2 are identified (30-2A and 30-2B). The RNase T₁ oligonucleotides that are enclosed in solid lines in panels A and B are shared by both MCF 247 and MCF 30-2. The putative positions of oligonucleotides absent from a particular fingerprint are indicated by a broken line. (E) Schematic representation of the genomic location of large RNase T₁-resistant oligonucleotides of MCF 30-2 and MCF 247. The box is a schematic diagram of a retroviral genome. Specific viral genes (*gag*, *pol*, *gp70*, and *Prp15E*) as well as regions of the LTR (U3, R, and U5) are shown. Sites of cleavage of restriction enzymes are indicated (*Sst*, *SstII*; *Xa*, *XbaI*; *P*, *PstI*). The numbers and locations of RNase T₁-resistant oligonucleotides unique to either MCF 30-2 or MCF 247 and shared by both are shown. The locations of the oligonucleotides were determined either by DNA sequencing or T₁ oligonucleotide mapping (29, 42). Oligonucleotides that have not been localized by either method are indicated in parenthesis (i.e., 30-2B and 107).

single copy of the enhancer sequence. These three clones were derived from two separate infections of *M. dunni* cells. It is therefore more likely that the original virus had a single copy of the enhancer sequence. Second, a Southern blot analysis of viral circular DNA intermediates isolated 16 h after infection of *M. dunni* cells with MCF 30-2 virus showed that the DNA contained one copy of the enhancer sequences (data not shown). Taken together, these data suggest that the original viral stock of MCF 30-2 was homogeneous and most viral genomes contained a single copy of the enhancer sequences.

Leukemogenicity of MCF 30-2 and MCF 247 recombinants.

To evaluate the effect of differences in the p15E genes and LTRs of MCF 247 and MCF 30-2 on the ability to accelerate leukemia, a series of molecular recombinants was constructed (Fig. 3). Restriction enzyme sites located in identical positions in both viral genomes were identified. We used those sites to exchange between clones of MCF 247 and MCF 30-2 a fragment from *XbaI* to *PstI* which encodes 80% of the p15E gene and 37 nucleotides of the U3 region of the LTRs (recombinants 1 and 2, Fig. 3). There are no nucleotide sequence differences between the viruses in the LTR portion of the *XbaI*-to-*PstI* fragment. In addition, greater than 95% of the amino acid differences in the p15E genes of

A

MCF247W protein	Glu	Pro	Val	Ser	Leu	Thr	Leu	Ala	Leu	Leu	Leu	Gly	Gly	Leu	Thr	Met	Gly	Gly	Ile	Ala	Ala	Gly	Val
MCF247W gene	GAA	CCC	GTC	TCA	CTA	ACT	CTG	GCC	CTA	CTA	TTA	GGA	GGA	CTC	ACT	ATG	GGC	GGA	ATT	GCC	GCT	GGA	GTA
MCF30-2 gene	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	**G
MCF30-2 protein	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
MCF247W protein	Gly	Thr	Gly	Thr	Thr	Ala	Leu	Val	Ala	Thr	Gln	Gln	Phe	Gln	Gln	Phe	Gln	Ala	Ala	Met	Gln	Asp	Asp
MCF247W gene	GGA	ACA	GGG	ACT	ACC	GCC	CTA	GTG	GCC	ACT	CAG	CAG	TTC	CAA	CAA	TTC	CAG	GCT	GCC	ATG	CAG	GAT	GAC
MCF30-2 gene	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	G**	**C	***	***
MCF30-2 protein	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	Leu	***	***	***	Val	His	***
MCF247W protein	Leu	Lys	Glu	Val	Glu	Lys	Ser	Ile	Thr	Asn	Leu	Glu	Lys	Ser	Leu	Thr	Ser	Leu	Ser	Glu	Val	Val	Leu
MCF247W gene	CTT	AAA	GAA	GTT	GAA	AAG	TCC	ATC	ACT	AAT	CTA	GAA	AAA	TCT	TTG	ACC	TCC	TTG	TCC	GAA	GTA	GTG	TTA
MCF30-2 gene	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
MCF30-2 protein	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
MCF247W protein	Gln	Asn	Arg	Arg	Gly	Leu	Asp	Leu	Leu	Phe	Leu	Lys	Glu	Gly	Gly	Leu	Cys	Ala	Ala	Leu	Lys	Glu	Glu
MCF247W gene	CAG	AAT	CGT	AGA	GGC	CTA	GAT	CTA	CTA	TTC	CTA	AAA	GAG	GGA	GGT	TTG	TGT	GCT	GCC	TTA	AAA	GAA	GAA
MCF30-2 gene	***	***	***	***	***	***	***	**G	**G	***	***	***	**A	***	**A	***	**A	***	***	C**	***	***	***
MCF30-2 protein	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
MCF247W protein	Cys	Cys	Phe	Tyr	Ala	Asp	His	Thr	Gly	Leu	Val	Arg	Asp	Ser	Met	Ala	Lys	Leu	Arg	Glu	Arg	Leu	Ser
MCF247W gene	TGC	TGT	TTC	TAT	GCC	GAC	CAC	ACA	GGA	TTG	GTA	CGG	GAT	AGC	ATG	GCC	AAA	CTT	AGA	GAA	AGA	TTG	AGT
MCF30-2 gene	***	***	***	***	**A	***	***	**T	**C	G**	***	**A	***	***	***	***	***	**A	***	**G	C**	G**	**C
MCF30-2 protein	***	***	***	***	***	***	***	***	***	Val	***	***	***	***	***	***	***	***	***	***	***	***	Asn
MCF247W protein	Gln	Arg	Gln	Lys	Leu	Phe	Glu	Ser	Gln	Gln	Gly	Trp	Phe	Glu	Gly	Leu	Phe	Asn	Lys	Ser	Pro	Trp	Phe
MCF247W gene	CAG	AGA	CAA	AAG	CTC	TTT	GAA	TCC	CAA	CAA	GGG	TGG	TTT	GAA	GGG	CTG	TTT	AAT	AAG	TCC	CCT	TGG	TTC
MCF30-2 gene	***	**G	***	**A	T**	***	**C	***	***	***	***	***	**G	**A	***	**A	***	**C	**G	***	**A	***	***
MCF30-2 protein	***	***	***	***	***	***	***	***	Gly	***	***	***	***	***	Arg	***	***	***	Arg	***	***	***	***
MCF247W protein	Thr	Thr	Leu	Ile	Ser	Thr	Ile	Met	Gly	Pro	Leu	Ile	Ile	Leu	Leu	Leu	Ile	Leu	Phe	Gly	Pro	Cys	
MCF247W gene	ACC	ACC	CTG	ATA	TCC	ACC	ATC	ATG	GGT	CCC	CTG	ATA	ATC	CTC	TTG	TTA	ATT	TTA	CTC	TTT	GGG	CCT	TGT
MCF30-2 gene	**G	***	***	***	***	***	**T	***	**C	**T	***	***	G**	**T	**A	***	**C	C**	***	C**	**A	**C	**C
MCF30-2 protein	***	***	***	***	***	***	***	***	***	***	***	***	Val	***	***	***	***	***	***	***	***	***	***
MCF247W protein	Ile	Leu	Asn	Arg	Leu	Val	Gln	Phe	Val	Lys	Asp	Arg	Ile	Ser	Val	Val	Gln	Ala	Leu	Val	Leu	Thr	Gln
MCF247W gene	ATT	CTC	AAT	CGC	CTG	GTC	CAG	TTT	GTA	AAA	GAC	AGA	ATT	TCG	GTG	GTG	CAG	GCC	CTG	GTT	CTG	ACC	CAA
MCF30-2 gene	***	***	**C	***	T**	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
MCF30-2 protein	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***
MCF247W protein	Gln	Tyr	His	Gln	Leu	Lys	Ser	Ile	Asp	Pro	Glu	Glu	Val	Glu	Ser	Arg	Glu	-					
MCF247W gene	CAG	TAT	CAC	CAA	CTC	AAA	TCA	ATA	GAT	CCA	GAA	GAA	GTA	GAA	TCG	CGT	GAA	TAA					
MCF30-2 gene	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***					
MCF30-2 protein	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***					

B

MCF247W	TGAAAGACCCACCATAAGGCTTAGCAAGCTAGCTGCAGTAACGCCATTTTGCAAGGCATGAAAAAGTACCAGAGCTGAATTCTCAAAG
MCF2471b	*****
MCF30-2	*****G*****
MCF247W	TCACAAGGAAGTTTAGTTAAAGAATAAGGCTGAACAAAACCTGGGACAGGGCCAAACAGGATATCTGTGGTCGAGCACCTGGGCCCGGC
MCF2471b	*****
MCF30-2	*****
MCF247W	TCAGGGCCAAGAACAGATGGTACTCAGATAAAGCGAAACTAGCAACAGTTTCTGGAGAGTCCACCCCTTCCAAACAGGATATCTGTGGT
MCF2471b	-----
MCF30-2	-----
MCF247W	CGAGCACCTGGGCCCGGCTCAGGGCCAAGAACAGATAGTACTCAGATAAAGCGAAACTAGCAACAGTTTCTGGAAAGTCCACCCCTCAGT
MCF2471b	*****G*****
MCF30-2	*****G*****
MCF247W	TTCAAGTTCGCCAAAAGACCGGGAAAACCCCAAGCCTTATTAAACTAACCAATCAGCTCGCTTCTCGCTTCTGTAAACCGCGCTTTATG
MCF2471b	*****
MCF30-2	*****A*****T**
MCF247W	CTCCCCAGCCCTATAAAAAGGGTAAAAACCCACACTCGGGCGCCAGTCCCTCCGATAGACTGAGTCGCCCGGGTACCCGTGTATCCAAT
MCF2471b	*****
MCF30-2	*****
MCF247W	AAAGCCTTTTGCTGTTCATCCGAATCGTGGTCTCGCTGATCCTTGGGAGGGTCTCCTCAGAGTGATTGACTGCCAGCCTGGGGTCTT
MCF2471b	*****
MCF30-2	*****A*****
MCF247W	TCA
MCF2471b	**
MCF30-2	**

FIG. 2. Nucleotide sequences of the p15E genes and the LTRs of MCF 247 and MCF 30-2. (A) The nucleotide and predicted amino acid sequences of the p15E envelope gene of MCF 30-2 were determined and compared with those of MCF 247 (29). (B) The LTR sequences of separate clones of MCF 247 (p247-W) and MCF 30-2 were determined and compared with those published previously (29) for a clone of MCF 247 (p247-1b). *, Nucleotide sequences or amino acids identical to those shown on the top line. Nucleotide sequence differences are indicated. Dashes indicate the absence of a nucleotide. ↓, Sequences present as a direct repeat in MCF 247-W. Boxes enclose a site with homology to the simian virus 40 enhancer core identified in Moloney MuLV as the binding site for NF-KB (45, 48).

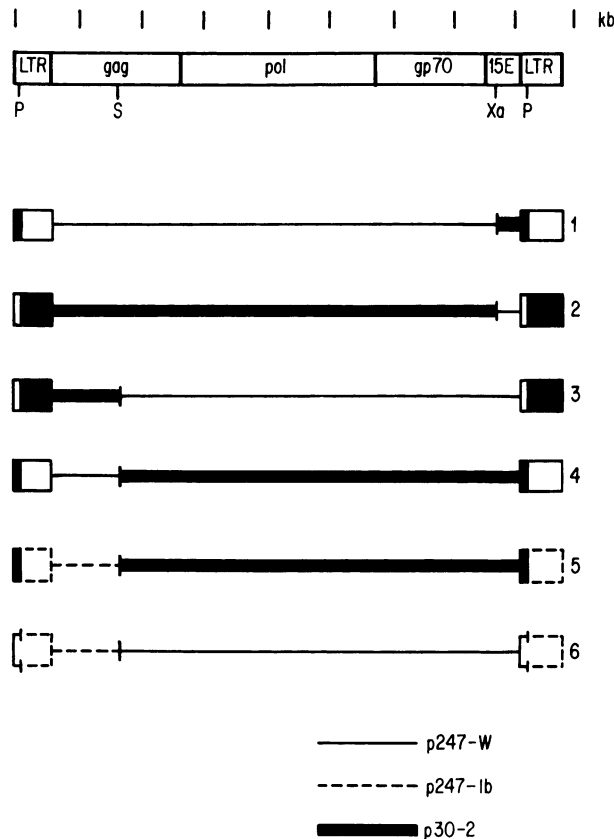


FIG. 3. Viral genomes of recombinants. Schematic representation of molecular recombinants. Restriction sites shared by DNA clones of MCF 247 and MCF 30-2 were used to generate recombinants (P, *Pst*I; S, *Sst*II; Xa, *Xba*I) and are shown at the top relative to the retroviral genome. Sequences were derived from one of two clones of MCF 247 or from a clone of MCF 30-2 (■, p30-2). One clone of MCF 247 contains two copies of the enhancer-containing sequences (—, p247-W); a second clone of MCF 247 contains one copy (---, p247-1b). Molecular recombinants are numbered.

MCF 247 and 30-2 are contained on this *Xba*I-to-*Pst*I fragment. Thus, recombinants 1 and 2 effectively exchange the p15E sequence differences shown in Fig. 2.

To evaluate the effect of the MCF 30-2 and MCF 247 LTRs on thymotropism and pathogenesis, we exchanged a fragment between the *Pst*I and *Sst*II sites of MCF 247 and MCF 30-2 (Fig. 3, recombinants 3, 4, 5, and 6). The fragment contains most of the LTRs of the viruses and the 5' noncoding region of the viral genome, including the primer-binding site and a portion of the coding region of two *gag* genes (p15 and p12). The *Pst*I-to-*Sst*II fragment was obtained from each of two clones of MCF 247. The sequences of the LTRs of these two clones are shown in Fig. 2B. One, p247-W, contains two copies of the enhancer sequences present as a direct repeat. The second, p247-1b contains a single copy of this sequence. In addition, since MCF 247 contains an oligonucleotide (i.e., 107; Fig. 1A) absent from MCF 30-2, the nucleotide sequences of the genomes of MCF 30-2 and MCF 247 between the end of the LTR and the *Sst*II site were determined and compared. The leader regions of the genomes have three isolated nucleotide sequence differences, but the amino acids of the *gag* genes of the two viruses are identical (data not shown). Thus, the major difference between the two viruses in the *Pst*I-to-*Sst*II fragment is the

number of copies of the enhancer sequence in the LTRs. Sequencing data shown in Fig. 2B establishes that the LTR of one clone of MCF 247 (p247-1b) is identical to another (p247-W) except that the second contains two copies of the direct repeat (p247-W). We considered these clones as variants and have used these two clones of MCF 247 to assess the importance of the number of copies of the enhancer sequences. In addition, since both MCF 247 clones differ from MCF 30-2 in other LTR sequences, we used these MCF 247 variants to assess the importance of those nucleotide sequence differences as well (see Fig. 2B).

Viruses obtained after transfection of the molecular clones of MCF 247, MCF 30-2, and the recombinants shown in Fig. 3 were injected intrathymically into 45-day-old AKR mice. Leukemogenicity of the viruses was evaluated by determining the percentage of animals that developed thymic lymphoma by 180 days of age (Fig. 4, Table 1). After 180 days, control mice injected with media developed tumors spontaneously (Fig. 4a) and 90% of the animals died from thymic lymphoma by 300 days of age. Nearly 100% of the animals injected with virus derived from transfection of the molecular clone of MCF 247 developed tumors by 180 days of age compared with 7% of the mice injected with MCF 30-2-derived virus. The recombinant composed predominantly of MCF 247 but with the p15E region of MCF 30-2 (recombinant 1, Fig. 3 and Fig. 4b) accelerated leukemia almost as rapidly as MCF 247. Moreover, the MCF 247 p15E region did not significantly change the nonleukemogenic phenotype of MCF 30-2 (recombinant 2, Fig. 3 and Fig. 4b). Thus, the differences in the p15E genes of MCF 30-2 and MCF 247 (Fig. 2A) are not sufficient to explain the differences in their pathogenicity.

The results obtained by injecting recombinants containing different LTRs (Fig. 3, recombinants 3, 4, 5, and 6) were quite different. The LTR of MCF 30-2 dampened the ability of MCF 247 to accelerate leukemia (Fig. 4, recombinant 3). Conversely, the LTR of MCF 247 made MCF 30-2 a more leukemogenic virus (Fig. 4, recombinant 4). These data suggest that the LTR of MCF 247 is the major determinant of the difference in the leukemogenic potential of these two MCF viruses. We noted that the incidence of leukemia at 180 days of recombinant 4 (Table 1; 75%) was lower than that for MCF 247 itself (Table 1; 90%). In addition, the latent period of recombinant 4 (Table 1; 170 ± 35) was longer than that of MCF 247 (Table 1; 127 ± 32). These results imply that there are other viral sequences in addition to the LTR that differ between MCF 247 and MCF 30-2 and contribute, at least in part, to the leukemogenic phenotypes of the viruses.

We noted that the clone of MCF 247 (p247-W) used to donate the LTR to recombinant 4 contained two copies of the enhancer sequences (Fig. 2B). To determine whether the difference in leukemogenic potential of MCF 247 and MCF 30-2 was due to the presence of two copies of the enhancer sequences in the MCF 247 LTR relative to the single copy in the MCF 30-2 LTR, we constructed recombinant 5 (Fig. 3). Recombinant 5 is identical to recombinant 4, except that the *Pst*I-to-*Sst*II fragment of the recombinant used to replace the LTR of MCF 30-2 was obtained from a molecular clone of MCF 247 (p247-1b) with a single copy of the enhancer. As a control, the same fragment was attached to the genome of MCF 247 obtained from p247-W (recombinant 6). Each of these was tested for the ability to accelerate the onset of leukemia. The incidence of leukemia at 180 days in animals injected with recombinant 6 was considerably lower (Table 1; 35%) than the incidence for MCF 247 itself (Table 1; 90%). These data show that the nucleotide sequence differences

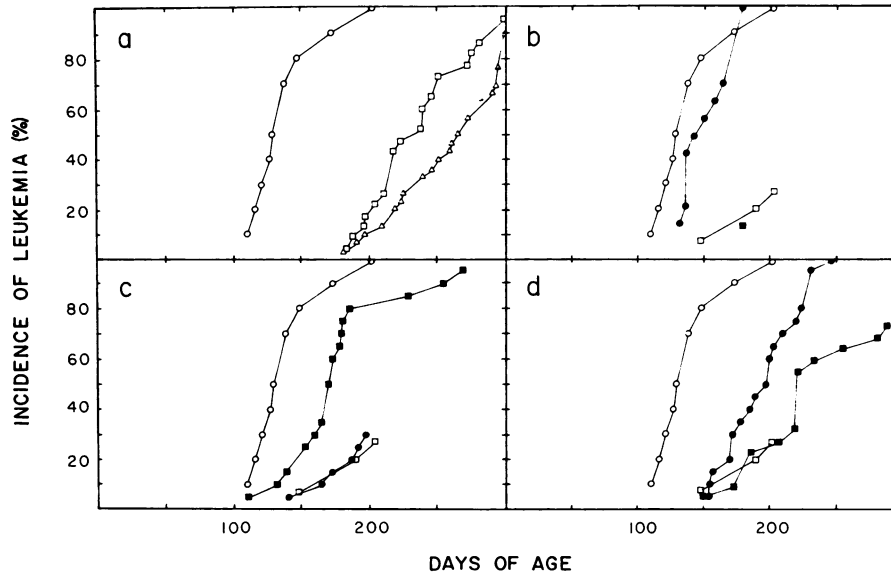


FIG. 4. Incidence of leukemia in AKR/J mice after intrathymic injection of parental and recombinant viruses. (a) The development of leukemia in mice injected with MCF 247W (○) or MCF 30-2 (□) virus is compared with the incidence of leukemia in control mice injected with media (△). (b) The incidences of leukemia in mice injected with parental viruses (MCF 247W [○]; MCF 30-2 [□]) are compared with those for viruses with recombinant p15E genes. Recombinant 1 contains the p15E gene derived from MCF 30-2 (●). Recombinant 2 expresses the MCF 247W p15E gene (■). (c and d) The leukemogenic potential of recombinant viruses with different LTRs is shown. Recombinant 3 (●) and recombinant 4 (■) have an MCF 30-2 and an MCF 247-W LTR, respectively. (d) Recombinant 6 (●) has an LTR derived from MCF 247-1b. Recombinant 5 has the same LTR as recombinant 6, but the structural genes are derived from MCF 30-2 (■). The data in panel a and in panels b, c, and d were derived from separate experiments. The numbers of mice in each group are given in Table 1.

between MCF 247-1b and MCF 247 (Fig. 2B) dramatically reduce the leukemogenic potential of MCF 247. In addition, the recombinant composed of MCF 30-2 but with the MCF 247 LTR with one copy of the enhancer (recombinant 5) produced approximately the same incidence of leukemia at 180 days (Table 1; 9%) as MCF 30-2 itself (Table 1; 7%). These data suggest (i) that the nucleotide sequence differences in the LTRs of MCF 30-2 and one of the clones of MCF 247 (p247-1b) do not dramatically affect the leukemogenic phenotypes of the viruses and (ii) that the presence of the duplicated enhancer sequences of MCF 247 (p247-W) contributes to the ability of this virus to accelerate leukemia.

The enhancer-containing sequences of the two parental clones of MCF 247 (p247-W and p247-1b) were aligned with those of MCF 30-2 (Fig. 5). We sequenced and compared the first copy of the direct repeat of MCF 247-W with that of the second as well as with those of MCF 247-1b and MCF 30-2. There are four nucleotide sequence differences among the enhancer sequences. The first copy of the direct repeat in MCF 247-W has a nucleotide transition (A to G). This transition occurs in the sequence which in Moloney MuLV binds the B-cell-specific enhancer-binding protein NF-KB (45, 48). In addition, the first direct repeat of MCF 247-W has three additional nucleotides compared with the second copy

TABLE 1. Acceleration of leukemia in AKR/J mice injected with recombinant and MCF viruses

Virus (no. ^a)	Structure	(Diseased mice)/ (injected mice) ^b	Latent period ^c	Incidence of leukemia (%) ^d
MCF 247-W		9/10	127 ± 32	90
MCF 30-2		1/15	148	7
1		14/14	137 ± 24	100
2		2/15	180	13
3		3/21	165 ± 16	14
4		15/20	170 ± 35	75
5		2/22	163 ± 11	9
6		7/20	170 ± 12	35

^a Numbers refer to genomes of viruses generated after transfection of molecular clones diagrammed in Fig. 3.

^b The number of mice with disease is compared with the total number of mice injected with a specific virus.

^c The latent period was calculated as the mean of all animals observed with disease at less than 180 days ± standard error.

^d Calculated as (number of mice with disease at <180 days)/(number of mice injected) × 100.

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1 dr MCF 247W CCAAAACAGGATATCTGTGGTCGAGCACCTGGGCCCGGCTCAGGGCCAAGAAGACAGATGGTACTCAGATAAAGCGAAACTAGCAACAGTTTCGGAGAGTCCACCCCTTC
2 dr MCF 247W .....A.....A.....
MCF 2471b .....A.....
MCF 30-2 .....A.....
    
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FIG. 5. Nucleotide sequences of enhancers of MCF viruses. The duplicated repeat region of the leukemogenic virus MCF 247W is compared with the single copy present in the clones of MCF 247-1b and MCF 30-2.

of the direct repeat in MCF 247-W. The second copy of the direct repeat in MCF 247-W is identical to that of MCF 247-1b and MCF 30-2. Our data does not exclude the possibility that these nucleotide sequence differences in the enhancer sequences contribute to the leukemogenic phenotype of MCF 247.

Thymotropism of the molecular recombinants. Studies on avian leukosis viruses demonstrate that the U3 region of the LTR is an important determinant of the growth rate of avian leukosis virus in fibroblasts (41, 49). More recent studies demonstrate that the LTR functions to determine the leukemogenic potential of the viruses in vivo by mediating the rate of infection of the bursal target stem cell (2). The timing of infection of thymocytes appears to affect the acceleration of leukemia in AKR mice as well (16).

To determine whether the number of copies of the enhancer sequence affects the ability of MCF viruses to replicate in thymocytes, we quantitated and compared the number of infectious viral particles produced by thymocytes isolated from animals 48 days after injection with either MCF 30-2, MCF 247, or recombinant viruses. Since the assay for thymotropism was performed 48 days after intrathymic injection of the viruses, we recognize that the viruses or the recombinants might replicate to different titers early after injection. Figure 6 shows that MCF 30-2, MCF 247, and most of the recombinant viruses were thymotropic as measured by infectious center assay on S⁺L⁻ mink cells. The only possible exception was recombinant 5 (30-2 + 247 1b LTR). Thymocytes isolated from three animals injected with recombinant 5 produced a low titer of virus. Thymocytes isolated from a fourth animal from that group produced a titer of virus approximately equal to that of the parents or other recombinants. We conclude from these data that MCF 30-2, MCF 247, and all the recombinants, including recombinant 5, are thymotropic.

In all cases, the frequency of infectious centers in thymocyte suspensions was low (0.01 to 0.1%), yet no infectious centers were detected in thymuses of control mice injected with tissue culture medium (<0.1/10⁶ thymocytes). Despite the low level of productive infection, flow cytometric analysis of thymocytes stained with a monoclonal antibody that reacts with both ecotropic and MCF MuLV gp70s showed that, on the average, 64% of thymocytes expressed high levels of gp70 compared with thymocytes of control mice. The majority of thymocytes in MCF 30-2, MCF 247, and recombinant virus-injected mice were, in fact, infected at day 48 as judged by expression of viral antigen, a known characteristic of MuLV-infected lymphoid cells (19, 20, 36, 37). These data confirm previous reports which demonstrate that the viral phenotypes of thymotropism and pathogenicity are clearly separable (37). In the present work, we have demonstrated that oncogenicity is dependent on the number of copies of the retroviral enhancer sequences or on their specific sequences.

DISCUSSION

Evidence from studies of several retroviruses supports the hypothesis that the ability of a virus to replicate in the target

tissue as well as the transformation potential of MuLVs are both controlled, at least in part, by sequences within the retroviral LTR (3, 5, 6, 12-14, 17, 24, 32, 33, 43, 46, 52). The results of experiments presented here demonstrate that the enhancer sequences of MCF viruses are important determinants of the disease-inducing potential in these viruses. These studies extend previous reports demonstrating that the LTR of MCF 247 controls an in vitro tissue culture host range property of the virus that is correlated with the in vivo thymotropic phenotype (25). Specifically, these data show that a single copy of the enhancer sequence is sufficient to establish the thymotropic phenotype of MCF 30-2 and that two copies of the enhancer or a specific enhancer sequence are required for an MCF virus to accelerate the onset of leukemia in AKR mice. Although the isolation of a leukemogenic MCF virus (MCF 13-81) with a nonduplicated enhancer has been reported (26), the sequence of the enhancer of MCF 13-81 differs from that of MCF 30-2 and the number of enhancers in the viruses found in tumors have not been examined.

The hypothesis that two copies of the enhancer are an essential feature of a leukemogenic virus is supported by the recent finding that spontaneous tumors of AKR mice contain viruses with duplicated enhancer sequences even though the identified endogenous virus which is the U3 donor for leukemogenic MCF viruses (BXV-1) contains a single copy

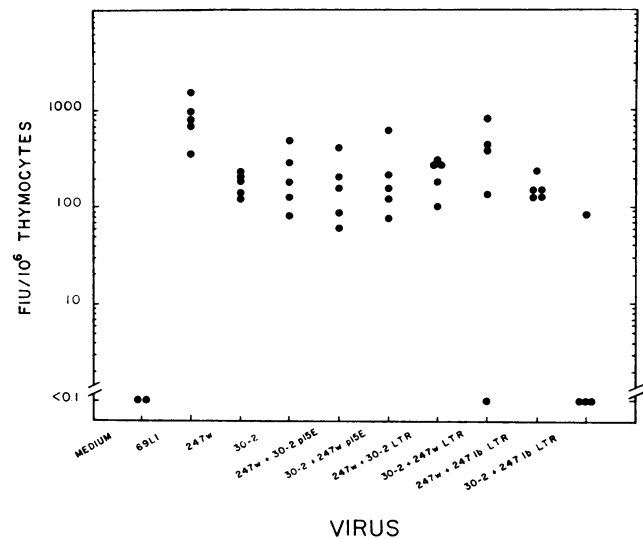


FIG. 6. Thymotropism of parental and recombinant viruses in AKR/J mice. The number of infectious centers per 10⁶ thymocytes was scored in an S⁺L⁻ mink focus induction assay. Thymocytes were obtained from animals at 48 days after intrathymic injection with medium, a virus to serve as a positive control (MCF 69L1), parental (MCF 247W or MCF 30-2), or recombinant viruses (recombinant 1, 247W + 30-2 p15E; recombinant 2, 30-2 + 247W p15E; recombinant 3, 247W + 30-2 LTR; recombinant 4, 30-2 + 247W LTR; recombinant 6, 247W + 2471b LTR; recombinant 5, 30-2 + 2471b LTR). Virus injections were normalized with respect to reverse transcriptase activity.

of the enhancer (J. Stoye, C. Moroni, and J. Coffin, personal communication). These data argue that two copies of the enhancer sequences are functionally important to the molecular mechanism of transformation by MCF viruses.

The presence of two copies of the enhancer sequences in the virus which is the proximal leukemogenic agent in AKR mice might influence the transformation process at a variety of levels and may be important to achieve high levels of either viral or cellular gene transcription. A direct effect on viral transcription may result in an increase either in the number of infected target cells or in the number of integrated proviruses per cell. Alternatively, the presence of two copies of the enhancer sequence creates a unique DNA sequence at the junction of the 3' end of the first copy of the enhancer and the 5' end of the second copy. This DNA sequence is unique and could be used as a new binding site for a *trans*-acting protein. Evidence for the existence of multiple, cooperative, or overlapping protein-binding sequences in the enhancer elements of Moloney (48) and Friend (N. Manley, P. Sharpe, and N. Hopkins, personal communication) MuLV, Rous sarcoma virus (30), simian virus 40 (39, 53), and polyomavirus have been presented previously (40, 44, 50).

The number of enhancer sequences produces differential effects in MuLVs with distinct oncogenic or tissue tropic properties. It has been demonstrated that Friend or Moloney MuLV with one copy of the enhancer sequence induces disease with a longer latent period compared with viruses with two copies of the enhancer (17, 33, 47). Conversely, separate experiments show that neither the number of copies nor the exact sequence of the enhancer determines either the latent period or the tissue specificity of spleen focus-forming virus (SFFV) (51). Neither the disease specificity nor the latency of the disease was affected by the number of copies of the direct repeat or by the origin of the direct repeat (from Friend MuLV, Friend MCF, or Moloney MuLV). These experiments and others suggest that the *env* gene of SFFV is the primary determinant of the leukemogenicity of SFFV (51). Taken together with our data, these results suggest that MCF viruses and some other replication-competent retroviruses transform the target cell by a different mechanism than that of SFFV. The mechanism of transformation of the replication-competent retroviruses might be dependent on a specific enhancer-controlled function, whereas viral genes of SFFV and other oncogene-containing replication-defective viruses transform the target cell more directly.

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